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TAMPERE UNIVERSITY OF TECHNOLOGY

MARJA SALO

BIOLOGICAL SULFATE REDUCTION AND RECOVERY OF ELEMENTAL SULFUR FROM MINING WASTE WATERS

Master of Science Thesis

Examiners: Professor Jukka Rintala,
Assistant Professor Aino-Maija
Lakaniemi
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ABSTRACT

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Sulfate in waste waters, especially in effluents of the mining industry, is a growing concern in environmental protection. The conventional methods are limited in terms of sulfate removal efficiency, and new processes are needed for decreasing sulfate emissions to water systems. Biological removal by sulfate reduction to sulfide is one alternative for efficient sulfate removal. The possibility of combining sulfate reduction and sulfide oxidation to elemental sulfur is a comprehensive process for the removal of sulfur compounds, as well as a way to create profit from sulfate containing waste streams. This work investigates a continuous biological sulfate removal from real mine drainage with cow manure as the main carbon source and electron donor. Batch experiments for elemental sulfur recovery were also performed. The bacterial communities present in the effluents of the sulfate-reducing reactors were analysed and their influence in the process is discussed.

Biological sulfate removal was tested with three upflow anaerobic sludge blanket reactors with different additional inocula. The highest stable sulfate removal efficiency was 60% with lactate as a co-substrate. Sulfide concentration in the effluents was low, but sulfide oxidation experiments indicated elemental sulfur formation, so the waste water treatment principles of this work could be applied to actual mining sites. The DNA analyses showed a wide range of bacterial groups present in the reactor effluents. The bacterial communities developed and the amount of sulfate-reducers grew during the operation. These microbial analyses allow a uniquely continuous peek inside the biological process, offering knowledge in the interactions of different bacterial groups and their effect on sulfate removal efficiency.

TIIVISTELMÄ

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Jätevesien, erityisesti kaivosteollisuuden jätevirtojen, sisältämä sulfaatti on kasvava huoli ympäristönsuojelussa. Perinteisten menetelmien sulfaatinpoistotehokkuus on rajoittunutta, ja uusia prosesseja tarvitaan sulfaattipäästöjen vähentämiseksi vesistöihin. Sulfaatin biologinen pelkistys sulfidiksi on yksi vaihtoehto tehokkaaseen sulfaatinpoistoon. Kun yhdistetään sulfaatin pelkistys ja sulfidin hapetus alkuainerikiksi, saadaan sekä kokonaisvaltainen prosessi rikkiyhdisteiden poistamiseksi että keino hyötyä taloudellisesti sulfaattia sisältävistä jätevesistä. Tässä työssä tutkitaan jatkuvatoimista biologista sulfaatinpoistoa aidosta kaivosvedestä käyttäen lehmän lantaa pääasiallisena hiilen ja elektronien lähteenä. Lisäksi suoritettiin panoskokeita alkuainerikin talteenottamiseksi. Sulfaattia pelkistävien reaktoreiden lähtövesistä tutkittiin bakteeripopulaatiota ja niiden vaikutusta prosessiin pohditaan.

Biologista sulfaatinpoistoa tutkittiin kolmella anaerobisella lietepatjareaktorilla, joissa käytettiin erilaisia lisäymppejä. Korkein vakaa sulfaatinpoistotehokkuus oli 60%, kun laktaattia käytettiin lisäsubstraattina. Reaktoreiden lähtövesien sulfidipitoisuus oli matala, mutta sulfidinhapetuskokeet viittasivat alkuainerikin muodostumiseen, joten tämän työn jätevesien käsittelyperiaatteita voisi soveltaa myös aidoissa kaivosympäristöissä. DNA-analyysit paljastivat monia erilaisia bakteeriryhmiä reaktoreiden ulostulovesissä. Bakteeripopulaatiot kehittyivät ja sulfaatinpelkistäjien määrä kasvoi kokeen aikana. Nämä mikrobianalyysit mahdollistavat ainutlaatuisen jatkuvan katsauksen biologiseen prosessiin ja tarjoavat tietoa eri bakteeriryhmien vuorovaikutuksista sekä niiden vaikutuksesta sulfaatinpoistotehokkuuteen.

PREFACE

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LIST OF TERMS AND ABBREVIATIONS

Abiotic	Without living organisms
AMD	Acid mine drainage
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
FBR	Fluidized bed reactor
HRT	Hydraulic retention time
Inoculum	Sample containing microorganisms, transferred to a growth medium or a reactor in order to induce microbial growth
Mesophile	Organism that thrives at $25 - 40$ °C
Methanogen	Methane producing microorganism
OTU	Operational taxonomic unit
Psychrophile	Organism that thrives below 10 °C
qPCR	Quantitative polymerase chain reaction
Q_s	Influent flow rate
Redox potential	Oxidation-reduction balance in an environment, described as volts (V)
SRB	Sulfate-reducing bacteria
Thermophile	Organism that thrives at $40 - 122$ °C
TOC	Total organic carbon
TS	Total solids
UASB	Upflow anaerobic sludge blanket (reactor)
V	Sludge volume
VS	Volatile solids

1. INTRODUCTION

The greatest environmental impacts of mining usually rise from the utilization of metal sulfide ores, for example millerite (NiS), chalcopyrite ($CuFeS_2$) and sphalerite ((Zn,Fe)S) (Hytönen 1999). In Finland, most of the old and still active metal mines are based on the use of these sulfide minerals (Toropainen 2006). In 2015, the total production of metal ore in Finland was nearly 17 Mt with over 35 Mt of waste rock generated, and these quantities are on the rise (Geological Survey of Finland 2015). Besides the physical factors related to all mines, such as noise and generation of dust, mining and storing the sulfidic rock material can have effects on the environment, as the unearthed bedrock and rock piles together with air, water and microorganisms can create a pathway to acidic effluents containing high concentrations of both heavy metals and sulfate (Heikkinen et al. 2005; Toropainen 2006). If the effluents are not properly managed, they can affect the natural ecosystems and may also have an impact on the recreational activities as well as the availability of drinking water (Heikkinen et al. 2005). As the ore production grows and general environmental concern and knowledge in the migration, transformations and effects of different substances increase, the environmental permits for mines can be expected to become more stringent in the future.

Sulfate (SO_4^{2-}) is a common anion in seawater (Lens 2009), but when introduced in elevated concentrations to fresh water environments, it can cause major shifts in ecosystem balance and consequently impair the natural habitat of many local species (Roden and Edmons 1997; Soucek and Kennedy 2005; Kauppi et al. 2013). Besides mining activities, other sectors creating waste waters with high concentrations of sulfate are for example tanneries and pulp processing (Hulshoff Pol et al. 1998). For sulfate removal there are several options which may vary greatly in treatment efficiency. The methods include traditional lime treatment as well as newer alternatives which are based on, for example, membrane technology, ion-exchange or utilisation of sulfate-reducing microorganisms. Especially bioreactors capable of efficient sulfate removal and allowing the recovery of metals as sulfides are considered potential. (Mitchell 2000; International Network for Acid Prevention 2003)

In biological sulfate removal, sulfate-reducing microorganisms consume organic matter in anaerobic environments while reducing sulfate to sulfide (aqueous HS^- or gaseous H_2S). The source of energy can be an inorganic combination of hydrogen

(H_2) and carbon dioxide (CO_2) (Davidova and Stams 1996; Liamleam and Annachhatre 2007), simple organic compounds, such as ethanol (Sahinkaya et al. 2011; Rodriguez et al. 2012) or lactate (Kaksonen et al. 2003b; Zhao et al. 2010), or more complex waste products, such as cellulosic plant material or livestock manure (Gibert et al. 2004; Choudhary and Sheoran 2011; Zhang and Wang 2014). The produced sulfide can be precipitated with metals, and recover the additional metals from the mining effluent (Boonstra et al. 1999), or oxidized to elemental sulfur (S^0), which can then be used in chemical industry, for example in the production of fertilizers (Lens 2009).

This work investigates the applicability of biological sulfate removal on waste water from a Finnish mining site. Another waste stream, cow manure, was utilised as the main carbon source and electron donor in upflow anaerobic sludge blanket (UASB) reactors operated in continuous mode. Three different microbial enrichments containing sulfate reducers were used as additional inocula in separate reactors. The aim was to acquire both efficient and reliable treatment of mining effluent. What separates this work from most other studies related to biological sulfate reduction, is that there were practically no metal ions present in the effluent, so no metal precipitates were formed with the sulfide. This in turn enabled the recovery of elemental sulfur after the reduction of sulfate, thus creating a side stream with possible economic value. Sulfide oxidation to elemental sulfur was studied in batch experiments. The transformations of different sulfur species in the processes of this work are compiled in Figure 1.1.

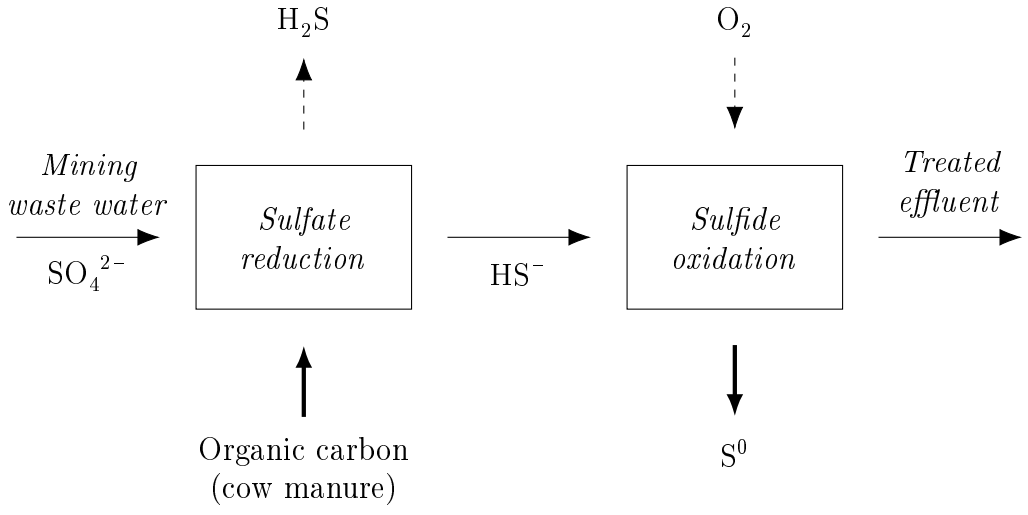

Figure 1.1 *The chain of sulfur reactions related to this thesis. Aqueous fractions are illustrated with thin arrows, gaseous compounds with dashed arrows and solid fractions with thick arrows.*

The changes in microbial communities inside the sulfate-reducing reactors were also monitored extensively throughout the experiment. A quantitative analysis of the

sulfate-reducing bacteria as well as a qualitative analysis of the whole bacterial domain were conducted on samples taken from reactor effluents. Based on these results, the interactions between different bacterial groups were studied and compared with reactor operation. A similar, long-term bacterial community analysis was not found from the available literature.

2. MINING WASTE WATERS

2.1 Formation of acid mine drainage

Sulfur compounds are generally present in bedrock in stable forms, commonly as metal sulfides. If left undisturbed in the earth, they remain inert. However, when the bedrock is exposed to air and water, for example during mining activities, sulfide minerals can undergo oxidation and various compounds can dissolve into the aqueous phase. (Toropainen 2006; Madigan et al. 2015)

One typical sulfide mineral in mining environments is pyrite (FeS_2). When this compound comes in contact with air and water, the following reactions are known to occur:

$$2\,FeS_2 + 7\,O_2 + 2\,H_2O \longrightarrow 2\,Fe^{2+} + 4\,{SO_4}^{2-} + 4\,H^+ \tag{2.1}$$

$$2\,Fe^{2+} + 0.5\,O_2 + 2\,H^+ \longrightarrow 2\,Fe^{3+} + 2\,H_2O \tag{2.2}$$

$$2\,FeS_2 + 14\,Fe^{3+} + 8\,H_2O \longrightarrow 15\,Fe^{2+} + 2\,{SO_4}^{2-} + 16\,H^+ \tag{2.3}$$

The first reaction (Equation 2.1) (Sawyer et al. 2003) produces soluble metal ions, sulfate and acidity. Although this reaction is relatively slow when occurring abiotically (Mitchell 2000), the biological effect becomes a major part of the process in the next step, when ferrous iron (Fe^{2+}) is transformed to ferric iron (Fe^{3+}) by iron oxidizing microorganisms, for example *Acidithiobacillus ferrooxidans* (Equation 2.2) (Dold 2010; Madigan et al. 2015). The formed Fe^{3+} acts as a stronger oxidant for pyrite than oxygen, and sulfate and acidity are formed at an accelerated pace (Equation 2.3) (Madigan et al. 2015). These reactions catalysed by microorganisms form a vicious circle that can generate highly acidic, sulfate-rich and heavy metal containing acid mine drainage (AMD). (Lens 2009; Madigan et al. 2015)

During mining activities AMD can originate from several sources besides the mine pit, such as waste rock piles, tailing ponds and ore stock deposits (Salomons 1995; Toropainen 2006). An important physical factor that affects the generation of acidic

waters is the permeability of the rock piles, as coarse particles allow oxygen diffusion and water penetration deeper into the pile, whereas finely ground material is more prone to be exposed to oxidation only from the surface. Several chemical and biological factors also have a major influence on the process, including pH, temperature, exposed surface area of the material, microbial populations and their growth rate as well as availability of nutrients. (Salomons 1995) One way to minimize the generation of AMD is to position the piles at the mining site in a way that the oxidation of the material could be avoided and possible effluents are prevented to enter the water systems (Toropainen 2006). The formation of AMD can occur within one year from the start of mining operations or only after several years (Salomons 1995). Thus, the generation of AMD is important to take into account when a mine is closed and it fills with water, as the pollution capacity may continue for years (Mitchell 2000; Johnson and Hallberg 2005).

Even though the pH of a typical AMD can be low (usually in the range of $1.5 - 4.0$ (Dold 2010)), some mining effluents may be close to neutral or even basic, depending on the dissolved minerals and the biological activity in the surroundings (Johnson and Hallberg 2005). For example carbonate minerals in soil are essential in raising the pH of AMD. A common carbonate compound calcite ($CaCO_3$) creates alkalinity (Equation 2.4) and lowers the acidity of mine waters. (Dold 2010)

$$CaCO_3 + H^+ \longrightarrow Ca^{2+} + HCO_3^- \tag{2.4}$$

Although the reactions related to the formation of AMD can be detrimental when occurring in the environment in an uncontrolled way, in biohydrometallurgy the oxidation ability of microorganisms can be used beneficially to extract minerals from ores for industrial purposes. This is a controlled and efficient way of utilising for example ores with a low content of valuable metals. (Lens 2009)

2.2 Environmental effects of sulfate and permitted limits

While flowing in the natural water systems and transporting different soluble compounds, AMD can alter the balance of ecosystems. The oxidized Fe^{3+} and acidity together in AMD are effective in dissolving other metal sulfides from rock, and thus increasing the heavy metal and sulfate load in the surroundings (Schippers and Sand 1999). The precipitation of iron hydroxides ($Fe_x(OH)_y$), when Fe^{3+} reacts with water, will also increase the acidity in the receiving waters (Dold 2010). Even though sulfate has been considered to be less harmful than the Fe^{3+} content and acidity of AMD (Kauppi et al. 2013), and it is the most stable sulfur compound in aerobic surroundings (Lens 2009), sulfate can have various effects on natural environments

(Table 2.1).

Table 2.1 *The main environmental impacts caused by sulfate in natural water systems, divided into chemical, physical and biological impacts.*

Chemical Ref. [1-2]	**Physical** Ref. [3-5]	**Biological** Ref. [3-6]
Acidity production	Water layering	Toxic to aquatic life
Increased metal solubility	Oxygen depletion	Eutrophication
Generation of metastable products		Brackish water systems

References: [1]=Dold (2010), [2]=Cravotta (2006), [3]=Blomqvist et al. (2004), [4]=Roden and Edmons (1997), [5]=Kauppi et al. (2013), [6]=Soucek and Kennedy (2005)

When soluble iron reacts with sulfate and water, metastable products such as jarosite ($KFe_3(SO_4)_2(OH)_6$) and schwertmannite ($Fe_8O_8(OH)_6SO_4$, one of several forms) are generated, and simultaneusly hydrogen ions are produced (Equation 2.5) (Regenspurg et al. 2004; Dold 2010). When these compounds transform to others, such as goethite (FeO(OH)), sulfate is liberated again and more acidity is produced (Equation 2.6) (Dold 2010).

$$8\,Fe_3{}^{+} + SO_4{}^{2-} + 14\,H_2O \longrightarrow Fe_8O_8(OH)_6SO_4 + 22\,H^{+} \tag{2.5}$$

$$Fe_8O_8(OH)_6SO_4 + 2\,H_2O \longrightarrow 8\,FeO(OH) + SO_4{}^{2-} + 2\,H^{+} \tag{2.6}$$

The pH of AMD is the determining factor in these transformations. Bigham et al. (1996) studied this relationship, and discovered that jarosite is present only in rather low pH area ($1 - 2.5$) before it dissolves. Schwertmannite precipitates at pH 3.0 and gives the water stream a typical yellowish orange colour. This compound is stable until the pH increases to 5.0, after which it dissolves to form other compounds, such as ferrihydrite ($5\,Fe_2O_3 \cdot 9\,H_2O$) and goethite. It should be noted that these pH ranges are not exact, as high concentrations of iron and sulfate can affect the stability of these compounds. (Bigham et al. 1996)

The increased dissolution of metals can also be induced by high sulfate concentration. For example in the case of aluminium, at low pH (less than 5.0) the formation of aluminium sulfates ($AlSO_4{}^{+}$ and $AlHSO_4{}^{+}$) increase the amount of soluble aluminium (Nordstrom 2004; Cravotta 2006). As the pH increases or dilution causes the sulfate concentration to decrease, aluminium is more prone to precipite as hydroxide mineral ($Al(OH)_3$). Similar enhancing effect of metal-sulfate complexes on dissolution has been found with zinc (Webster et al. 1998) and ferric iron, but with ferrous iron and manganese the sulfate concentration did not have any effect, as

the equilibriums are controlled by formation of carbonates ($FeCO_3$ and $MnCO_3$) at higher pH values (above 6.0) (Cravotta 2006). Barium dissolves less with increasing sulfate concentration, as the insoluble barite ($BaSO_4$) is formed at low pH. The concentration of soluble lead also correlates inversely with sulfate concentration, and possibly precipitates together with barite. Based on this, sulfate can also prevent metal dissolution and decrease the mobility of harmful substances. (Cravotta 2006)

Sulfate can also have toxic impacts on living creatures. The lethal concentration of sulfate in which 50% of the tests subjects die in a specific time period (LC50), has been studied for different freshwater organisms, such as crustaceans and shellfish (Soucek and Kennedy 2005). The LC50 values obtained by Soucek and Kennedy (2005) values varied between $512 - 14000$ mg/l of sulfate depending on the species. Increasing the amount of hardness (Ca^{2+} and Mg^{2+}) and chloride in the water increased the LC50 values, as these ions protected the organisms from the osmoregulatory stress caused by the sulfate ion. The same phenomenon of protective hardness was noted with aquatic moss (Davies 2007). The combined influence of all ions present in the water should be taken into account when examining the effect of high sulfate concentration on the local aquatic life.

When sulfate containing water flow meets fresh water, it can cause layering, as water with high sulfate concentration will settle at the bottom. This can effectively prevent the natural mixing of water and cause oxygen depletion, in addition to changing the ecosystem from a fresh water into a brackish water environment. Sulfate can also cause eutrophication, as it transforms and reacts with iron in anaerobic sediments, and the phosphorus normally bound by iron is released. (Roden and Edmons 1997; Lamers et al. 2002; Blomqvist et al. 2004; Kauppi et al. 2013; Lehtoranta and Ekholm 2013)

For a long time the effects of sulfate were not considered important, and since not much research had been conducted in northern countries, there were no limits for sulfate in mining effluents in Finland (Kauppi et al. 2013). Authorities do provide recommendations for sulfate concentration in drinking water. Although sulfate has no acute toxic effects for humans, its excessive consumption may have cathartic impacts (Sawyer et al. 2003). However, already lesser concentrations of sulfate are known to cause corrosion in pipes. In Finland, the limit for sulfate in drinking water is 250 mg/l, although concentrations below 150 mg/l are recommended to prevent corrosion (Finlex 2000). However, the environmental accident in Talvivaara mine in 2012 was probably the trigger to improve the monitoring of effluents as well as tightening the limits of different pollutants, including sulfate (Kauppi et al. 2013). For example, the new environmental permits for the Finnish mines Suurikuusikko (Aluehallintovirasto 2013) and Kevitsa (Aluehallintovirasto 2014) dictate that the new limit for sulfate in effluents is 2000 mg/l, but a value of 1000 mg/l is to be aimed

for. This more stringent level of sulfate removal to 1000 mg/l cannot be achieved with conventional methods (e.g. lime treatment) (Boonstra et al. 1999), so there is an urgent need for new processes. Other countries in the European Union share the same recommended limit of 1000 mg/l for sulfate discharge (Reinsel 2015).

3. REMOVAL OF SULFUR COMPOUNDS FROM WASTE WATERS

3.1 The conventional lime treatment and upcoming abiotic processes

A common way to treat mine waters characterized by acidity and high concentration of both heavy metals and sulfate is to use different forms of lime, for example $Ca(OH)_2$ (hydrated or slaked lime). This alkalinic compound neutralizes the solution and precipitates the metals as hydroxides and sulfate as gypsum ($CaSO_4 \cdot 2\,H_2O$) (Equation 3.1). (Boonstra et al. 1999; Geldenhuys et al. 2003)

$$2\,Ca(OH)_2 + Fe^{2+} + 2\,{SO_4}^{2-} + 2\,H^+ + 2\,H_2O \longrightarrow Fe(OH)_2 + 2\,CaSO_4 \cdot 2\,H_2O \quad (3.1)$$

According to Boonstra et al. (1999), metals in mining waste waters can be decreased to 0.5 mg/l and sulfate to 1500 mg/l. The metal hydroxides mix with the gypsum sludge, so the metals cannot be recovered separately, and today practically the only option for the sludge mix is to dispose it in a landfill. As high amount of sludge is generated with this process, the disposing costs can be high. (Boonstra et al. 1999)

Many new chemical and physical processes that can treat sulfate-rich waters are under research and development. These include different technologies based on, for example, membrane filtration, chemical precipitation and ion-exchange. Membrane technologies include reverse osmosis (creating concentrates utilizing a semi-permeable barrier), electrodialysis (use of electric current to enhance the separation of cations and anions through membranes) and filtration. Chemical precipitation can be salt precipitation either as barite or ettringite ($Ca_6Al_2(SO_4)_3(OH)_{12} \cdot 26\,H_2O$). In ion-exchange the sulfate ions are immobilized to a material surface. Depending on the location and waste water characteristics, all of these methods could be used for sulfate removal from mine waters. (For a review, see Bowell 2004)

3.2 Biological sulfate reduction

Microorganisms are an important part of the natural sulfur cycle, and they can be used beneficially in treating mining waste waters containing sulfur compounds. In anaerobic conditions, sulfate-reducing microorganisms use sulfate to oxidize organic compounds (or hydrogen) and consequently reduce sulfate to sulfide (Equation 3.2). (Sawyer et al. 2003; Lens 2009)

$$SO_4^{2-} + organic\ matter \longrightarrow HS^- + OH^- + CO_2 \tag{3.2}$$

The acidity of the waste stream is neutralized in the reactions, and if there are metals present, these will react with sulfide and precipitate as metal sulfides (for example FeS_2, NiS, ZnS). As metals are the only compounds precipitating, there is a possibility to recover metals from the sulfide sludge. Compared to lime treatment, the amount of sulfide sludge generated is smaller, metal removal is more efficient and sulfate and metal concentrations in the treated effluent can be lower with biological sulfate reduction (Table 3.1) (Boonstra et al. 1999). It should be noted that waters with lower sulfate content than 1500 mg/l cannot even be treated with lime, as the dissolution and precipitation of gypsum are at equilibrium below this value (Geldenhuys et al. 2003). Generally biological sulfate reduction can produce effluents with only $100 - 200$ mg/l of sulfate and a total reduction of $85 - 95\%$ in continuous systems treating (real or synthetic) mine waters (Kaksonen et al. 2003b; Oyekola et al. 2010; Sahinkaya et al. 2011; Rodriguez et al. 2012), but near 100% sulfate removal has also been reported (Sarti et al. 2010). The sulfate load (described as $mgSO_4^{2-}$/l*d) to the reactor and the initial sulfate concentration of the feed are important factors when comparing different studies. However, unlike with lime treatment, there is no chemical saturation limit with biological sulfate reduction, and low sulfate concentrations can be achieved.

Table 3.1 *Comparison of mine water treatment with lime precipitation and biological sulfate reduction.*

	Sulfate removal	Sludge generation	Metal recovery	References
Lime precipitation	Not below 1500 mg/l	High	Difficult	[1,2]
Biological reduction	Near 100% removal possible	Low	Possible	[2-5]

References: [1]=Geldenhuys et al. (2003), [2]=Boonstra et al. (1999), [3]=Kaksonen et al. (2003b), [4]=Rodriguez et al. (2012), [5]=Sarti et al. (2010)

The benefit of metal sulfide precipitation in biological sulfate reduction compared to hydroxide precipitation with lime, is that metal sulfides have lower solubility and a wider pH range of stability than their metal hydroxide counterparts (Figure 3.1). Most metal hydroxides are only stable at pH values above 9.0, so even a small increase in acidity causes the precipitates to dissolve again, whereas for example ZnS is in solid form in a wider pH range of approximately $5.8 - 11.0$.

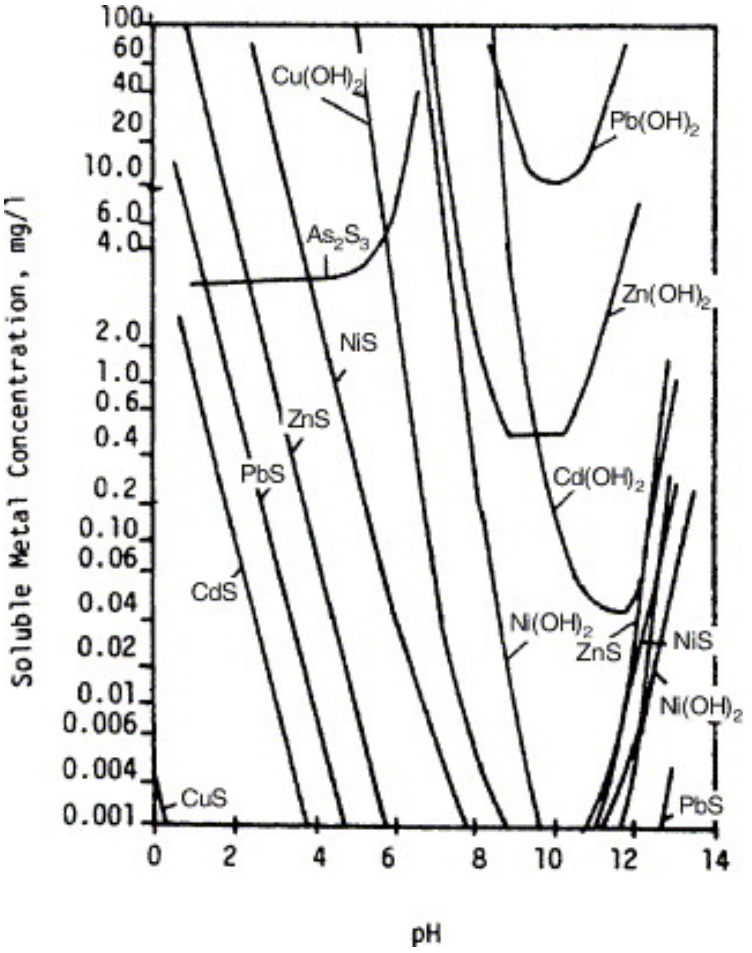

Figure 3.1 *The solubilities of metal sulfides and metal hydroxides at different pH values (Huisman et al. 2006). Low soluble metal concentration indicates a more stable precipitate.*

If a limited concentration or no metals are present, most of the sulfide remains free in the solution. Sulfide can exist in three forms depending on the pH of the solution (Figure 3.2). At a pH below 7.0, sulfide is mainly present in its undissociated form H_2S, which easily becomes gasified from the solution. Gaseous H_2S is toxic and has the odor of bad eggs. (Sawyer et al. 2003) A short exposure (less than 15 minutes) to 10 ppm (14 mg/m^3) of H_2S is considered dangerous, and is the upper limit at workplaces in Finland (Sosiaali- ja terveysministeriö 2014). The other forms of sulfide, HS^- and S^{2-}, are nonvolatile compounds (Madigan et al. 2015), and dominate when pH is above neutral (Figure 3.2).

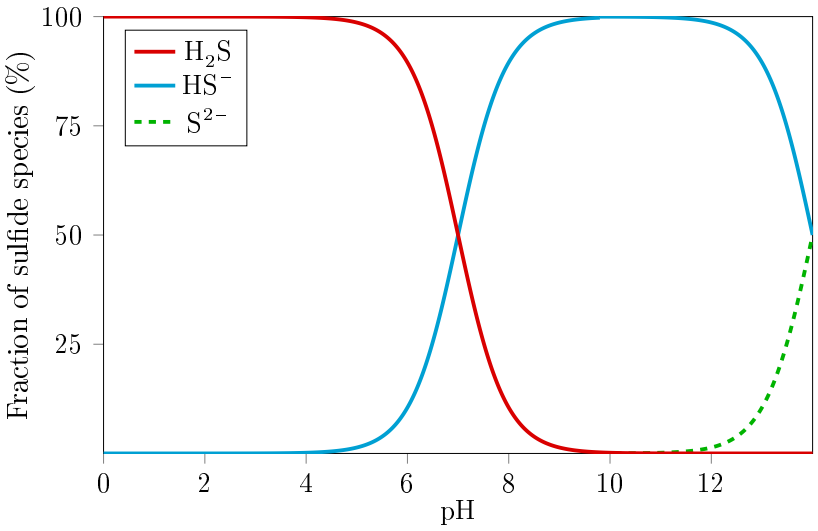

Figure 3.2 *The presence of sulfide species in an aqueous solution at different pH values at 30°C (modified from Moosa and Harrison 2006).*

The temperature has an effect on the sulfide speciation. As temperature decreases, the relative amount of H_2S grows compared to HS^- and vice versa. At 30 °C and pH 7.0, the molar ratio of H_2S/HS^- is approximately 50/50 (Figure 3.2), while for example at 10 °C the molar ratio has changed to 65/35. (Nevatalo 2010).

3.3 Sulfur recovery

One way to remove the sulfide produced from the reduction of sulfate is to chemically oxidize it to elemental sulfur (Equation 3.3) (Chen and Morris 1972).

$$2\,HS^- + O_2 \longrightarrow 2\,S + 2\,OH^- \tag{3.3}$$

The elemental sulfur remains in the solution as an inert and insoluble compound (Madigan et al. 2015). Sulfide oxidation to elemental sulfur is simple (only air needed as the oxidant), no unwanted chemical sludge is produced, the oxidation has a low energy need and the utilization of elemental sulfur is possible (Buisman et al. 1991). However, if excess oxygen is available, the oxidation can continue further and sulfide can be transformed back to sulfate (Equation 3.4) (Chen and Morris 1972).

$$2\,HS^- + 4\,O_2 \longrightarrow 2\,SO_4{}^{2-} + 2\,H^+ \tag{3.4}$$

This reaction is unwanted because of sulfate and acid production. If the HS^-/O_2 ratio in the solution is above the stoichiometrical need of Equation 3.3, elemental sulfur is the dominating product, and with a lower ratio the production of sulfate

increases (Janssen et al. 1998). However, it has been postulated that the oxidation of sulfide to sulfate is much slower than that of sulfide to elemental sulfur (Buisman et al. 1991; Janssen et al. 1998). Moreover, it is possible that the oxidation route of sulfide to sulfate always includes the formation of elemental sulfur as an intermediate. The transformations between different sulfur species are therefore dictated by reaction kinetics rather than equilibrium thermodynamics. (Lewis et al. 2000)

At pH $7.0 - 8.0$, the oxidation of sulfide is fast and occurs spontaneously with oxygen dissolved in the solution (Madigan et al. 2015). However, there are microbial genera such as *Acidithiobacillus* (Lens 2009; Madigan et al. 2015) capable of catalyzing these reactions. Microbial conversion is only notable compared to chemical oxidation if oxygen is not evenly mixed in the liquid, and the microorganisms are able to work in the borderline of the aerobic and anoxic phases (Madigan et al. 2015).

When the target is elemental sulfur production, it is important that the amount of dissolved oxygen (DO) is low, approximately 0.1 mg/l (Vannini et al. 2008). This can create challenges in reactor configuration, as the aeration has to be controlled in order to achieve the desired oxygen concentration. A pH below neutral is also unfavourable as it makes sulfide appear in H_2S form, which escapes from the solution and less sulfur can be generated (Figure 3.2). The sulfide content in the water should be as high as possible to secure a high HS^-/O_2 ratio and to increase the product yield. If biological means are used, there should be minimal amount of organic compounds present, so that the chemolithoautotrophic sulfide oxidizing microorganisms can prosper and sulfur production is at maximum. (Vannini et al. 2008)

The main use for elemental sulfur is the production of sulfuric acid (H_2SO_4), which can then be used in various applications including the manufacture of industrial chemicals, pharmaceuticals, cosmetics and pigments, to name a few. Elemental sulfur and its derivatives are increasingly important in fertilizer industry, as in many parts of the world the soil is deprived of sulfur compounds, both because of intensive farming and the decrease of sulfur emissions (for example SO_2) to the atmosphere, as well as the exclusion of sulfur from many commonly used fertilizers. Sulfur compounds together with other nutrients are essential in increasing the yield and quality of farmed crops. (Scherer 2001; The Sulphur Institute 2016)

3.4 Biological sulfate removing technologies

Depending on the location, the desired treatment efficiency, the available funds and the possible economic value of the end-products, several possibilities exist for biological sulfate reduction in the treatment of mining waste waters. Passive options, for example reactive barriers and constructed wetlands or ponds, are suitable for

ground or surface water when minimal labour is required. Even though a passive system is a low-cost option, the time needed for treatment can be long and the area large. Active options are a compact and efficient way of handling waste streams and provide good control and predictability. However, these processes are more labour-intensive and have higher operational costs. (For a review, see Kaksonen and Puhakka 2007)

Active systems may vary tremendeously in terms of flow direction, unit numbers and process complexity (Figure 3.3). In a continuously stirred tank reactor (CSTR) (Figure 3.3 (a)) the thorough mixing of biomass and substrate is ensured with an external stirrer (Oyekola et al. 2010), though the system requires high energy input as well as an additional unit for the settling and recovering of biomass (Bijmans et al. 2011). An UASB reactor (Figure 3.3 (b)) is built so that the waste water flows upwards in a reactor through a sludge blanket where the sulfate reduction occurs. This system can hold plenty of sludge without any inert supporting material. (Rodriguez et al. 2012) The direction of the feed flow can also be downwards instead of upwards (Zhang and Wang 2014). This system requires good granulation properties from the sludge (Bijmans et al. 2011). A fluidized bed reactor (FBR) (Figure 3.3 (c)) utilizes the recycling of liquid inside the reactor to make the biomass carrier float. This allows an efficient contact between substrate and biomass as well as prevents clogging of the reactor, if a suitable carrier material is found (Kaksonen et al. 2003a).

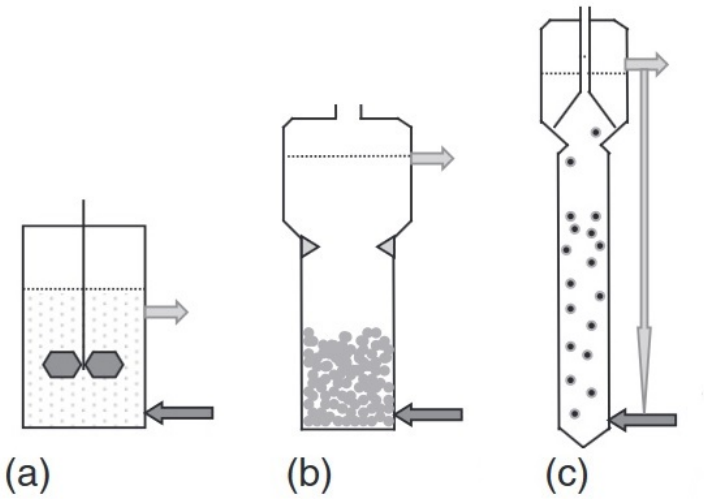

Figure 3.3 *Example reactor types used in biological sulfate reduction: (a) continuously stirred tank reactor (CSTR), (b) upflow anaerobic sludge blanket (UASB) reactor with stationary biomass, and (c) fluidized bed reactor (FBR), where biomass is bound to a carrier material and the sludge is fluidized with recycle flow (modified from Bijmans et al. 2011).*

One commercially used process in biological sulfate removal from mining waste wa-

ters is Sulfateq™ by Paques (Paques Ltd 2016). It consists of an anaerobic reactor for sulfate reduction and metal precipitation, followed by an aerobic bioprocess for sulfur recovery (Figure 3.4).

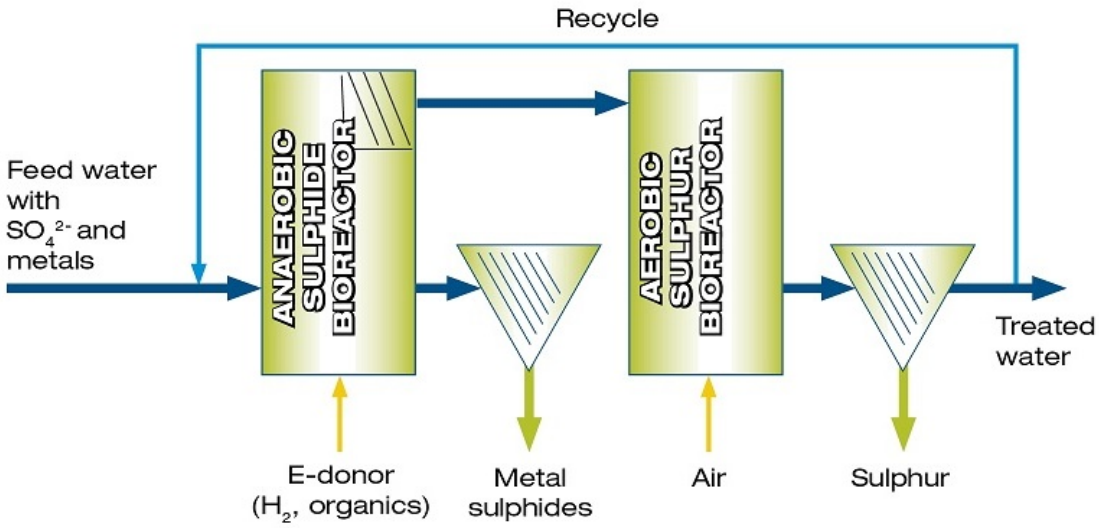

Figure 3.4 *Configuration of Sulfateq™ process (Paques Ltd 2016).*

The substrate used is either hydrogen or organic compounds. The process can treat waste waters with sulfate concentrations of $1000 - 25000$ mg/l and remove sulfate to less than 300 mg/l. Sulfateq™ is used for example in Nyrstar zinc refinery in the Netherlands (Table 3.2), where currently no solid waste is being produced as all generated zinc compounds and elemental sulfur are recycled in the process. (Paques Ltd 2016)

Table 3.2 *Sulfate and metal removal from Nyrstar waste water with Sulfateq™ process (adapted from Boonstra et al. 1999).*

	Influent (mg/l)	Effluent (mg/l)
Sulfate	1000	< 200
Zinc	100	< 0.05
Cadmium	1	< 0.001

4. SULFATE-REDUCING MICROORGANISMS

4.1 General characteristics

Because of abundant supplies of sulfur compounds in the early times of Earth (approximately $3.3 - 3.7$ billion years ago) and simple yet exergonic reactions involved, sulfur chemistry may be one of the oldest in microbial metabolism, and thus the utilisation of sulfur compounds is very diverse. As a macronutrient, sulfur is needed for certain amino acids and vitamins, but elemental sulfur can also serve as a long-term energy storage inside microbial cells. For example some sulfide-oxidizing microorganisms rely on this energy storage once the sulfide in the surroundings has been exhausted, and oxidize sulfur to sulfate to generate energy for growth. Sulfate is the most oxidized species of sulfur, and it is used as terminal electron acceptor by specialized obligately anaerobic microorganisms, which reduce it to sulfide. Sulfate reducers are plentiful for example in marine sediments, which often release the odour of H_2S resembling bad eggs. (Madigan et al. 2015)

Even though sulfate-reducing microorganisms are strictly anaerobic organisms, some can still function in the presence of oxygen and they can be isolated from sources that are temporarily exposed to air (Barton 1995; Lens 2009; Madigan et al. 2015). Some of the various sulfate-reducing microbial genera discovered in anaerobic reactors and mine sites include *Desulfovibrio*, *Desulfobulbus* and *Desulfotomaculum* (Figure 4.1) (Kaksonen et al. 2004a,b; Lens 2009).

Sulfate-reducing microorganisms can be divided into complete and incomplete oxidizers according to their utilisation of substrate. Complete oxidizers are able to transform organic carbon sources to CO_2 and incomplete oxidizers can produce only acetate (CH_3COO^-) from organic compounds (Madigan et al. 2015). The bacterial genera mentioned earlier, *Desulfovibrio*, *Desulfobulbus* and *Desulfotomaculum*, are all incomplete oxidizers (Figure 4.1). (Madigan et al. 2015)

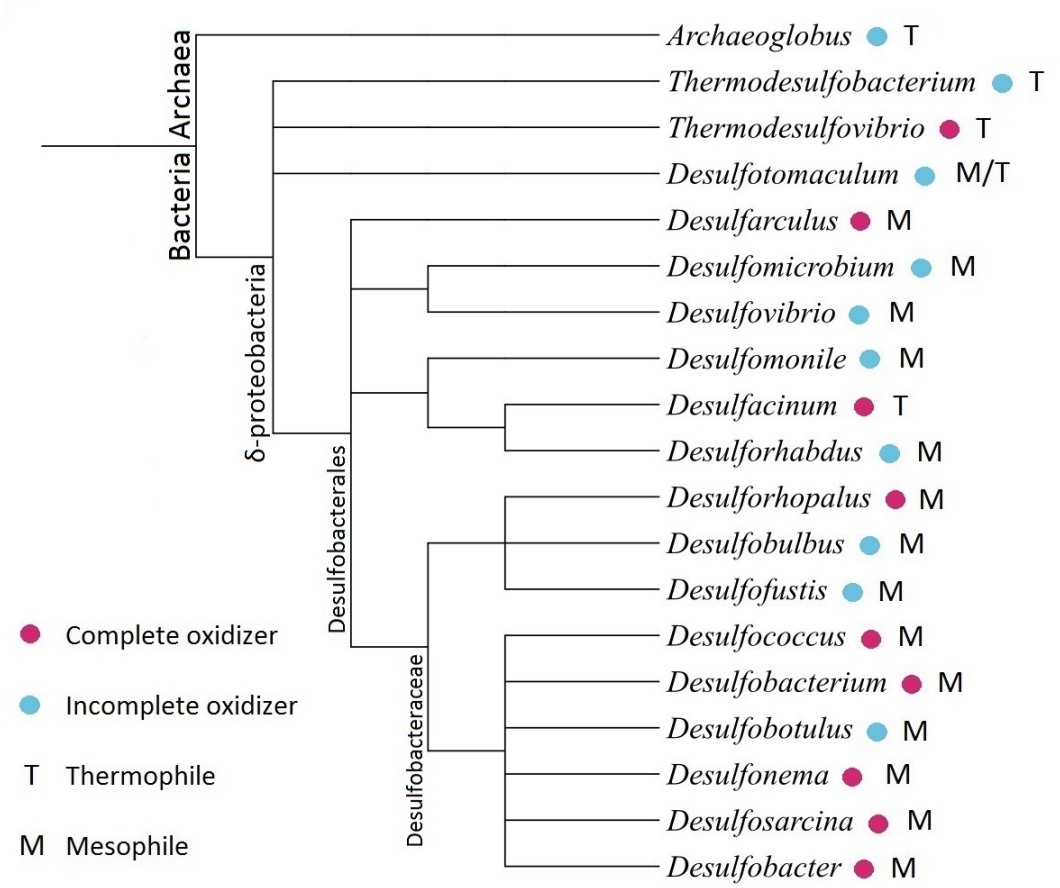

Figure 4.1 *Phylogenetic tree including some known sulfate-reducing genera. With incomplete oxidizers, the substrate oxidation stops at acetate, whereas complete oxidizers can transform acetate further to CO_2. Thermophilic sulfate reducers thrive in environments where temperature is above 40 °C, and mesophilic genera prefer moderate temperatures of 20 – 40 °C. Information on the genera was collected from Madigan et al. (2015) and Castro et al. (2000). The tree was generated with phyloT online tool based on taxonomy provided by National Center for Biotechnology Information (NCBI) (Biobyte Solutions GmbH 2016).*

4.2 Living requirements

4.2.1 Temperature and pH

Each sulfate-reducing microorganism has an optimum temperature where sulfate reduction and cell growth are at their maximum. Usually the performance is improved as the temperature increases up to 35 °C, after which the activity decreases and ultimately stops. Therefore most sulfate-reducing reactors are operated in mesophilic conditions (20 – 45 °C). (Bijmans et al. 2011) However, besides a few thermophilic sulfate reducers commonly found from thermal springs (Madigan et al. 2015), some psychrophilic (cold environment thriving) species have been isolated by Knoblauch and Jørgensen (1999) from arctic marine sediments, where temperature is constantly

below zero. The optimal temperatures for the growth of these psychrophilic species in laboratory conditions varied from 7 to 18 °C, though the highest sulfate reduction rates were reached at $2-9$ °C above the optimal growth temperatures. The highest sulfate reduction rates were $10-50\%$ greater than the rates at optimal growth temperatures. When operating a reactor at low temperatures (below 8 °C), the system requires external alkalinity, as acetate oxidation is slow (Sahinkaya et al. 2007).

Usually pH values near neutral are optimal for sulfate-reducers, but exceptions do exist. Waste water streams are often either below or above neutral, so the process should be adjustable to these situations as well. (Bijmans et al. 2011) A pH of $7.0-8.0$ is often considered the best for sulfate reduction. One reason for this may be because when the pH is above neutral, the HS^- concentration is higher than the concentration of more toxic H_2S (Figure 3.2). (Moosa and Harrison 2006)

4.2.2 Redox potential

Redox potential is the state of oxidation-reduction balance in a certain environment; more specifically it describes the availability of electrons. In oxidation, electrons are removed from a compound, and the oxidation number of the compound increases. In reduction the compound receives electrons and its oxidation number deceases. Redox potential describes the possibility of these reactions happening, and can be used to estimate the stability of a certain compound. However, specific interpretations of the balance of a known redox pair can only be done in pure systems, not in mixed environments as nature or real waste waters. The potential is a mix of all couples present weighed with the respective concentrations. Temperature and pH of the surroundings can also affect the redox potential. (Delaune and Reddy 2005)

In an anaerobic environment the redox potential is negative, as there are plenty of electron donors but electron acceptors are scarce. In an aerobic environment the redox potential is positive, as oxygen, a strong oxidizer, is present but the system lacks electron donors. The reduction of all compounds present in a certain environment proceeds in a specific order (Table 4.1). For example nitrate (NO_3^-) and manganese (Mn^{4+}) are reduced first in reducing surroundings and only after a larger decrease in the redox potential can sulfate be reduced. In sulfate reduction, much of the oxidation energy obtained is used in moving the electrons to sulfate, thus less energy is obtained for the microorganisms' own needs. This is why sulfate reducers prosper in relatively reducive environments, where sulfate is unstable and accepts electrons more easily. Sulfate acts as the electron acceptor when redox potential is -100 – -200 mV. At this stage sulfate becomes unstable and can be transformed to sulfide (Table 4.1). (Delaune and Reddy 2005) According to Barton (1995), sulfate reducers require a negative redox potential of at least -150 mV for

sulfate reduction to occur.

Table 4.1 *Required redox potentials for common reactions to occur in soils and sediments at pH 7.0 (adapted from Delaune and Reddy 2005).*

Redox couple	Critical redox potential (mV)
$O_2 + 4\,e^- + 4\,H^+ \longrightarrow 2\,H_2O$	$\leq +400$
$2\,NO_3^- + 10\,e^- + 12\,H^+ \longrightarrow N_2 + 6\,H_2O$	$\leq +300$
$MnO_2 + 2\,e^- + 4\,H^+ \longrightarrow Mn^{2+} + 2\,H_2O$	$\leq +250$
$Fe(OH)_3 + e^- + 3\,H^+ \longrightarrow Fe^{2+} + 3\,H_2O$	$\leq +100$
$SO_4^{2-} + 8\,e^- + 8\,H^+ \longrightarrow S^{2-} + 4H_2O$	≤ -100
$CO_2 + 8\,e^- + 8\,H^+ \longrightarrow CH_4 + 2\,H_2O$	≤ -200

4.2.3 Carbon and electron sources

As mentioned in Section 3.2, a carbon source/electron donor is required for biological sulfate reduction, and this is usually an organic compound (with the exception of using the combination of hydrogen and CO_2). As mining waste waters typically contain only little or no organic matter, an external carbon source is needed (Kolmert and Johnson 2001), and some possible substrates are presented in Table 4.2. The ratio of added substrate to sulfate is important. If the amount of substrate is lower than what would be stoichiometrically required, sulfate reduction is decreased. In the case of excess substrate, methanogenic microorganisms can begin a competition for substrate and dominance with sulfate reducers. When describing the organic content of a substrate with chemical oxygen demand (COD), the optimal mass ratio of COD to sulfate is 0.67, when all COD is used for sulfate reduction. (Lens et al. 1998) In addition to a carbon source, some additional nutrients should be available for sulfate reducers. These include nitrogen and phosphorus, which are important compounds for example in nucleic acids and other cell components (Madigan et al. 2015). According to Gerhardt (1981), an optimal ratio for carbon, phosphorus and nitrogen (C:N:P) is 110:7:1. Small amounts of metals, such as nickel and iron, are also needed as cofactors for enzymes (Barton 1995; Madigan et al. 2015).

Table 4.2 *Some examples of substrates used in biological sulfate reduction.*

	Chemistry	Advantages	Disadvantages	References
Hydrogen	$4\,H_2 + {SO_4}^2 + H^+ \longrightarrow HS^- + 4\,H_2O$	+ Wide suitability + Does not dilute reactor liquid	− Separate carbon source needed − High capital input	[1-3]
Lactate	$2\,CH_3CHOHCOO^- + {SO_4}^{2-} \longrightarrow 2\,CH_3COO^- + 2\,{HCO_3}^- + H^+ + HS^-$	+ Wide suitability + Supports biomass growth well + Great alkalinity production	− Expensive in large scale use	[4-6]
Ethanol	$2\,C_2H_5OH + {SO_4}^{2-} \longrightarrow 2\,CH_3COO^- + H^+ + HS^- + 2\,H_2O$	+ Low-cost + Safe to use	− Limited alkalinity production − Risk of acetate accumulation	[2,4,5,7]
Cellulosic waste and manure	${SO_4}^{2-} + \textit{organic matter} \longrightarrow HS^- + OH^- + CO_2$	+ Low-cost + Sustainable + Contains nutrients + Can function as inoculum	− Availability and quality may vary − Contains non-degradable matter	[3,8,9]

References: [1]=Liamleam and Annachhatre (2007), [2]=Boonstra et al. (1999), [3]=Bijmans et al. (2011), [4]=Nagpal et al. (2000a), [5]=Kaksonen et al. (2004a), [6]=Zhao et al. (2010), [7]=Davidova and Stams (1996), [8]=Gibert et al. (2004), [9]=Choudhary and Sheoran (2011)

Hydrogen is a widely used, high-energy substrate for biological sulfate reduction (Equation 4.1) (Liamleam and Annachhatre 2007).

$$4\,H_2 + {SO_4}^2 + H^+ \longrightarrow HS^- + 4\,H_2O \tag{4.1}$$

Sulfate reducers are considered to consume hydrogen more efficiently than methanogens, so it may be advantageous to use hydrogen as an electron donor instead of organic matter (Davidova and Stams 1996). In addition, hydrogen gas fed into the reactor does not dilute the waste water inside and the substrate not removed from the reactor with the effluent (Bijmans et al. 2011). Still, a carbon source, for example CO_2, is needed for the growth of sulfate reducers (Liamleam and Annachhatre 2007; Boonstra et al. 1999). However, using CO_2 can lower the pH of the reactor to undesired levels, so careful pH monitoring is required (Liamleam and Annachhatre 2007). The production and handling of hydrogen gas may increase the capital costs of sulfate reduction compared to the use of liquid substrates, so hydrogen is most economic when treating waste waters with high sulfate loads in large scale applications (Boonstra et al. 1999; Bijmans et al. 2011).

Lactate is a good source of energy for sulfate reducers and improves biomass growth more than many other substrates (for example hydrogen) (Nagpal et al. 2000a), and has been shown to enable an efficient sulfate reduction from the very beginning of the reactor start-up (Kaksonen et al. 2004a; Zhao et al. 2010). Lactate oxidation generates a lot of alkalinity, and is thus good in neutralizing acidic waste waters (Equations 4.2 and 4.3) (Nagpal et al. 2000a; Kaksonen et al. 2004a).

$$2\,CH_3CHOHCOO^- + {SO_4}^{2-} \longrightarrow 2\,CH_3COO^- + 2\,{HCO_3}^- + H^+ + HS^- \tag{4.2}$$

$$CH_3COO^- + {SO_4}^{2-} \longrightarrow 2\,{HCO_3}^- + HS^- \tag{4.3}$$

However, lactate is such an expensive substrate, that in large-scale processes it is feasible only in the beginning when the target is to generate plenty of biomass for efficient sulfate reduction. (Nagpal et al. 2000a; Kaksonen et al. 2004a)

Another frequently used substrate is ethanol. Compared to lactate, the oxidation of ethanol does not produce as much alkalinity and so there is a higher risk of acetate accumulation if the pH remains unfavourable for sulfate reducers (Equation 4.4) (Nagpal et al. 2000a).

$$2\,C_2H_5OH + SO_4^{2-} \longrightarrow 2\,CH_3COO^- + H^+ + HS^- + 2\,H_2O \qquad (4.4)$$

Ethanol oxidation produces alkalinity (in the form of bicarbonate, HCO_3^-) only after complete oxidation of the produced acetate (Equations 4.4 and 4.3). Still, when treating waste waters with moderate sulfate content in large-scale processes, ethanol is cost-effective, safe to use, and has proved to be a potential electron donor for sulfate reduction (Davidova and Stams 1996; Boonstra et al. 1999; Kaksonen et al. 2004a).

The possibility of using different types of organic wastes, such as compost, cellulosic material (e.g. straw) and manure, as substrate for sulfate reduction is intriguing yet challenging. Although in some cases it may be a cheap and sustainable option, the availability and quality of the material may vary. (Bijmans et al. 2011) Manures from different origins have proved to be promising substrates for sulfate reducing bioreactors (Choudhary and Sheoran 2011; Zhang and Wang 2014).

The key factor of any complex organic substrate is the chemical composition of the material. Gibert et al. (2004) found that the amount of lignin is one important parameter, as low lignin content indicated higher biodegradability and better support for microbial activity. Manure had the lowest amount of lignin when compared to municipal compost and oak leaves. Even though the plant material contained more carbon than manure, the availability of this carbon to microorganisms was poorer. Manure contained the highest amount of easily degradable matter and supported a high sulfate removal efficiency (99% in batch experiments). However, with complex organic materials such as manure, the residence times in continuous systems may have to be prolonged to achieve notable treatment results. (Gibert et al. 2004)

When compared to cellulosic wastes, manures tend to be better in raising the pH and lowering the redox potential of the system, creating more favourable conditions for sulfate reduction (Choudhary and Sheoran 2011; Zhang and Wang 2014). In addition, manure is practical in the sense that it can be used both as substrate and as inoculum for reactors, as manure naturally contains sulfate reducers (Choudhary and Sheoran 2011). Manure contains high amounts of necessary nutrients for microbial growth, so extra nitrogen or phosphorus additions may not be necessary (Gibert et al. 2004; Choudhary and Sheoran 2011).

Nevertheless, it can be challenging to use organic wastes such as manure as substrate for biological sulfate reduction. The availability and the quality of the material is not constant, it may contain only complex organic compounds that are slowly degradable and the amount of organic matter may be low, which forces the reactors to be large. If a stable organic waste stream close to the sulfate reduction site is found, it could be both low-cost and sustainable option for substrate. (Bijmans et al. 2011)

4.3 Inhibition

Many circumstances and compounds can affect the growth and activity of sulfate-reducing microorganisms. For example, low pH and excess concentrations sulfate, sulfide or acetate are common factors inhibiting the reactor performance (Table 4.3). It should be noted though, that most often it is not only one parameter that determines the magnitude of inhibition, but the synergy of different factors together, as will be described in detail in this section.

Table 4.3 *Different factors causing inhibition in biological sulfate-reducing systems.*

	Inhibiting limits	References
pH	$1-3$	Elliott et al. (1998), Lu et al. (2011)
Sulfate	$>$ 4000 mg/l *	Al-Zuhair et al. (2008)
Sulfide	$>$ 500 mg/l **	Reis et al. (1991), O'Flaherty and Colleran (1999)
Acetate	$>$ 800 mg/l *	Koschorreck et al. (2004), Nagpal et al. (2000b)

* Exact limits dependent on reactor parameters and microbial consortia

** pH dependent

If the waste water pH is much below neutral, it may hinder sulfate reduction by lowering the pH inside the reactor. This can become a problem when treating AMD. Elliott et al. (1998) gradually lowered influent pH from 4.5 to 3.0 in an UASB reactor, and discovered that the sulfate reduction results with pH values between 4.5 and 3.3 did not greatly differ from each other (from 45% to 35% sulfate reduction efficiency). However, the more the pH was lowered, the longer adaption period the microorganisms needed to regain their sulfate reducing capacity. With influent pH 3.0 the sulfate reducers did not fully recover anymore, and sulfate removal efficiency remained at 14%. The reduction of sulfate produces alkalinity, so the microorganisms can control the pH inside the reactor to a certain extent (Equation 3.2). In Elliott et al. (1998), only with influent pH 3.0 the acidity was too great for the microorganisms to continue sulfate reduction and produce alkalinity. Santos and Johnson (2016) performed a long term acid tolerance experiment in a continuous flow bioreactor. During 462 days, the reactor pH was mostly kept at 4.0 and raised to 5.0 for the last 100 days of the experiment, while the temperature was altered in the range of $30-45\,^{\circ}\mathrm{C}$. No remarkable changes in performance were noticed when altering the pH or temperature, as microbial populations soon adjusted to new con-

ditions with a shift in the dominating species.

Lu et al. (2011) verified the biological recovery of sulfate reducers from changes in media pH at an even lower range of $3.0 - 1.0$ in batch experiments. At an extremely acidic environment (pH 1.0) the sulfate reduction efficiency remained low (10% reduction), but a change of pH to 2.0 or 3.0 enhanced the performance remarkably, as nearly all sulfate was reduced within two months in batch experiments. In a continuous column experiment the pH of the feed was altered in a sequence of 3.0 - 1.0 - 3.0 - 2.0. Sulfate reduction was poor only at pH 1.0, and the performance was rapidly recovered after reverting to more moderate conditions, and no significant differences in sulfate reduction efficiency was noticed between pH values of 2.0 and 3.0. The operating conditions greatly affect the capability of sulfate reducers to cope in extremely acidic conditions, as with Lu et al. (2011) the influent sulfate concentration was lower and the residence time in the reactor was longer (8 days) than with Elliott et al. (1998) (3 days), and no batch experiments were conducted in the latter case.

Sulfate reduction is also affected by sulfate concentration in the influent. Oyekola et al. (2010) reported that the reduction rate was decreased when gradually increasing the sulfate load in two reactors (influent sulfate 2.5 g/l and 5 g/l). This may be because high sulfate concentration has been experimentally shown to have a lowering effect on pH and an increasing effect on redox potential, which lowers the sulfate reduction potential by allowing other types of microorganisms to prosper (White and Gadd 1996). However, both Moosa et al. (2002) and Oyekola et al. (2010) discovered that biomass concentration and sulfate reduction rate increased with increasing sulfate concentration, and in two reactors by Oyekola et al. (2010) the reduction rate increased with higher sulfate loadings (influent sulfate 1 g/l and 10 g/l), even though the sulfate removal decreased. Al-Zuhair et al. (2008) studied the effect of sulfate concentration on biomass growth in batch tests with initial sulfate concentration ranging between $500 - 4000$ mg/l. The results showed that biomass growth accelerated as the initial sulfate concentration increased to 2500 mg/l. With the highest sulfate concentration of 4000 mg/l, the biomass growth was the slowest of all concentrations tested. Even though 2500 mg/l was the optimal concentration for biomass growth, no data for thorough comparison of sulfate reduction efficiency with different initial sulfate concentrations was presented. Based on these studies, it could be concluded that the reactor performance ultimately depends on the microbial species and their interactions, and that inhibition by sulfate is not necessarily straightforward.

Excess sulfide has an impact on reactor performance as well, although the exact mechanism of this is still unclear (Sheoran et al. 2010). Different theories exist including whether sulfide inhibition is a reversible process where sulfide passes through

microbial cell membranes and disturbs protein synthesis (Moosa and Harrison 2006) and cell respiration (Madigan et al. 2015), or rather a consequence of the precipitation of important trace metals for the microorganisms in their surroundings (Loka Bharathi et al. 1990; Barton 1995). Whether it is only the undissociated form of sulfide (H_2S) or the total sulfide that causes the inhibition is not entirely certain, but Moosa and Harrison (2006) stated that in their experiment the effect of H_2S was more significant, as the sulfate reduction improved with decreasing H_2S concentration, even though the total sulfide concentration increased. As for the limit of inhibition, Moosa and Harrison (2006) showed that a total sulfide concentration above 750 mg/l significantly affected the performance of an acetate fed reactor at pH 7.8, when the measured H_2S concentration was 110 mg/l. This fraction of gaseous H_2S is expected from the total amount of sulfide at the given pH according to Figure 3.2.

In a study by O'Flaherty and Colleran (1999), a reactor fed with a substrate mixture of propionate, butyrate and ethanol was inhibited by a total sulfide concentration of 1000 mg/l at pH 8.0, which would correspond to a H_2S concentration of 70 mg/l. The proof of sulfide inhibition was attained when the reactor sulfide concentrations were lowered with a nitrogen gas scrubber, and the sulfate reduction was improved. Interestingly, according to Reis et al. (1991) and Reis et al. (1992), a sulfide concentration of approximately 550 mg/l is completely inhibitory at pH $6.2-6.7$, where sulfide is mostly in the form on H_2S (approximately 470 mg/l). Based on these studies, it would seem that lower pH raises the limit of inhibition by H_2S, but the tolerance towards total sulfide compounds increases with increasing pH. The operating pH is therefore the dominating factor when estimating the inhibitory effect of sulfide concentration.

The effect of excess acetate is especially clear when using ethanol as substrate (Equation 4.4). If the oxidation stops at acetate, the reactor pH lowers due to the production of protons, as no alkalinity is produced at this stage. A pH below 5.0 enables acetate to diffuse more intensively through the cell membrane and this allows more protons to enter the cytoplasm, which effectively acidifies the cytoplasm and ceases growth when acetate concentration in the medium is high (Baronofsky et al. 1984). Koschorreck et al. (2004) tested the limits for acetate inhibition when using ethanol as substrate. According to their batch experiments, the inhibition started between $880-5500$ mg/l of acetate (at pH 6.0), but no specific concentration was presented. According to Nagpal et al. (2000b), the estimated limit for acetate inhibition in their ethanol-fed system was 7000 mg/l (pH $6.9-7.4$), which was not verified experimentally. As both of these studies were conducted above pH 5.0, other mechanisms besides cell membrane diffusion are likely affect acetate inhibition (Baronofsky et al. 1984; Koschorreck et al. 2004).

The reason for accumulated acetate can be both the absence of complete oxidizers or other acetate users, as well as the slow oxidation rate of acetate (Nagpal et al. 2000b; Kaksonen et al. 2003a; Koschorreck et al. 2004). The exact reason cannot be thorougly explained, as in some studies the acetate oxidation occurred (Winfrey and Ward 1983; Kaksonen et al. 2003a), whereas in others it did not (Nagpal et al. 2000b; Koschorreck et al. 2004). Both acetate and sulfide inhibition can affect acetate consumption, but also the competition between complete and incomplete oxidizers for sulfate can be a determining factor (Nagpal et al. 2000b).

4.4 Competition and co-operation with other microorganisms

The key issue in any biological sulfate reduction reactor is to create an environment where the growth of sulfate reducers is enhanced and the other microbial species are suppressed. Acidogenic (acid-producing), acetogenic (acetate-producing) and methanogenic (methane-producing) microorganisms are known to compete with sulfate reducers for substrates. The end-products of the process are defined by which one of the microbial groups will dominate others. (Hulshoff Pol et al. 1998) For example, Oyekola et al. (2010) reported differences in results when increasing the sulfate load in their reactors. They concluded that reactors resulting with a lower sulfate redution rates may have been affected by a mechanism change of substrate utilisation between oxidation and fermentation. According to the results from different reactors, sulfate reducers used the substrate more efficiently with limiting (1 g/l) and intense (10 g/l) sulfate concentrations in the feed compared to fermenters (Oyekola et al. 2010).

Even the use of pure microbial cultures in extremely isolated systems may not necessarily guarantee the existence of only one species inside a reactor. Attaining this level of purity would include the sterilization of substrate and feed, which would be neither practical nor economic in industrial scale. However, there is no need for such precautions, as having several types of microorganisms in one reactor is often considered to be beneficial in order to have the best possible treatment results (Oyekola et al. 2010). For example, many microorganisms can utilise simple carbon sources such as lactate, but with more complex compounds like glucose or molasses, the efficient co-operation between different species is more crucial (Zhao et al. 2010). First the starting compound is fermented to more simple products, which are then used as carbon source by sulfate reducers (Maree et al. 1987; Liamleam and Annachhatre 2007). The substrate plays an important role in the formation of microbial communities and affects the reactor performance. A mixed population of sulfate reducers (incomplete oxidizers) and acetate using methanogens could also help to prevent acetate accumulation and the consequent inhibition (Nagpal et al. 2000b).

5. MATERIALS AND METHODS

5.1 Sulfate reducing bioreactors

5.1.1 Reactor configuration

The experiment used three identical laboratory scale UASB reactors (Figures 5.1 and 5.2). Each reactor was composed of a glass column (inner ⊘ 50 mm, height 400 mm, volume 0.7 l) equipped with a feed line to the bottom of the column from a bottle containing the feed, an effluent line from the top of the column to a waste canister and a recycle line with a separate pump. The top was closed with a rubber stopper which had an inlet for a gas bag. The bag was partly filled with nitrogen gas and it collected all possibly forming gases and balanced pressure in the reactor. A marble was placed at the bottom inlet of the column to hold the sludge bed in place. The reactors were operated at room temperature ($20 - 22\,^{\circ}\mathrm{C}$).

The feed water was kept in a 5 liter bottle at room temperature ($20 - 22\,^{\circ}\mathrm{C}$), sealed with Parafilm M (Bemis) and refilled before exhaustion. Before a new batch was introduced to the system, the water was purged with nitrogen gas for at least 15 minutes to remove oxygen. The line from the feed bottle to the reactor was operated with a Watson Marlow 205S/CA pump and the tube used was Tygon R3607 (⊘ 1.14 mm).

After leaving the reactor, the effluent went through an air-lock, which was a smaller glass column equipped with a rubber stopper on the top. The height difference between the inflow and outflow channels prevented air leakage to the reactor. From the air-lock the effluent continued into a waste canister, in which $Fe_2(SO_4)_3$ was added to precipitate the sulfide in the effluent according to Equations 5.1 and 5.2 (Firer et al. 2008).

$$8\,Fe^{3+} + HS^- + 4\,H_2O \longrightarrow 8\,Fe^{2+} + {SO_4}^{2-} + 9\,H^+ \tag{5.1}$$

$$Fe^{2+} + 2\,HS^- + 0.5\,O_2 \longrightarrow FeS_2 + H_2O + H^+ \tag{5.2}$$

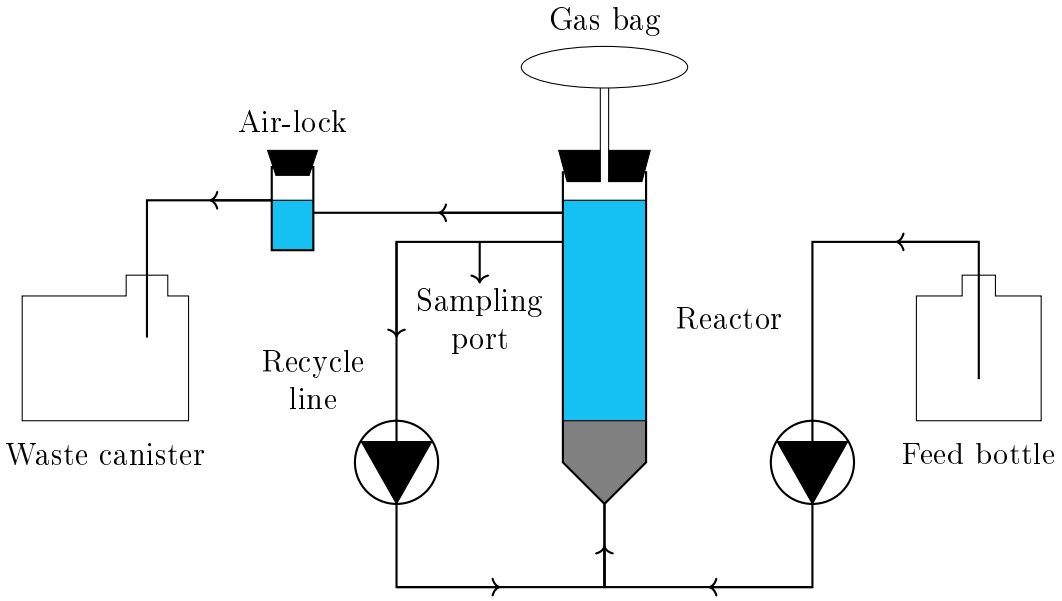

Figure 5.1 *Configuration of the UASB reactors.*

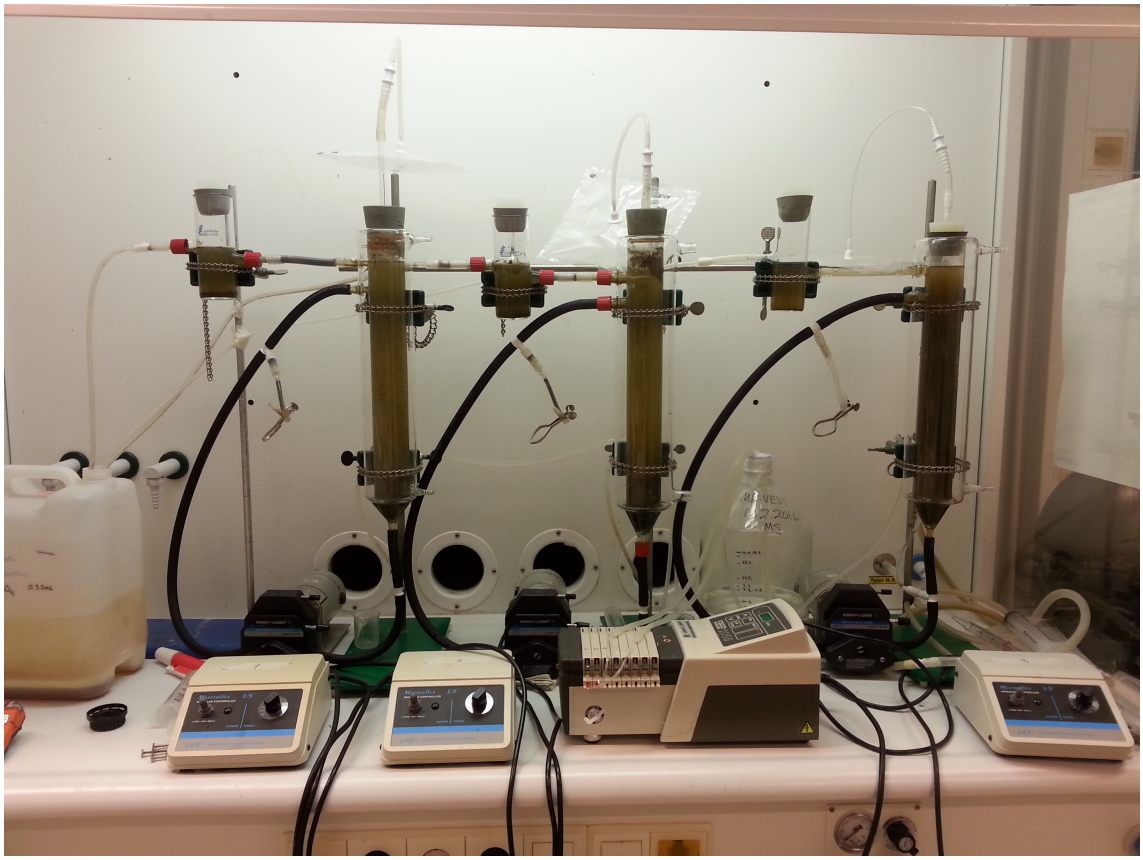

Figure 5.2 *Photograph of the three reactors in operation. From left to right: reactor 1, reactor 2 and reactor 3.*

By calculation, approximately 38 g of $Fe_2(SO_4)_3$ in a 10 l waste canister was needed to precipitate all sulfide, that could in theory be generated from the feed sulfate. NaOH pellets were added to raise the pH to above 12.0. These chemical additions were done to avoid the generation of toxic hydrogen sulfide gas (H_2S) in the waste canister.

The recycle line was made from Masterflex Norprene 06404-36 (⊘ 9.7 mm) and the pump used for recycling was a Cole-Parmer Masterflex model 77201-62 operated with a Cole-Parmer Modular Controller. Other tube lines in the reactor configuration, which were not directly attached to the pumps, were silicone tubes with diameters between 3 to 9 mm. Teflon tape was used in connectors to ensure tight junctions.

5.1.2 Feed and substrate

In reactors 1 and 2, the sulfate concentration of the feed in the initial batch mode was 2125 mg/l, but the feed was changed to drainage water with approximately 1100 mg/l of sulfate after the start of continuous feed on day 15. The drainage water was from a Finnish mine, and the pH of the drainage water was near constant with values between $7.4 - 7.9$. The analysis for the chemical composition of the water showed only small traces (< 150 µg/l) of metals, such as iron, zinc and nickel, capable of precipitation as sulfides, so these compounds were expected not to have a major influence in the reactions inside the reactors, such as precipitating with the produced sulfide.

The substrates used were cow manure obtained from Viikki Research Farm (University of Helsinki), and sodium lactate (50% solution, Merck). The cow manure was analysed to have a total solids (TS) value of 14% and the fraction of volatile solids (VS) of TS was 87%. The amount of total organic carbon (TOC) in a fresh sample was 6.7% by mass, which was used for calculating the needed cow manure additions in Section 5.1.3. The C:N:P ratio of cow manure was 100:5:2. This is close to the optimal C:N:P ratio of 110:7:1 for sulfate-reducing microorganisms (Gerhardt 1981), so no extra nutrient additions were considered necessary.

5.1.3 Reactor operation

In the beginning, the reactors were filled with mine drainage water and cow manure was added to obtain sludge beds with volumes of approximately 100 ml. Sulfate load for the sludge volume (V=100 ml) was kept approximately 1000 mg/l*d during the continuous operation. Hydraulic retention time (HRT) and influent flow rate (Q_s) were calculated according to Equations 5.3 and 5.4.

$$HRT\,(h) = \frac{{SO_4}^{2-}\ concentration\,(mg/l) * 24\,(\frac{h}{d})}{{SO_4}^{2-}\ load\,(mg/l * d)} \tag{5.3}$$

$$Q_s\,(ml/h) = \frac{V\,(ml)}{HRT\,(h)} \tag{5.4}$$

The acquired HRT was 27 h with an influent sulfate concentration of 1140 mg/l, and the influent pump was adjusted according to Q_s value of 3.7 ml/h. This influent velocity was kept constant throughout the experiment, as the measured sulfate concentration of the influent varied only little after the start of continuous operation ($990 - 1160$ mg/l; Appendix A).

The mass of TOC needed in sulfate reduction was calculated from Equation 5.5 (Vestola and Mroueh 2008).

$$2\,CH_2O + {SO_4}^{2-} \longrightarrow H_2S + 2\,{HCO_3}^- \tag{5.5}$$

Based on masses of carbon and sulfate in Equation 5.5 (24 g and 96 g, respectively), the required TOC is roughly one fourth of the sulfate reduced. As sulfate in the feed was approximately 1100 mg/l, the stoichiometric amount of TOC needed for total sulfate reduction was 275 mg per liter of the feed water, and 50% excess (i.e. 413 mg of TOC in total per liter of feed) was used to ensure enough TOC for sulfate reduction. The cow manure additions to reactors were adjusted according to the cow manure's TOC content (6.7%) and the feed Q_s of 3.7 ml/h, though a portion for several days (usually $3 - 4$ days) was introduced at once, as continuous cow manure addition would have caused clogging of the tubes. The cow manure used as substrate was stored in a freezer (-20 °C) and thawed in a refrigerator (4 °C) before use. The required amount of cow manure was diluted to a practical concentration of 1:10 with deionized water to ease the dosing with syringe. The substrate additions to the reactors were conducted through the sampling ports with a syringe twice per week. During the summer months, the substrate additions in reactor 3 were done once per week from day 78 onwards.

Because of sulfate reducers naturally present in the cow manure (Choudhary and Sheoran 2011), sulfate reduction occurred right from the beginning of the operation, although the reduction efficiency increased only slowly. To create more favourable starting conditions for sulfate reducers, the pH was raised to near neutral with an addition of 1.2 g of $NaHCO_3$ (as 6 wt-% solution), which was fed directly to all three reactors. The addition was done in two parts on days 24 and 28 for reactors 1 and 2 and in one part on day 34 for reactor 3 (Table 5.1). To enhance the sulfate reduction efficiencies and compare microorganisms from different sources,

three different inocula (100 ml) were added to the reactors after 32 days (reactors 1 and 2) or 17 days (reactor 3) of operation (Table 5.1).

Table 5.1 *Detailed operation of the sulfate-reducing reactors in this study. The lactate feed percentage values describe the fraction of carbon need covered by lactate, while the rest is covered by cow manure*

Day	Reactors 1 and 2	Reactor 3
0	Batch mode	Batch mode
15	Start of continuous operation	
17		Inoculum addition
22	Start of cow manure feed	
24	Addition of 0.6 g of $NaHCO_3$	
27		Start of continuous operation
28	Addition of 0.6 g of $NaHCO_3$	
30		Start of cow manure feed
32	Inoculum addition and batch mode	
34		Addition of 1.2 g of $NaHCO_3$
35	Start of continuous operation	
45	Batch mode	
53	Start of continuous operation	
74	Lactate feed (50%)	
77	Lactate feed (40%)	
80	Lactate feed (30%)	
84	Lactate feed (20%)	
87	Lactate feed (10%)	
91	Lactate feed (25%)	
127		End of operation
133	End of operation	

At times, the reactors were kept in batch mode (no influent feed, but slow continuous pumping of the reactor liquid through the recycle line) to acclimatize the microbial communities and increase biomass concentration inside the system (Table 5.1). For reactors 1 and 2, the days in batch mode were $0-15$ (for biomass increase in the beginning), $32-35$ (for acclimatizing the added inocula) and $45-53$ (attempt to increase biomass and improve the sulfate reduction efficiency), for reactor 3 the days $0-27$ (for biomass increase in the beginning). From day 59 onwards the redox potential started to increase above -100 mV and pH was decreasing from above 7.5 to near 7.0 in reactors 1 and 2. Lactate was added as substrate together with cow manure to reactors 1 and 2 from day 74 onwards to ensure the efficient operation of the sulfate reducing bioreactors (Table 5.1). The ratio of lactate and cow manure

started from a mass ratio of 50/50 of the total carbon need of the reactors, and the ratio for lactate was gradually lowered to find the minimum amount of lactate needed to enhance sulfate reduction. After day 91, 25% of the carbon need (by mass) was permanently covered with lactate in reactors 1 and 2 (Table 5.1).

The reactor liquid was sampled twice a week from a sampling port in the recycle line (Figure 5.1). Microbial samples (1.5 ml) and other measurements (pH, redox potential, sulfate and sulfide concentration) were taken from this effluent sample. Each reactor was operated approximately 130 days, although reactor 3 was started later than the others because of delays in equipment deliveries.

5.2 Sulfur recovery experiment

Sulfide oxidation to elemental sulfur, and subsequent recovery, were done in batch experiments. These were performed in two different configurations: with addition of air and using a magnetic stirrer (Setup A) (Figure 5.3) or in a shaker (Setup B).

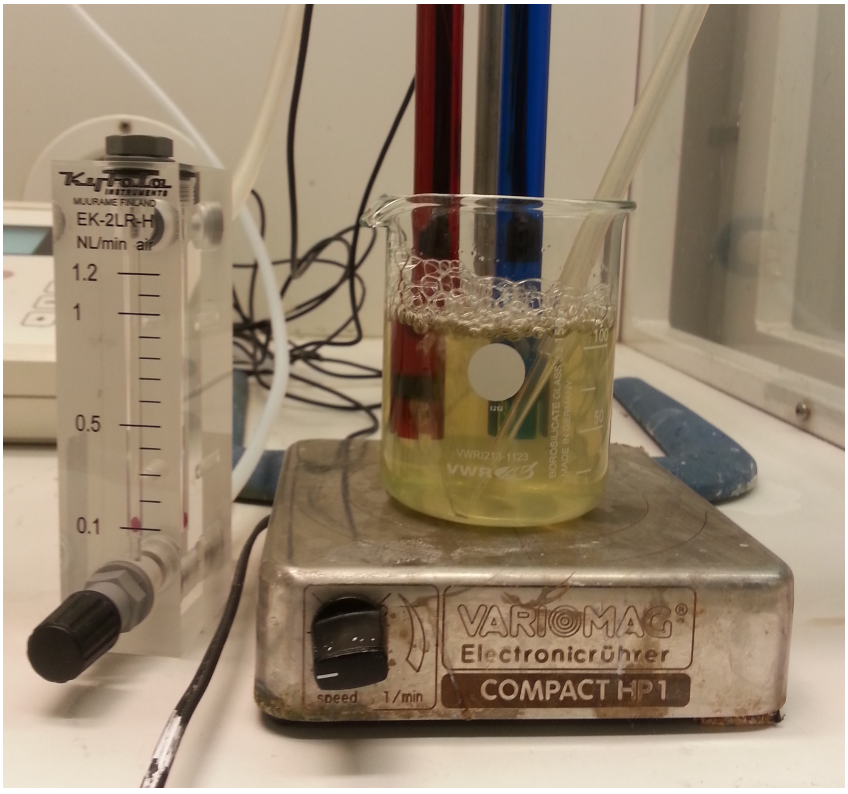

Figure 5.3 *Sulfur recovery experiment with setup A.*

In Setup A, approximately 100 ml of effluent from the UASB reactors was collected in a small beaker with electrodes for measuring pH and redox potential. Compressed air was fed into the beaker through a pipette head producing small air bubbles. The air flow was controlled with a rotameter (Kytola Instruments, model EK-2LR-H)

and a magnetic stirrer was used for mixing the solution. The liquid was purged with air until the pH did not increase significantly anymore (increasing pH indicates sulfide oxidation to sulfur as shown in Equation 3.3), after which the air feed was ceased and the solution was only mixed with the magnetic stirrer. Samples for sulfate measurements were taken every $10-15$ minutes. When sulfide in the solution was less than 2 mg/l, the experiment was stopped and the solution was filtrated with a 0.45 µm pore size membrane filter (PALL Nylaflo) and the precipitate was dried at 50 °C overnight.

In Setup B, loosely capped bottles with 10 ml of effluent were placed in a shaker (65 rpm) without active air feed. This could allow a more even dissolution of oxygen into the effluent. The bottles were in the shaker until no sulfide was detected from the solutions. Redox potential and pH were monitored as in Setup A. Samples for sulfate measurements were taken in the beginning and in the end of the experiment.

The effect of active sulfur-oxidizing microorganisms in sulfur recovery was studied. In Setup A, 5 ml of sulfur-oxidizing inoculum was added to 100 ml of effluent and in Setup B 1 ml of inoculum was added to 10 ml of effluent. Setup B experiment was also conducted with growth media without inoculum as a negative control.

5.3 Inocula

In the sulfate-reducing reactors, three different additional inocula were used besides the original fresh cow manure. Hereafter these are referred to as inocula. The inoculum for reactor 1 was enriched from effluent of another anaerobic sulfate reducing reactor, which had been treating mine water with cow manure as substrate for several months (Mintek, South Africa). The inoculum for reactor 2 was enriched from a tailings sample from a holding pond of a Finnish mine. The inoculum for reactor 3 was enriched from cow manure from Viikki Research Farm. All the inocula samples were grown and maintained anaerobically in two different media suitable for sulfate reducers (Table 5.2) and added to the reactors only after the colour of the growth media in batch bottles had turned black, indicating sulfate reduction. The growth medium 1 was used in South Africa for inoculum maintenance outside reactors, so the same medium was used in the inoculum for reactor 1 in this study as well. The inocula for reactors 2 and 3 were cultivated in DSMZ Medium 63 (Leibniz-Institut DSMZ GmbH 2015d), a commonly used growth medium for sulfate reducers at VTT Technical Research Centre of Finland Ltd, where the research was conducted.

Table 5.2 *The growth media used for sulfate-reducing inocula in this study. Medium 1 was used in the inoculum for reactor 1 and medium 63 in the inocula for reactors 2 and 3.*

Reagent	Medium 1	Medium 63 *
NH_4Cl	1 g/l	1 g/l
Na_2SO_4	3 g/l	1 g/l
$CaCl_2 \cdot 2H_2O$	0.2 g/l	0.1 g/l
$MgSO_4 \cdot 7H_2O$	1 g/l	2 g/l
$FeSO_4 \cdot 7H_2O$	0.08 g/l	0.5 g/l
Ethanol	7.5 ml/l	-
NaCl	0.1 g/l	-
KH_2PO_4	0.5 g/l	-
K_2HPO_4	-	0.5 g/l
DL-Na-Lactate	-	2 g/l
Yeast exctract	-	1 g/l
Resazurin	-	1 mg/l
Na-thioglycolate	-	0.1 g/l
Ascorbic acid	-	0.1 g/l

* Leibniz-Institut DSMZ GmbH (2015d)

The sulfur-oxidizing inoculum for elemental sulfur recovery experiments was prepared from a 2 ml sample from reactor 2 after approximately 100 days of operation. This inoculum was maintained in a 10 ml medium described in DSMZ 486 (Appendix Table B.1) and was kept at room temperature ($20 - 22\,^{\circ}C$) and shaked occasionally. The used medium allowed the naturally occurring sulfur oxidizers in the effluent to enrich in the culture. The propagation of the inoculum took place at approximately 1 week intervals, when 1 ml of the old growth solution was added to 9 ml of fresh medium. As described in Equation 3.4, the oxidation of sulfur to sulfate produces acidity, and this change in pH is indicated by a colour shift from purple to yellow (bromocresol purple).

5.4 Analytical methods

All pH and redox potential values were measured with a Consort multi-parameter analyser C3040 equipped with Van London-pHoenix Co. electrodes (Ag/AgCl in 3 M KCl). Sulfate, sulfide, phosphate and ammonium analyses were conducted with Hach Lange cuvette tests and measured with a Hach Lange DR 3900 spectrophotometer. The tests were conducted according to the manufacturer's instructions and the cuvettes were labelled with barcodes, which the spectrophotometer recognized and adjusted the wave length automatically for the absorbance measurement. The analyses were conducted with Hach Lange kits LCK 353 (sulfate; barium sulfate

method), LCK 653 (sulfide; dimethyl-p-phenylenediamine method), LCK 349 (phosphate; phosphormolybdenum blue method) and LCK 304 (ammonium; indophenol blue method). Other analyses included the measuring of nitrite (SFS 3029), nitrate (inner method, Aquakem), total nitrogen (SFS-EN ISO 11905-1), TOC (SFS-EN 1484:1997) and acetate (SFS-EN ISO 10304-1:2009). The analyses for the chemical composition of the feed were conducted with inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma optical emission spectrometry (ICP-OES) and ion chromatography (IC) (SFS-EN-ISO 10304-1:en).

For all the chemical measurements, except for sulfide, the samples were filtered using PALL Nylaflo 0.45 µm Nylon membrane filters or Whatman FP30/0.45 CA-S syringe filters before analysing. Sulfide measurement was done within 1 minute of taking the sample from the reactor, as sulfide is eager to escape from the solution as gaseous sulfide (H_2S).

The total and volatile solids (TS and VS) were measured according to standard SFS-3008 (Finnish Environment Institute SYKE 2000). TOC (SFS-EN 13137 method A 2001), phosphate (SFS-EN ISO 6878:2004) and nitrogen compounds of the cow manure were examined in Metropolilab Oy, Helsinki.

5.5 Microbial analyses

In order to examine the relationship between the reactors' performance and microbial communities over time, samples for the microbial assays were taken throughout the experiment. A next generation DNA sequencing method utilising the Ion Torrent PGM platform was used in this work to characterize the range of microbial groups, as well as to estimate the relative abundances of their populations in the reactors. A quantitative polymerase chain reaction (qPCR) method was used to estimate the abundance of sulfate-reducing bacteria in the reactors.

5.5.1 DNA extraction

Microbial samples taken from the reactor effluents were stored in a freezer (-20 °C) until the DNA extration process. The DNA extraction was done in a laminar flow cabinet which was first disinfected with UV light (approximately 15 minutes of radiation) and wiped with ethanol (70%). Before extraction the samples were thawed, centrifuged to collect the microbial cells (10 minutes at 20800 g) and the supernatant was sterilely removed, leaving the solid material containing the biomass to be analysed. The DNA was extracted from the biomass using a Nucleospin Soil DNA extraction kit (Macherey-Nagel GmbH & Co KG 2015) according to the man-

ufacturer's instructions. In this work, lysis buffer SL1 and the Enhancer buffer was used, and the extracted DNA was finally eluted to 100 µl of elution buffer. The acquired DNA was stored in a freezer (-20 °C) until further processing through PCR and DNA sequencing.

5.5.2 Quantitative analysis of the sulfate-reducing bacteria

The concentration of sulfate-reducing bacteria (SRB) in the effluent samples was analysed with quantitative polymerase chain reaction (qPCR). A dilution series of plasmids containing $5.84 \times 10^7 - 5.84 \times 10^1$ copies of the *dsr*B gene fragment of *Desulfobulbus propionicus* (E-06220 VTT culture collection) was used as standard for the assay, as described in Foti et al. (2007). The qPCR reactions were run in 10 µl volumes using a KAPA Sybr Fast qPCR Kit including Master mix 2x for LightCycler 480 (Kapa Biosystems). The primers used were DSRp2060F (5'-CAA CAT CGT YCA YAC CCA GGG-3') and DSRS4R (5'-GTG TAG CAG TTA CCG CA-3') (Foti et al. 2007). All equipment and deionized water were kept in an UV oven (Stratalinker) for 30 minutes before use, and the laminar flow cabinet was first treated with UV light for 15 minutes. To 500 µl of 2x mastermix, 25 µl of each primer was added, the mixture was diluted with 350 µl of deionized water and 8.5 µl of this mixture was added to each well in a 12 × 8 well plate, followed with 1 µl of the DNA extract to be analysed. The analyses were conducted with three parallel reactions per sample and three wells served as negative controls with the additions of sterile deionized water. The qPCR was conducted with LightCycler 480 (Roche Diagnostics). The amplification program was divided into initial denaturation (15 minutes at 95 °C), amplification (45 cycles with 10 seconds at 95 °C, 35 seconds at 55 °C and 30 seconds at 72 °C), final extension (3 minutes at 72 °C), a melting curve analysis (10 seconds at 95 °C, 1 minute at 65 °C and continuously at 95 °C) and a cooling period (15 minutes at 40 °C). The SRB concentration of a sample was calculated from the average of three reactions, including the determination of standard deviation and standard error.

5.5.3 Ion Torrent sequencing

The PCR protocol for the Ion Torrent sequencing was conducted as described in Rajala et al. (2016). The samples were sent to Bioser, Oulu, for sequencing. The results were processed and analysed according to the sequences' operational taxonomic units (OTUs) with QIIME-software, described in more detail in Rajala et al. (2016).

6. RESULTS AND DISCUSSION

6.1 Sulfate-reducing bioreactors

Redox potential, pH, acetate concentration, sufate reduction and sulfide concentration were monitored in all reactors during the 130 days of operation to obtain information on their relation to each other and their effect on sulfate removal efficiency (Figures 6.1 and 6.2 and Appendix Tables A.1, A.2 and A.3). The other compounds measured from the effluents (ammonium, phosphate and TOC) were monitored mainly to ensure that the carbon and nutrient feed was sufficient although not optimized (Appendix Tables A.1, A.2 and A.3).

The pH was less than 6.7 when continuous operation was started (day 15 for reactors 1 and 2, day 27 for reactor 3), and increased only slowly to above 7.0 in all reactors (Figure 6.1), probably when acetate started to oxidize to bicarbonate (Equation 4.3). As the acetate concentration decreased, the pH increased in all reactors. After the final addition of $NaHCO_3$ to reactors 1 and 2 (after day 28), the pH values remained mostly above 7.0, with the exception of the last phase of reactor 2 (days $116-133$). In reactor 3, the pH values declined more quickly to below 7.0 (from day 76 onwards) after the addition of $NaHCO_3$, but between days 106 and 127 the pH steadily increased from 6.7 to 6.9.

Even though the feed pH was generally above 7.5, the reactor effluents had lower pH values ($6.7-7.5$). This may have happened because the system was not completely anaerobic, and small amounts of air could have penetrated through the tubes and junctions. As the feed bottle was not sealed gas-tightly, some oxygen had been dissolved into the feed and therefore transported into the reactors. This could cause the generated sulfide to be oxidized back to sulfate inside the reactor and result in a pH drop as well as lower sulfate reduction efficiency (Equation 3.4). The cow manure had a pH of $7.0-8.0$, so it did not directly lower the reactor pH.

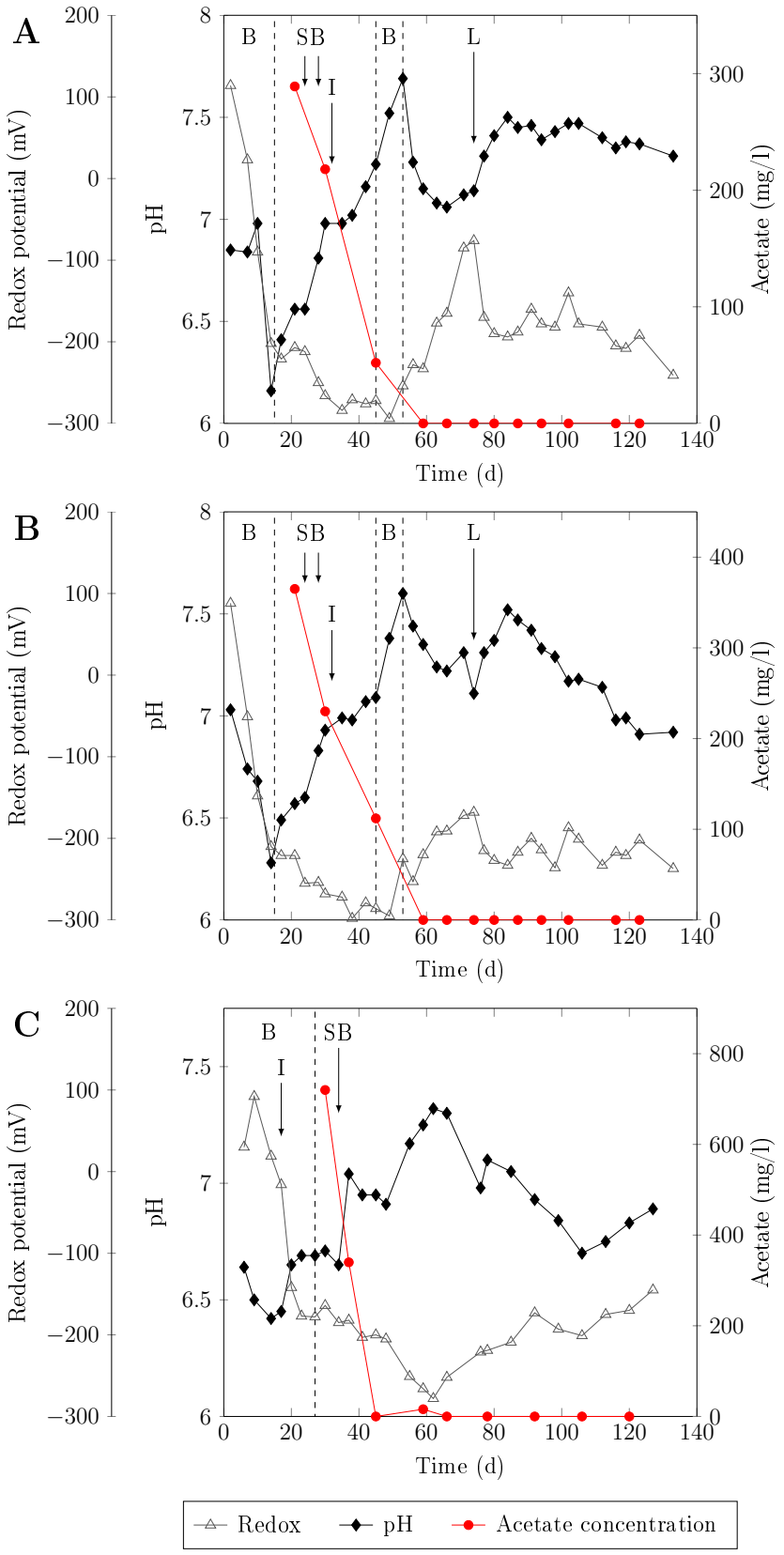

Figure 6.1 *The redox potential, pH and acetate concentration in reactors 1 (A), 2 (B) and 3 (C) during the experiment. Symbols: B = batch mode (dashed lines indicating the beginning/end), SB = sodium bicarbonate addition, I = inoculum addition and L = start of lactate feed. Batch mode after inocula additions to reactors 1 and 2 is not shown for clarity.*

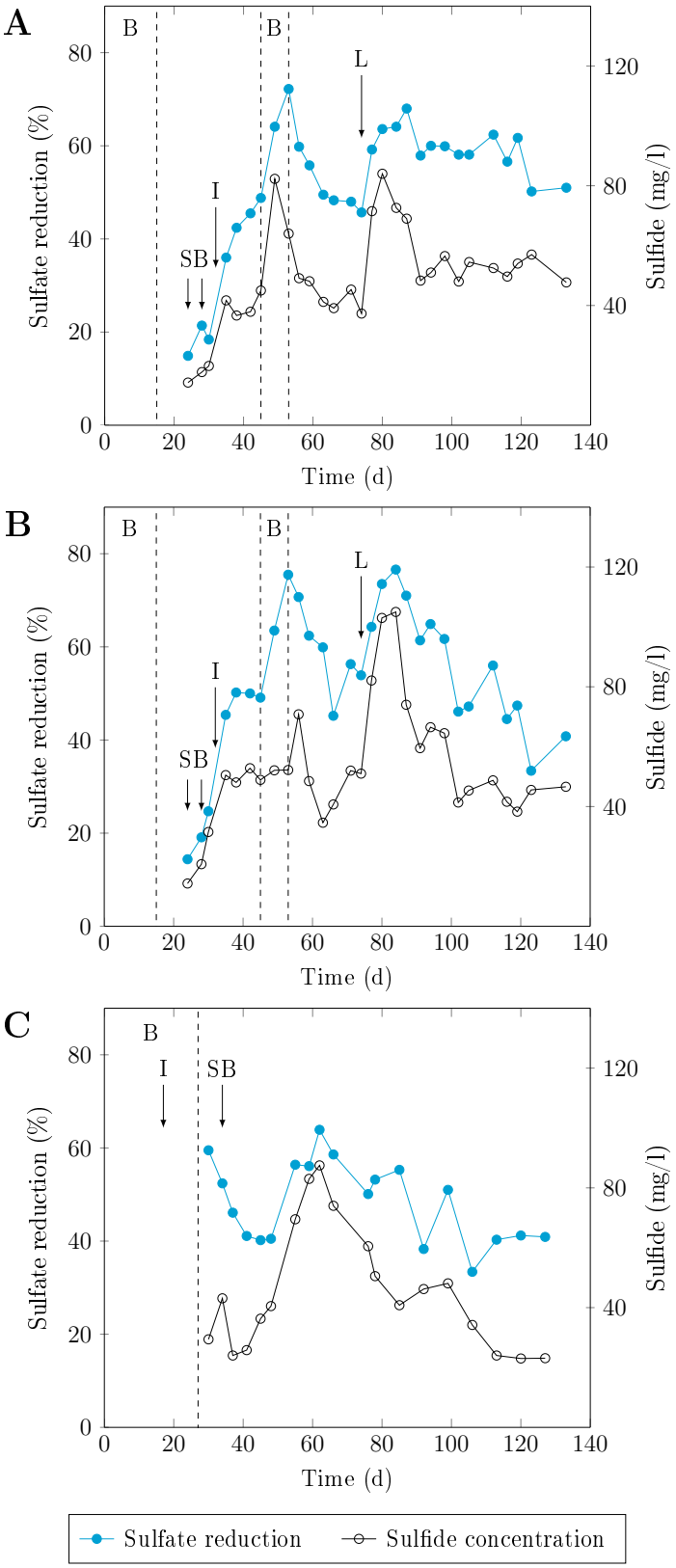

Figure 6.2 *The sulfate reduction efficiency and sulfide concentration in reactors 1 (A), 2 (B) and 3 (C). Symbols: B = batch mode (dashed lines indicating the beginning/end), SB = sodium bicarbonate addition, I = inoculum addition and L = start of lactate feed. Batch mode after inocula additions to reactors 1 and 2 is not shown for clarity.*

The redox potentials were usually low when pH was high and vice versa (Figure 6.1). The redox potentials decreased sharply below -200 mV in the beginning (days $0-50$) in reactors 1 and 2, and after day 74 the redox potential was quite stable in both reactors, presumably due to the addition of lactate. In reactor 3, it took 40 days for the redox potential to reach -200 mV, and after day 60 the redox potential increased quite steadily from -280 mV to -150 mV, so the redox conditions were not as favourable for sulfate reduction in reactor 3 as in reactors 1 and 2.

Acetate concentrations were examined to acquire more information on possible inhibition factors. In the beginning of the experiment the acetate concentrations were high ($300-700$ mg/l) (Figure 6.1) as the initial addition of cow manure contained plenty of TOC and possibly acetate producing fermenters (Madigan et al. 2015). However, in all three reactors the acetate was soon removed from the effluent. As the amount of acetate decreased, the sulfate reduction efficiency increased, so the depletion of acetate was favourable for sulfate reducers (Figures 6.1 and 6.2). But when the acetate was completely exhausted, the sulfate reduction efficiency decreased in all reactors. This may have indicated a scarcity of substrate and that the amount of available TOC could not support efficient sulfate reduction anymore.

Sulfate reduction efficiency increased from day 20 onwards in reactors 1 and 2, presumably because the sulfate reducers present in the cow manure started to become acclimatized to the conditions (Figure 6.2 A and B). Sulfate reduction efficiency increased further after adding the inocula on day 32, decreased between days 53 and 74, and increased again after the start of lactate addition on day 74. In the end of reactor operation (days $90-133$), reactor 1 stabilized to a sulfate removal efficiency of $50-60\%$ (reduction rate of $500-600$ mg/l*d), but the performance of reactor 2 declined steadily after day 80, resulting in a sulfate removal efficiency of $40-50\%$ (reduction rate of $400-500$ mg/l*d)(Figure 6.2 A and B). In reactor 3, the sulfate removal efficiency decreased after the start of continuous feeding on day 27, but then increased after day 41 until day 62, after which it declined (Figure 6.2 C), similarly to reactors 1 and 2. The reason for the decrease in efficiency after day 62 can be that the cow manure feed to the reactor was reduced to 1 time per week from the previous frequency of approximately 2 times per week during the summer months, which may have caused substrate depletion inside the reactor. In the end of the operation (between days 113 and 127) sulfate removal efficiency was near 40%, even though the redox potential increased to above -200 mV (Figure 6.1 C). Reactor 3 was not supported with lactate at any time.

There was a clear connection between sulfate removal efficiency and sulfide concentration in each reactor. When sulfate reduction efficiency was high, so was the concentration of sulfide (Figure 6.2). In reactor 2, the highest sulfate removal efficiency was almost 80% and the highest sulfide concentration was slightly over 100

mg/l. The addition of lactate increased sulfate removal efficiency and sulfide concentration from day 74 onwards, although only until day 84, after which the sulfate removal efficiency and sulfide concentration declined. When calculating the theoretical sulfide concentration based on the sulfate reduction efficiency in this study, the measured sulfide was always much less than anticipated, mostly only $20-30\%$ of the stoichiometric maximum (theoretical values ranging from 140 to 250 mg/l of sulfide). This is not unusual though, as many others have reported the same phenomenon. Elliott et al. (1998), Moosa and Harrison (2006), Oyekola et al. (2010) and Rodriguez et al. (2012) reported less sulfide than estimated. In these cases the reason was often thought to be the release of sulfide as gaseous H_2S or the formation of other sulfur compounds, especially elemental sulfur, inside the reactor. Both of these explanations could be applied to the reactors of this study as well, although the release of gases was difficult to detect as the gas bags slowly emptied by themselves because of pressure changes caused by the effluent flow. The formation of elemental sulfur was highly probable, as pale yellowish precipitate was formed in the upper parts of the reactors and effluent pipe lines. Similar precipitate was detected by van der Zee et al. (2007) and Brahmacharimayum and Ghosh (2014). In addition, some sulfide may have been lost as gaseous H_2S when taking the effluent sample from the reactors.

Results from this work and other studies related to biological sulfate reduction are compiled in Table 6.1. When looking solely at sulfate removal efficiencies, others such as Kaksonen et al. (2003b), Rodriguez et al. (2012) and Oyekola et al. (2010) have reached better sulfate removal rates and efficiencies ($840-1900$ mg/l*d, $82-85\%$). However, other studies utilizing waste material (either manure or other waste effluents) as substrate (Boshoff et al. 2004; Xingyu et al. 2013; Zhang and Wang 2014), have similar or even lower values either for sulfate reduction rate or sulfate removal efficiency ($200-600$ mg/l*d, $30-90\%$). The lower sulfate loadings and longer HRTs in these studies compared to this work are undoubtedly one reason for better sulfate reduction results. The highest sulfate reduction rates and efficiencies (up to 1900 mg/l*d and 85%) were obtained with lactate, as it is more easily utilized by sulfate reducers (Kaksonen et al. 2003b; Oyekola et al. 2010).

Table 6.1 *The operation, sulfate removal efficiencies and sulfate removal rates from selected sulfate-reducing bioreactor studies using similar reactor type or substrates as in this work. Sulfate removal values represent the best stable situation reported in each study.*

Reactor	Substrate	Temperature (°C)	Feed pH	Sulfate load (mg/l*d)	HRT (h)	Sulfate removal rate and efficiency	References
UASB	Cow manure + Lactate	20 – 22	7.4 – 7.9	1000	27	400 – 600 mg/l*d 40 – 60%	This work
UASB	Lactate	35	6.0 – 3.0 [GD]	max 2450 [GI]	80 – 15 [GD]	1900 mg/l*d 70 – 85%	Kaksonen et al. 2003b
UASB	Ethanol	[NR]	3.9	max 1060 [GI]	24	960 mg/l*d 82%	Rodriguez et al. 2012
Downflow column bioreactor	Chicken manure + Lactate	30	3.0 – 3.5	330	178	app. 280 mg/l*d 60 – 90%	Zhang and Wang 2014
CSTR	Lactate	35	8.0	max 1920 [GI]	120 – 24 [GD]	840 mg/l*d 85%	Oyekola et al. 2010
Pilot UASB (V = 1.5 m^3)	Tannery effluent	[NR]	7.5	app. 800	96	600 mg/l*d 70 – 80%	Boshoff et al. 2004
Pilot UASB (V = 3.0 m^3)	Activated sludge	[NR]	2.3 – 2.5	app. 830	72	app. 220 mg/l*d 30 – 50%	Xingyu et al. 2013

[GD] = Gradually decreased
[GI] = Gradually increased
[NR] = Not reported

In terms of feed pH, the above neutral feed in this study was favourable for sulfate reducers, whereas the sulfate reduction efficiencies or rates in some other studies may have been lowered by acidic feed (Table 6.1) (Xingyu et al. 2013; Zhang and Wang 2014). The operating temperatures in all other studies (when reported), however, were higher, which may have led to improved sulfate reduction process, as most sulfate reducers are mesophilic and thrive at temperatures above 30 °C (Figure 4.1, Table 6.1) (Kaksonen et al. 2003b; Oyekola et al. 2010; Zhang and Wang 2014). Increasing the operation temperature of the reactors could have improved the sulfate reduction efficiencies in this work.

The cow manure used in this study was quite dense, so the sludge blankets occasionally rose in the reactors. This could be avoided by mixing inert material with the sludge, for example silica sand (Zhang and Wang 2014) or small pebbles (Choudhary and Sheoran 2011), to increase permeability. The flotation of sludge could also be prevented by reactor design, as Rodriguez et al. (2012) had a narrowing separator in the upper part of their reactor restricting the movement of sludge. Elliott et al. (1998) has pointed out that most biomass in an upflow reactor reside further away from the base where the feed is introduced to the system. Thus, it is optimal to have as much sludge as possible to have a large and stable environment for the microorganisms to thrive. As in this work the sludge volume was only 100 ml, which is 1/7 of the reactor volume, there is a possibility to increase the sludge volume and therefore the sulfate reduction capacity.

Zhang and Wang (2014) reported a steep decrease in sulfate reduction efficiency and an increase in redox potential when the substrate was nearly completely exhausted, and the addition of lactate improved the sulfate reduction. In the reactors of this work, the decline in sulfate reduction efficiency in each reactor after approximately two months of operation may have happened because of the similar depletion of easily degradable organic matter, as cow manure contains complex compounds that degrade more slowly (Bijmans et al. 2011; Zhang and Wang 2014). The dosing of diluted cow manure may not have been enough to supply substrate for the microorganisms, and after the effluent TOC decreased below 50 mg/l (Appendix Tables A.1, A.2 and A.3), assuming linear decrease, the sulfate reduction suffered. Based on this, organic wastes such as cow manure should be provided in great quantity for the biological sulfate reduction to remain efficient. This could mean a partial replacement of sludge with fresh cow manure at regular intervals. However, as Zhang and Wang (2014) pointed out, a secondary treatment method to remove the excess organics from the effluent may be required in this case.

6.2 Quantitative analysis of the sulfate-reducing bacteria

The concentration of SRB in the reactor effluents was examined by qPCR of the *dsr*B gene (Figure 6.3). The SRB concentrations varied over time in a similar manner in reactors 1 and 2. The final SRB concentrations on day 133 were $6 * 10^7$ copies/ml in reactor 1 and $3 * 10^7$ copies/ml in reactor 2. However, at two occasions the differences were rather notable, as on day 21 the SRB concentration in reactor 1 ($1 * 10^9$ copies/ml) was over 10 times higher than in reactor 2 ($9 * 10^7$ copies/ml), although the SRB concentration was increasing in reactor 2 as well, and on day 53 the situation was reversed. On day 53, the SRB concentration in reactor 2 ($1 * 10^9$ copies/ml) was over 14 times higher than in reactor 1 ($7 * 10^7$ copies/ml). The first peaks in SRB concentrations may have resulted from the start of the continuous feed on day 15, which could have induced the sulfate reducers to multiply. The second peaks in SRB concentrations could have resulted from the switch to batch mode from day 45 onwards, which again offered a stable environment for the growth of sulfate reducers.

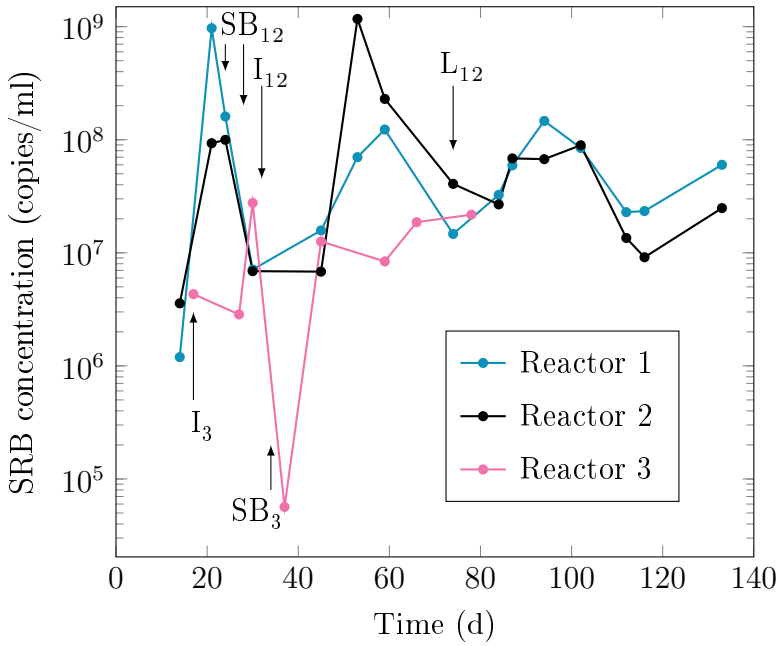

Figure 6.3 *SRB concentrations in the effluents of reactors 1, 2 and 3, based on the average of three parallel samples. Symbols: SB = sodium bicarbonate addition, I = inoculum addition and L = start of lactate feed. The subscripts describe the reactor(s) the symbol in question refers to. Samples from reactor 3 could be obtained only during the first 80 days of operation. The calculated standard errors for the concentrations were not visible in the graph and they were excluded from the figure.*

The SRB concentration in reactor 3 was monitored a shorter time than those of reactors 1 and 2, but the SRB concentration fluctuated in a similar way as in reactors

1 and 2 (Figure 6.3). The continuous feed seemed to be the enhancer of SRB growth in reactor 3 as well, as the SRB conentration increased from $3 * 10^6$ copies/ml to $3 * 10^7$ copies/ml from day 27 onwards. The addition of $NaHCO_3$ on day 34 did not enhance SRB growth, as there was a sharp decrease in SRB concentration from $3*10^7$ copies/ml to $6 * 10^4$ copies/ml 3 days after the addition of $NaHCO_3$. However, the SRB concentration reverted back to $1 * 10^7$ copies/ml on day 45, and afterwards the SRB concentration increased more steadily than with other reactors, reaching a SRB concentration of $2 * 10^7$ copies/ml. The more steady increase in SRB concentration in reactor 3 than in reactors 1 and 2 was probably because minimal alterations were done during the operation of reactor 3.

The SRB concentration trends in all reactors followed sulfate reduction efficiency; when the SRB concentration increased, the sulfate reduction efficiency usually improved (Figures 6.2 and 6.3). The start of lactate feed in reactors 1 and 2 from day 74 onwards had only a weak positive effect on SRB concentration. The SRB concentrations in all reactors seemed to slightly increase towards the end of operation, although the SRB concentrations fluctuated constantly. Similar fluctuating behaviour in SRB concentration was reported by Pruden et al. (2007). In this work, even the addition of inocula did not immediately increase the SRB concentrations (day 32 for reactors 1 and 2, day 17 for reactor 3), and the addition of $NaHCO_3$ (days 24 and 28 for reactors 1 and 2, day 34 for reactor 3) had a negative effect on the SRB concentrations. There may have been differences in sampling, for example the reactor effluent samples for microbial analyses may have varied a little in volume, so the sharp increases and decreases in SRB concentrations can be exaggerated from reality, but the overall trends can be regarded as correct based on the measured SRB concentrations. As the samples for qPCR were taken from the effluent and not from the sludge bed where most of the microorganisms reside, the true concentration of SRB inside the reactors is higher than presented in Figure 6.3.

6.3 Bacterial community characterization

The microbial analyses presented in this thesis cover only the bacterial domain. Using the Ion Torrent sequencing, sequence counts from 2912 to 13410 per sample were acquired with an average of 7092 counts per sample.

The bacterial populations in all reactors (Figure 6.4) consisted mainly (up to 90%) of the phyla Proteobacteria, Bacteroidetes and Firmicutes throughout the experiment. In reactors 1 and 2, some bacteria belonging to an undetermined candidate phylum WWE1 were discovered, although in reactor 3 this phylum was absent. Because of its unknown features, phylum WWE1 is not studied any further and its lower taxonomic ranks are excluded from Figure 6.5.

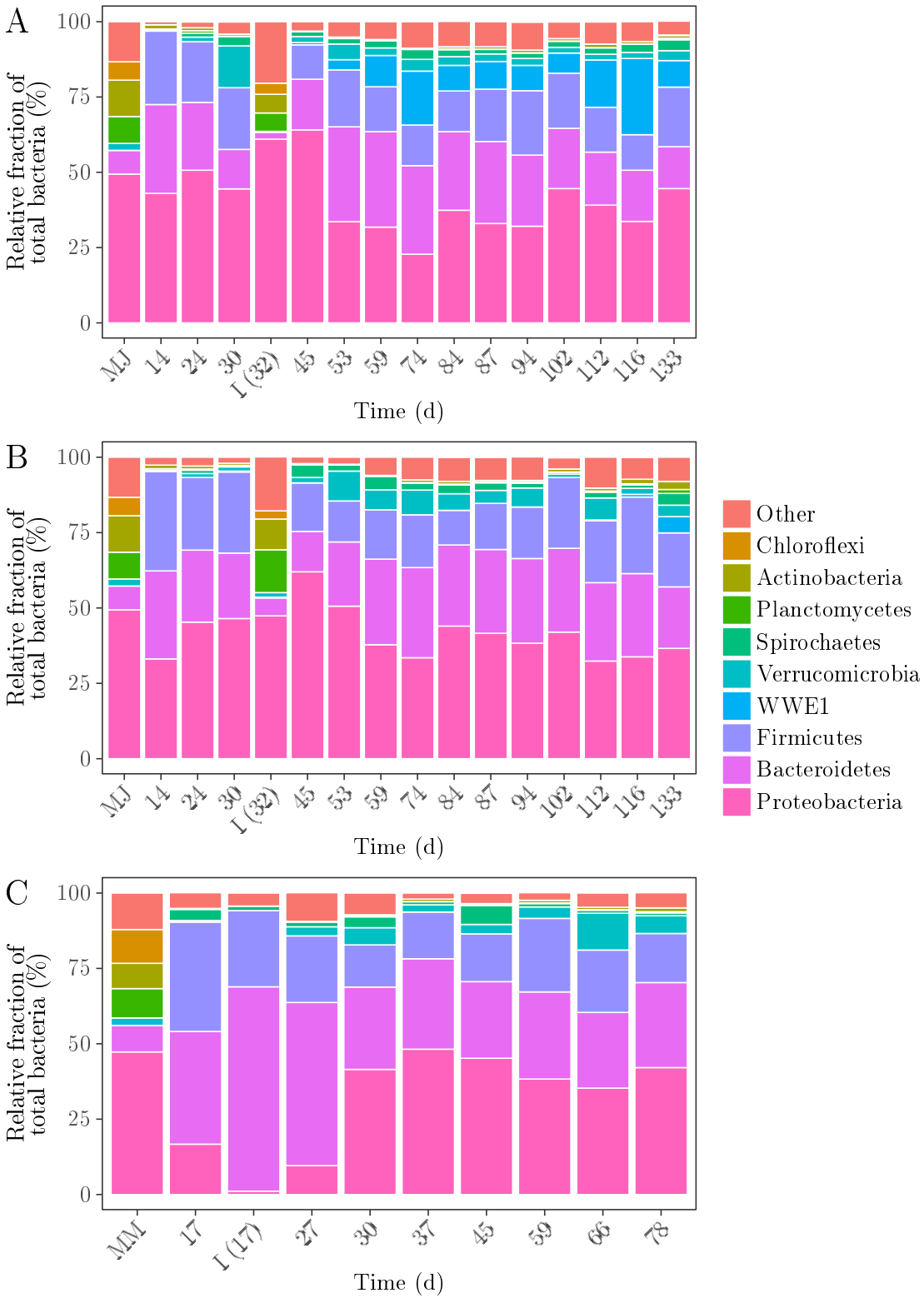

Figure 6.4 *Relative bacterial phylum distribution in reactors 1, 2 and 3 during the experiment. The analyses for cow manure (MJ and MM) and inocula (I + day of addition) are included in the time line. These samples were taken independently from their respective sources, not from the reactor effluents. Reactors 1 and 2 were started in January (MJ) and reactor 3 in March (MM), hence the slightly different compositions of the starting cow manure.*

In reactors 1 and 2 (Figure 6.4 A and B), the original cow manure and the inocula had similar compositions, and the dominant groups were Proteobacteria ($50-60\%$ in cow manure and both inocula), Planctomycetes ($6-14\%$) and Actinobacteria ($6-12\%$). Bacteroidetes ($2-8\%$) and Verrucomicrobia ($0.2-2\%$) were minorities in cow manure and both incula, but these groups were more abundant in the reactors during operation, in addition to Proteobacteria. The inoculum used for reactor 3 (Figure 6.4 C) contained only 1% of Proteobacteria, whereas Bacteroidetes was the dominating phylum with a fraction of nearly 70%. Firmicutes were abundant both in the inoculum (25%) and in the reactor ($15-30\%$). Still, the environment inside reactor 3 resulted in a similar composition in the bacterial phyla as in reactors 1 and 2.

The sediment sludge used by Zhao et al. (2010) as the inoculum in their bioreactors had some similarities to the cow manure in this work, with Proteobacteria as the largest phylum (42% of all bacteria) as well as the same dominating classes within this phylum, α-Proteobacteria and δ-Proteobacteria (class data not shown in this work). However, the fractions of Firmicutes and Chloroflexi (22% and 20%, respectively) were larger for Zhao et al. (2010).

Zhao et al. (2010) concluded that in their work a high relative amount of δ-Proteobacteria resulted in a high sulfate reduction efficiency in the reactors, especially in a lactate-fed reactor (95% of δ-Proteobacteria) compared to for example a reactor fed with ethanol and acetate (20% of δ-Proteobacteria). In this work, reactor 1 had the highest abundance of δ-Proteobacteria, approximately 10% during last sampling days (data not shown), whereas in reactors 2 and 3 the fractions were approximately $3-4\%$ and 2%, respectively (data not shown). Thus, lactate may improve sulfate reduction by favouring certain bacterial groups, but is not the only contributing factor, as the difference between reactors 2 (lactate feed) and 3 (no lactate feed) was not significant. Kaksonen et al. (2004a) also studied the differences between lactate and ethanol as substrates for biological sulfate reduction. Interestingly, they discovered that the ethanol-fed reactor had remarkably larger fraction of δ-Proteobacteria (57%) compared to the lactate-fed reactor (10%), although their performance in mine water treatment was similar. This difference between Zhao et al. (2010) and Kaksonen et al. (2004a) could have resulted from numerous reasons, including different reactor configurations (CSTR in Zhao et al. (2010) and FBR Kaksonen et al. (2004a)) and Zhao et al. (2010) using acetate as a co-substrate, which may have caused inhibition if complete oxidizing SRB were absent.

The DNA analyses in this work were able to differentiate the taxonomic rank of different families, but the specification of genera and species was often unclear. For this reason, a thorough presentation of different genera and species is excluded from

this work. The families which formed the majority of the reactor compositions (at least 50%) were examined in more detail in Figure 6.5.

Cow manure and both of the inocula in reactors 1 and 2 had similar types of bacteria (Figure 6.5 A and B), although the relative proportions varied. Inoculum 1 had nearly 5 times more Hyphomicrobiaceae (18%), over 2 times more Rhodobacteraceae (11%) and 6 times more Syntrophobacteraceae (6%) than inoculum 2 (4%, 5% and 1%, respectively), whereas Rhodospirillaceae and Flavobacteriaceae were more plentiful in inoculum 2 (6% and 3%, respectively) than in inoculum 1 (4% and 0.01%, respectively). The inoculum for reactor 3 (Figure 6.5 C) contained 2% of Bacteroidales, but in the reactor the fraction stabilized to approximately 13% of the whole bacterial population. A distinct feature of inoculum 3 was that its bacterial composition was more similar to other sampling days during the operation than inocula 1 or 2 and their respective other rector sampling days. So the microbial enrichment of the original cow manure describes most accurately the bacterial composition in all reactors during the operation.

During the experiment, the order of Bacteroidales was the most abundant type of bacteria in reactors 1 and 2 (majority belonging to an unspecified family) (Figure 6.5 A and B). The relative proportions varied mostly between 10% to 20%: in reactor 1 the fraction steadily decreased towards the end of the experiment and in reactor 2 the fraction fluctuated more. According to Krieg et al. (2010), the Bacteroidales order mostly comprises of anaerobic organisms, but sulfate-reducers are absent. Perhaps this order was the fastest to take control of an anaerobic environment, but as the fraction of sulfate reducers began to increase especially in reactor 1 (Desulfobacteraceae), the fraction of Bacteroidales started to decrease.

Most of the sulfate-reducers found in the reactors belonged to the family Desulfobacteraceae (Brenner et al. 2005a). In reactor 1 (Figure 6.5 A) the fraction steadily increased to 10%, but in reactor 2 (Figure 6.5 B) the final and highest fraction was only 3%. In reactor 3 (Figure 6.5 C), the maximum fraction was only 1.5% on days 30 and 78, while during the rest of the samplings days the fraction of Desulfobacteraceae remained below 1%. Interestingly, the fraction of Desulfobacteraceae was below detection limit in both cow manure samples and inocula 1 and 2. On the contrary, Syntrophobacteraceae was present in cow manure and inocula 1 and 2, but could not be detected from reactors during the operation, even though most members of this family are capable of reducing sulfate (Brenner et al. 2005a) (Figure 6.5 A and B). Somehow the conditions in reactor 1 favoured sulfate-reducers compared to reactors 2 and 3, since a steady and strongly growing Desulfobacteraceae population as that of reactor 1, was not established in these reactors.

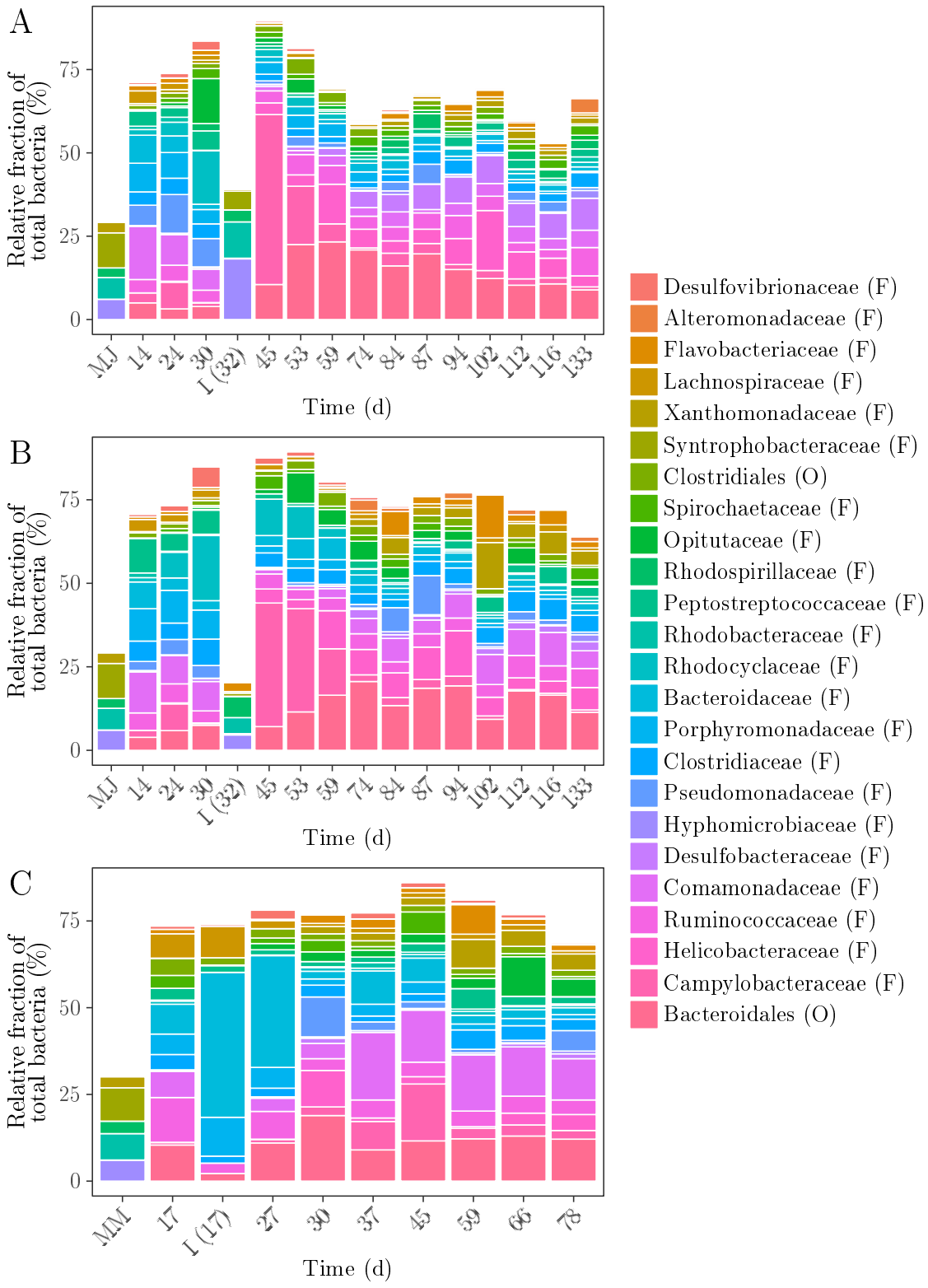

Figure 6.5 *Relative bacterial family distribution in reactors 1, 2 and 3 during the experiment. Symbols: MJ = cow manure in January, MM = cow manure in March, I (day) = inoculum + day of addition, (F) = bacterial family, (O) = bacterial order.*

Desulfovibrionaceae, another family of sulfate-reducers (Brenner et al. 2005a), was found abundantly from reactors 2 and 3, although this group was dominant only in the beginning of the experiment. The maximum fractions reached 6% on day 30 in reactor 2, and 2.6% on day 27 in reactor 3 (Figure 6.5 B and C).

The fraction Campylobacteraceae, a family containing microaerobic bacteria (Brenner et al. 2005a), was high in reactors 1 (51%) and 2 (37%) after adding the inocula, but steadily decreased in the reactors (Figure 6.5 A and B). Because the inocula did not contain any Campylobacteraceae, the nutritious growth media may have temporarily enhanced the growth of this family in the reactors. A similar increase and decrease of Campylobacteraceae was deteted in reactor 3, although the temporary increase in fraction occurred more slowly after the inoculum addition (Figure 6.5 C).

Another group that increased its share after inocula additions was Helicobacteraceae. This group's fraction was approximately $3-18\%$ of the total population in reactors 1 and 2 (Figure 6.5 A and B). In reactor 3, the fraction of Helicobacteraceae reached a peak of 10% on day 30, after which it lowered to 1% and increased again to 5% (Figure 6.5 C). The fractions alternated similarly in each reactor. The family was nearly equally divided between two genera, *Sulfuricurvum* and *Sulfurimonas* (data not shown). Bacteria belonging to the genus *Sulfuricurvum* are anaerobic or microaerobic and use sulfur compounds, such as elemental sulfur and sulfide, as electron donors, nitrate as electron acceptor and inorganic compounds as carbon sources (CO_2 and bicarbonate) (Kodama and Watanabe 2004). The same applies to many species in the *Sulfurimonas* genus, although many can utilise also organic carbon sources (Han and Perner 2015). The Helicobacteraceae increased when the sulfate reduction efficiency started to improve after day 40 in reactor 1 and 2, probably because there was more sulfide (and possibly elemental sulfur) to be utilised. In reactor 3, this suggested causality is less evident, perhaps because of differences in the microbial population and competition between microbial groups.

Families of anaerobic bacteria, which were present in all reactors in high fractions before the inocula additions included Porphyromonadaceae, Bacteroidaceae (Krieg et al. 2010) and Rhodocyclaceae (Brenner et al. 2005a) (Figure 6.5). Other stronger anaerobic groups probably replaced these families soon after inocula additions, whereas the anaerobic families of Ruminococcaceae and Clostridiaceae (De Vos et al. 2009) kept a steady share of the total population during the operation.

Aerobic groups of bacteria which fractions alternated during the operation in all reactors included Xanthomonadaceae, Pseudomonadaceae (Brenner et al. 2005b), Flavobacteriaceae (Krieg et al. 2010), Comamonadaceae and Hyphomicrobiaceae (Brenner et al. 2005a) (Figure 6.5). The presence of these families could indicate temporary oxygen accesses to the reactors. Most of these families had larger fractions in reactors 2 and 3 compared to reactor 1, indicating that anaerobic conditions were

probably maintained better in reactor 1.

No significant differences in bacterial diversity was noted between reactors, although the relative fractions of bacterial groups varied. Hiibel et al. (2011) reported that in their study the bacterial diversity was greater with complex, lignocellulosic substrates (like wood chips) compared to more simple ethanol. When comparing common simple substrates such as ethanol and lactate, both Kaksonen et al. (2004a) and Zhao et al. (2010) reported that the diversity in bacterial communities was greater in ethanol-fed reactors compared to lactate-fed reactors. Using cow manure as the substrate can be expected to increase the variety of bacterial species detected. Even though lactate was used in this work as a co-substrate, its fraction from the feed (25%) was not enough to greatly influence the bacterial diversity, although it affected the system performance.

The bacterial families including sulfate-reducing genera were examined more thoroughly (Figure 6.6). Most of the sulfate-reducers present in the samples of reactor 1 (Figure 6.6 A) belonged to the Desulfobacteraceae family, and the fraction steadily increased during the experiment reaching a final fraction of 8.5%. In reactors 2 and 3 (Figure 6.6 B and C), the fraction of Desulfobacteraceae increased as well, but more slowly and not in an even manner. In rector 2, the fraction increased quite rapidly to 2.5% by day 74, but decreased to less than 1% during the next sampling on day 84. The fraction continued to increase afterwards, but reached only 2.5% before the experiment was ended. Similar fluctuation occurred in reactor 3, as the fraction of Desulfobacteraceae peaked at 1.2% on day 30 and decreased below detection limit 7 days later. During the final sampling the fraction had increased to 1.3%. Most genera belonging to this family are complete oxidizers (Brenner et al. 2005a), so as their fraction increased, the concentration of acetate decreased in the reactors.

Most SRB in reactors 2 and 3 belonged to the *Desulfovibrio* genus (Figure 6.6 B and C), although the fraction decreased as the experiment continued and the fraction of Desulfobacteraceae increased. In reactor 1 (Figure 6.6 A), the *Desulfovibrio* was overcome by other groups more quickly. In reactor 2 the fraction was at maximum on day 30 (6.1%) before adding the inoculum. Afterwards the fraction started to gradually decline, although this genus was the dominant SRB group in the reactor until day 59. In reactor 3, the *Desulfovibrio* was the largest SRB group on all sampling days except day 30 and day 78. *Desulfovibrio* is an incomplete oxidizer (Figure 4.1), so the acetate production in the beginning of the experiment may have partly resulted from this genus.

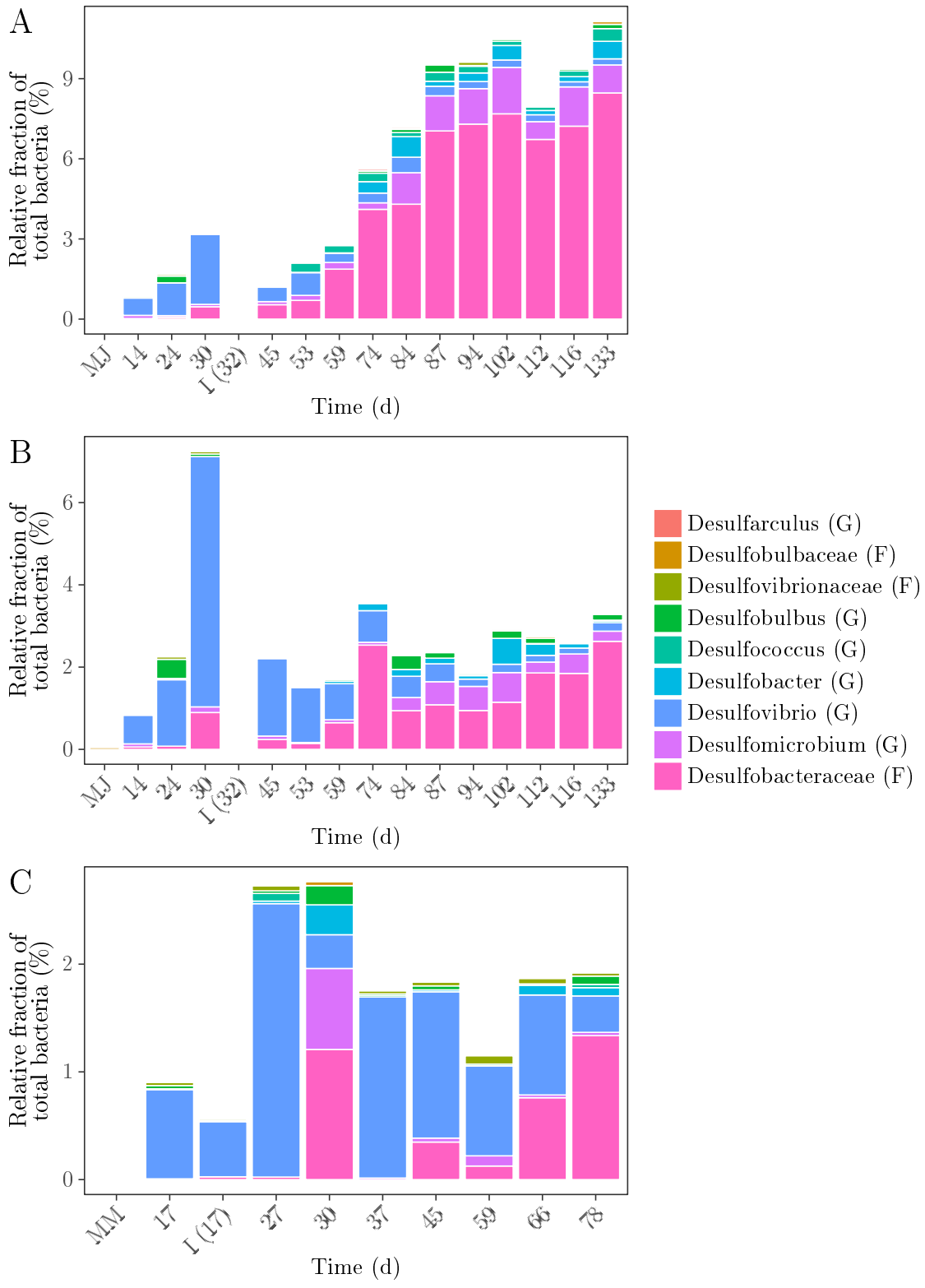

Figure 6.6 *Relative SRB distribution in reactors 1, 2 and 3 during the experiment. Symbols: MJ = cow manure in January, MM = cow manure in March, I (day) = inoculum + day of addition, (G) = bacterial genus, (F) = bacterial family. Note the different scaling in y-axes.*

Desulfomicrobium was the second most abundant group of SRB in reactor 1 (Figure 6.6 A), with a steady increase throughout the experiment, reaching a maximum fraction of 1.7% on day 102, and after a small decline the fraction increased to 1.1% on the final sampling day. In reactor 2 (Figure 6.6 B), the trend was similar and the maximum was also reached on day 102 (0.7%), although the *Desulfomicrobium* fractions in reactor 2 were approximately 1/3 of the fractions in reactor 1. *Desulfomicrobium* can incompletely oxidize lactate (Brenner et al. 2005a), which explains the increase after lactate addition. In reactor 3 (Figure 6.6 C), without any lactate feed, this genus was a minority on most sampling days (less than 0.1%). However, the *Desulfomicrobium* was abundant on day 30 (0.8%), although no profound reason for this was identified.

The fraction of *Desulfobacter* fluctuated in all reactors. The maximum values were 0.8% (day 84), 0.6% (day 102) and 0.3% (day 30) in reactor 1, 2 and 3, respectively (Figure 6.6). *Desulfococcus* was practically present only in reactor 1 (Figure 6.6 A), where the final fraction on day 133 was 0.5%. In other reactors the fraction of *Desulfococcus* was always below 0.1% and often below detection limit (Figure 6.6 B and C). The *Desulfobulbus* genus was a minor group in reactor 1 with fractions below 0.3% (Figure 6.6 A). In reactor 2 (Figure 6.6 B), the maximum of *Desulfobulbus* was on day 24 with 0.5%, and afterwards increased to above 0.3% only on day 84. In reactor 3 (Figure 6.6 C), the fraction of *Desulfobulbus* reached 0.2% on day 30, but otherwise stayed below 0.1%. The smallest groups of SRB in the reactors belonged to the families Desulfovibrionaceae and Desulfobulbaceae and the genus *Desulfarculus* (Figure 6.6).

In cow manure and inocula for reactors 1 and 2, nearly all SRB were below detection limit, excluding the 0.05% fraction of Desulfobulbaceae (Figure 6.6 A and B). The inoculum for reactor 3, however, contained 0.5% of *Desulfovibrio* in addition to small fractions of Desulfobacteraceae, *Desulfobacter* and Desulfovibrionaceae (Figure 6.6 C). The low fraction of sulfate-reducers in the inocula was surprising, because during inoculation the media was coloured black, indicating SRB growth and metal sulfide precipitation, and generated a strong smell of H_2S. Perhaps longer cultivation could have enriched more sulfate-reducers, as the samples from South Africa and the Finnish mine (inocula 1 and 2) grew in the media for approximately one week, whereas the cow manure enrichment inoculum for reactor 3 had been grown for over two weeks before inoculation. The inoculation caused the fraction of sulfate-reducers to decrease at first in reactors 1 and 2, perhaps because a proper SRB community had not been formed in the inocula and the added nutrients encouraged other bacteria to prosper inside the reactors (Figure 6.6 A and B). In reactor 3 (Figure 6.6 C), with a stronger SRB population, the inoculum managed to increase the fraction of sulfate-reducers, even though the increase ceased after day 30.

Cow manure as an inoculum in a passive biological sulfate reduction system was studied by Pruden et al. (2007). In their experiment the cow manure did not have much effect on sulfate reduction compared to more acclimated inoculum (from a previous sulfate-reducing reactor), and the performance was nearly the same as in a system without any inoculum. No SRB were detected from reactor samples (Pruden et al. 2007). The conditions were most likely unfavourable for the SRB in cow manure to grow enough biomass for effective sulfate reduction, whereas a previously adapted inoculum had an advantage. Mirjafari and Baldwin (2016) also questioned the applicability of cow manure as the inoculum, as the bacterial groups in cow manure were not abundant in reactors during the operation. However, in the case of this work, the fraction of some sulfate-reducers originating from the cow manure increased even before adding the enriched inocula in all reactors, so the operating conditions are crucial when considering the applicability of a certain inoculum. Of course, it would be ideal to use biomass from a steady-running reactor as the seed sludge without enrichment in growth media, but in this case the shipping time was too great (from South Africa to Finland) to ensure the activity of the microorganisms.

Lactate addition had different effects on reactors 1 and 2. For reactor 1, lactate seemed to enhance SRB growth and slightly improved sulfate reduction (Figure 6.6 A), which was reported also by Rasool et al. (2015), but for reactor 2 the effect was not so clear. The highest SRB concentration in reactor 2 after the inoculum addition was on day 74 (the start of lactate feed), and the situation did not improve even until the end of the experiment (Figure 6.6 B). The SRB groups were similar to reactor 1, but for some reason the relative fractions were considerably lower in reactor 2 despite the identical lactate feed.

Hiibel et al. (2011) compared ethanol and cellulosic materials as substrates in down-flow bioreactors. The SRB fraction was greater with ethanol (70%) than with other substrates (up to 5%). However, the absolute amount of bacteria detected with qPCR was lower with ethanol, probably because of less available support material for the bacterial biomass. As the sulfate reduction efficiency was similar in all reactors, Hiibel et al. (2011) concluded that in their work the absolute amount of SRB was more important for system performance than the relative fraction of all bacteria.

Direct comparison of microbial analyses in other studies to the ones in this work is not straightforward, as in most cases (Kaksonen et al. 2004a; Pruden et al. 2007; Sarti et al. 2010; Zhao et al. 2010; Hiibel et al. 2011; Rasool et al. 2015) the DNA is extracted from the carrier material or the biomass itself, whereas here the samples were taken from the mostly clear (though not filtrated) effluent. Whether the bacteria detected in the effluent had similar composition in the sludge bed could not

be confirmed with this sampling technique. However, a frequent removal of sludge from UASB for microbial analyses can impair the sulfate reduction, both because of oxygen access to the system and excess biomass removal. Probably for this reason as thorough and equally long-term study of bacterial communities and their transformations could not be found from the available literature.

To study the relations of different sulfate-reducing genera/families on other parameters measured from the reactors (e.g. pH, redox potential, sulfate reduction efficiency), the relative fractions of SRB were plotted against different parameters in Figure 6.7.

Desulfobacteraceae clearly dominated when the reactors had been operated for a longer time (over 80 days) (Figure 6.7 A), the pH was high (near 7.5) (Figure 6.7 B) and the SRB were abundant in the reactors (Figure 6.7 E). However, *Desulfovibrio* dominated during the first 60 days of operation (Figure 6.7 A) and at pH values below 7.0 (Figure 6.7 B). Interestingly, the fraction of *Desulfovibrio* was greater when the redox potential was the lowest (below -250 mV) (Figure 6.7 C). *Desulfovibrio* was also the largest group when sulfate reduction efficiency was the highest (near 80%) (Figure 6.7 D), but as the fraction of *Desulfovibrio* exceeded the fraction of Desulfobacteraceae in only one sampling point, no thorough conclusions on the sulfate-reducing capability between these two groups can be made. When sulfate reduction efficiency was $40-70\%$, Desulfobacteraceae was clearly the largest group, whereas *Desulfovibrio* was the dominating group when sulfate reduction efficiency was below 30% (Figure 6.7 D). Similarly with SRB concentrations, the fraction of *Desulfovibrio* was greatest at the highest concentration of 10^{9} SRB copies/ml, but an error in sampling is also possible.

Other SRB groups did not seem to be especially enhanced by any parameter, and the relative fractions remained low. However, when the fraction of Desulfobacteraceae started to increase, the fraction of *Desulfomicrobium* increased as well, even though the fraction of *Desulfomicrobium* stayed below 2% at all times (Figure 6.7). This could indicate a co-operative relationship between these two groups of SRB, whereas Desulfobacteraceae and *Desulfovibrio* are more likely to be competing groups of SRB, because of their shift in dominance depending on the reactor parameters.

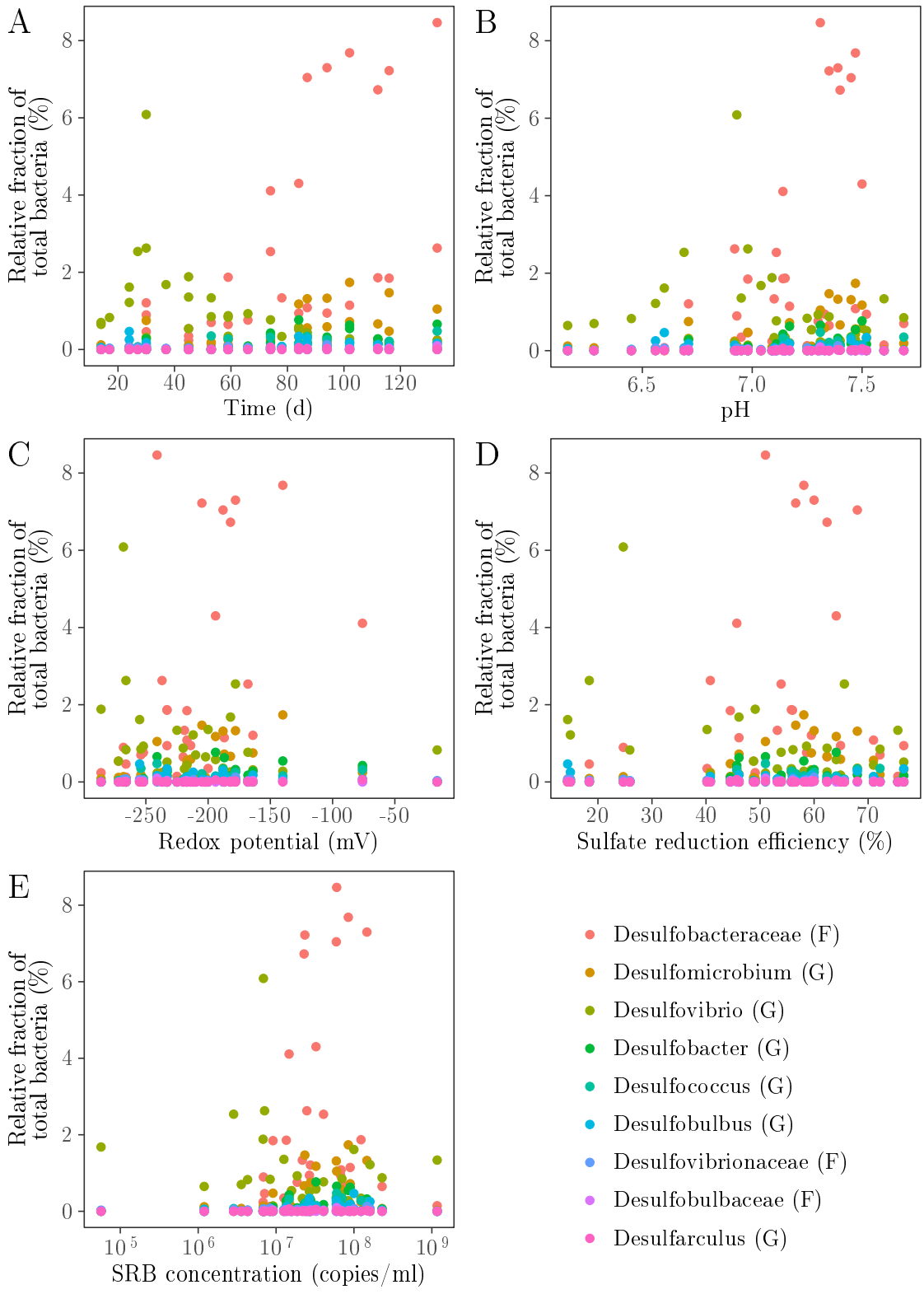

Figure 6.7 *Relative SRB distribution during the experiment compared to operation time, pH, redox potential, sulfate reduction and SRB concentration of the reactors. Symbols: (G) = bacterial genus, (F) = bacterial family. The figures include data from all three reactors.*

6.4 Sulfur recovery

In sulfide oxidation experiment 1, effluent from the UASB reactors (approximately 100 ml) was purged with air (0.1 l/min) until the pH did not increase significantly anymore (Figure 6.8).

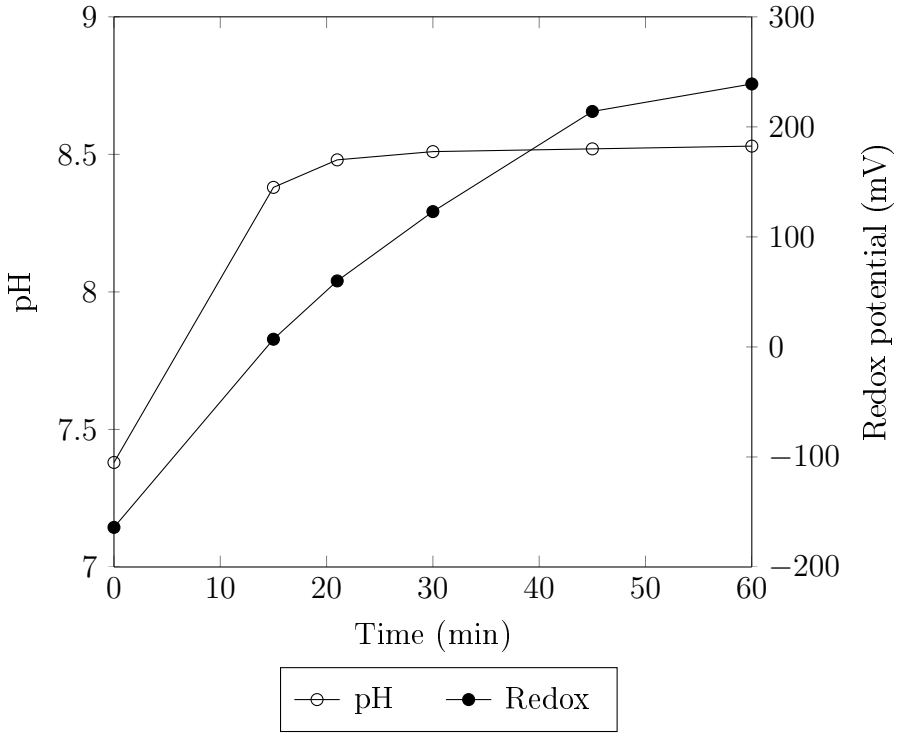

Figure 6.8 *The pH and redox potential values of sulfide oxidation experiment 1: effluent oxidation (Setup A).*

After 20 minutes the air flow was stopped as the pH of the solution had increased quickly to 8.5 but then remained at this level. This was expected as sulfide oxidation to elemental sulfur generates hydroxide ions (Equation 3.3). A decrease in pH would have indicated unwanted sulfate formation (Equation 3.4). The redox potential continued to increase even after ceasing the air flow because of mixing the solution with a magnetic stirrer and letting some oxygen to dissolve in the solution. After one hour the sulfide concentration had decreased from 48 mg/l to below detection limit and the experiment was ceased. As an indication of elemental sulfur formation, the solution that was clear in the beginning had turned more turbid. While filtering the final sample for the sulfate measurement, a clear resistance was felt when using a syringe filter compared to samples taken in the beginning of the experiment, which could have been caused by elemental sulfur particles. The sulfate concentrations remained nearly similar (from 550 mg/l to 460 mg/l), so sulfide was either oxidized to elemental sulfur or lost as gaseous H_2S. The exact amount of elemental sulfur formed was impossible to ascertain, as the starting sulfide concentration was very

low and only little precipitate could be collected onto a filter paper.

In sulfide oxidation experiment 2, inoculum containing enriched sulfide oxidizers from reactor 2 was added to reactor effluent (Figure 6.9).

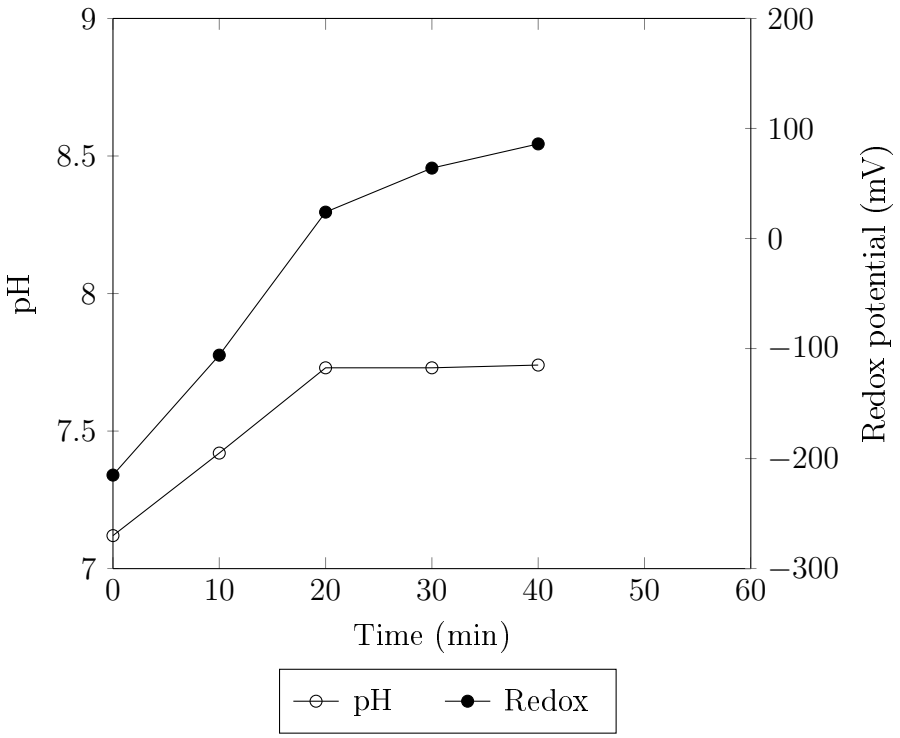

Figure 6.9 *The pH and redox potential values of sulfide oxidation experiment 2: effluent and inoculum oxidation (Setup A).*

As sulfur oxidation had been active in the growth medium, the sulfate concentration was high (2500 mgSO_4^{2-}/l) in the inoculum and the pH was low (yellowish colour of the inoculum). High sulfate concentration and a low pH had an immediate effect on the starting conditions of the experiment. The pH was a bit lower in the beginning (7.1) than in experiment 1 (Figure 6.8) and increased only to 7.7, even though the redox potential did not increase as much as in experiment 1 (Figure 6.8). In addition there was no resistance when filtering the final solution, and the sulfate concentration of the liquid increased a little towards the end (from 750 mg/l to 850 mg/l). Sulfide was oxidized to below detection.

A small part of sulfide was probably oxidized to elemental sulfur, because the pH increased, but mostly sulfide was either oxidized to sulfate or transformed to H_2S due to the decrease in pH caused by the inoculum addition. An interesting phenomenon during the experiment was that after 20 minutes some beige coloured, slowly settling precipitate was formed in the liquid. Most probably this was due to unknown chemical reactions between different compounds in the solutions, and not related to sulfide oxidation.

Sulfide oxidation experiment 3 was conducted with three samples: a sample with only effluent, a sample with effluent and inoculum, and a sample with effluent and uninoculated growth medium (Figure 6.10, Table 6.2).

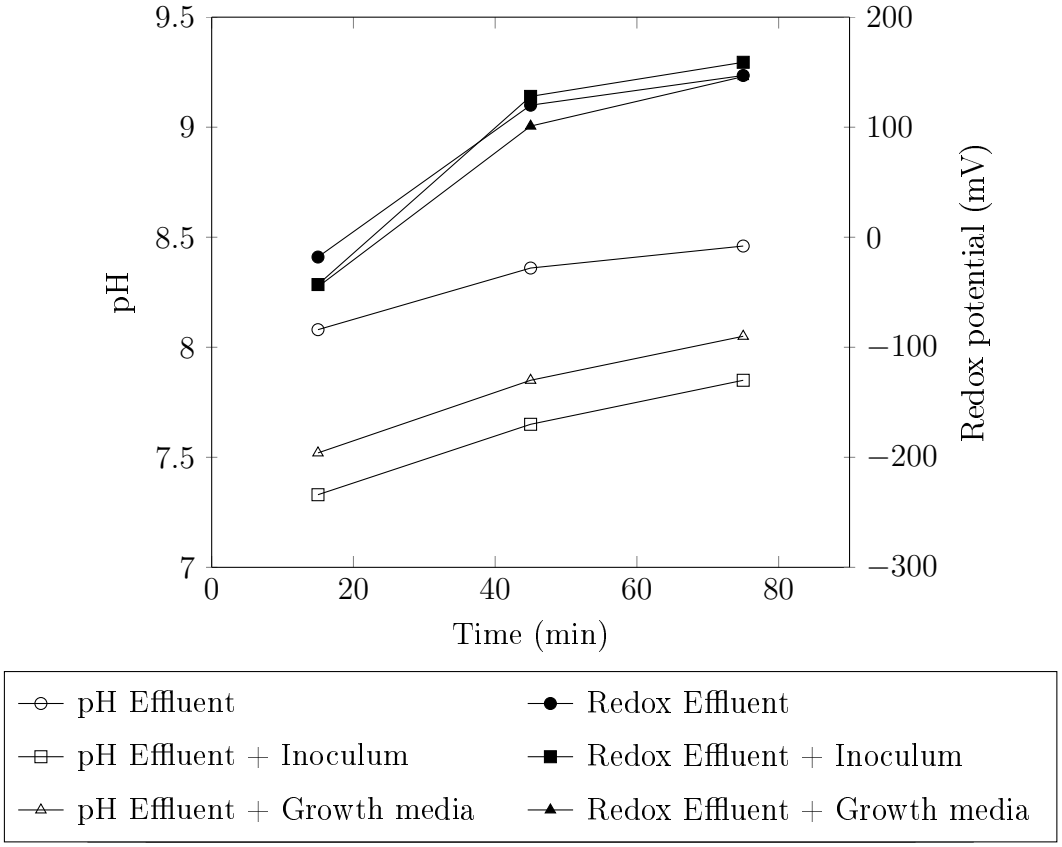

Figure 6.10 *The pH and redox potential values of sulfide oxidation experiment 3: effluent, effluent with inoculum and effluent with growth medium (Setup B).*

Table 6.2 *Samples used in sulfide oxidation experiment 3.*

	pH	Redox (mV)	SO_4^{2-} (mg/l)	HS^- (mg/l)
Effluent	7.3	-222	460	53
Inoculum	5.3	139	2400	-
Growth media	7.2	156	120	-

This was operated as setup B, where the samples were mixed in bottles in a shaker without active air feed. The experiment was ceased when the pH of pure effluent had increased to 8.5 as in experiment 1. Because of the small test volumes (10 ml), no sulfide measurements were conducted during this experiment.

The redox potential changes were near identical with all three samples (from -50 mV to 150 mV). The pH increased the most in the sample with only effluent (from pH 7.3 to pH 8.5), whereas the sample with inoculum had the lowest final pH

value of 7.9, followed closely by sample with growth medium (final pH 8.0). The sulfate concentrations increased in samples with inoculum (from approximately 640 mg/l to 800 mg/l) and growth medium (from approximately 430 mg/l to 640 mg/l). Similarly to experiment 2, unknown precipitate was formed in these two samples (Figure 6.11). The sample with only effluent turned more turbid and there was more resistance when filtering compared to other samples, so elemental sulfur was most probably formed in this solution.

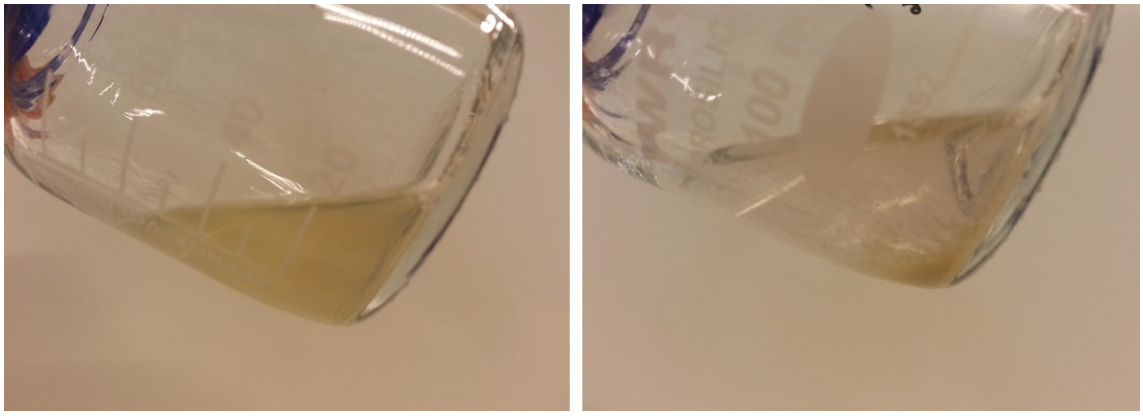

Figure 6.11 *Photos of two sample bottles after the sulfide oxidation experiment 3 with setup B. Left photo shows a sample with only effluent, right photo shows a sample with effluent and inoculum.*

Based on these batch experiments we could assume that chemical oxidation is better than biological oxidation, as elemental sulfur is formed and no sulfate is produced. With the addition of inoculum the sulfate concentrations increased and elemental sulfur formation was not detected. However, because growth media affects sulfide oxidation with partly unknown mechanisms, no profound conclusions can be made from biological oxidation. In addition the small test volume and low sulfide concentration could not result in high elemental sulfur yield. A continuous system could offer a more suitable platform to study the true potential of elemental sulfur formation and collection, as more sulfide can be oxidized. Even without the addition of inoculum, the resulting system would probably be a naturally enriched biological process, as sulfide oxidizers from sulfate reducing reactors would multiply when exposed to an aerobic environment. In a continuous system, the challenges would be to prevent the sulfur from oxidating to sulfate as well as to increase the size of sulfur flocks, for example with the addition of flocculant (Chen et al. 2016), to ease the filtration process.

7. CONCLUSIONS

Sulfate removal from real mine drainage water was studied in three continuously operated UASB bioreactors at $20-22\,^{\circ}C$ using cow manure as the main substrate. The microbial communities inside the reactors developed from the added fresh cow manure as well as the additional enriched inocula from different origins. Biological sulfate reduction produced sulfide, which in the absence of metal ions could be oxidized to elemental sulfur in batch experiments. The interactions of different bacterial groups as well as their contribution to the reactor performance was evaluated with DNA analyses.

The efficiency of sulfate reduction in different reactors varied between 40% to 60% with a sulfate reduction rate of $400-600$ mg/l*d (Table 7.1). During stable operation, the highest sulfate reduction efficiency was obtained with reactor 1, which contained additional inoculum enriched from a sulfate-reducing bioreactor operated with cow manure in South Africa (Table 7.1). The second highest sulfate reduction efficiency was obtained with reactor 2, with additional inoculum enriched from holding pond tailings of a Finnish mine (Table 7.1). Reactor 3, which was inoculated both with fresh cow manure and an additional enrichment culture of the same origin, had the lowest sulfate reduction efficiency (Table 7.1). With reactors 1 and 2, the highest sulfate reduction efficiencies results were obtained using lactate as a co-substrate with cow manure. Sulfide concentrations in the effluents were the highest with reactor 1 and the lowest with reactor 3 (Table 7.1).

Table 7.1 *A summary of the sulfate-reducing bioreactors and their results obtained in this thesis.*

	Inoculum	Substrate	Sulfate removal	Sulfide (mg/l)
Reactor 1	Operating bioreactor	Cow manure + Lactate	$500-600$ mg/l*d $50-60\%$	55
Reactor 2	Mine holding pond	Cow manure + Lactate	$400-500$ mg/l*d $40-50\%$	45
Reactor 3	Cow manure	Cow manure	400 mg/l*d 40%	30

The periodical substrate feed combined with the flow-through (no inner recycling of liquid) configuration of the UASB reactors did not enable efficient sulfate removal. In the beginning the easily degradable fraction of cow manure ensured a proper start to sulfate reduction, but the diluted substrate probably did not provide enough accessible carbon to sustain operation. Instead, a CSTR could be more practical when using organic wastes such as cow manure. In that case, the solid substrate could be provided in greater quantity to compensate for the slowly degradable fraction. Other option could be the circulation of liquid inside the UASB reactor through a recycle line, to increase the availability of substrate to microorganisms in the sludge bed. Nevertheless, a continuous substrate feed would be optimal for efficient sulfate removal, and this may be difficult to obtain with cow manure, because of both the availability of cow manure near a mining site and the difficulty of dosing a solid substrate. The use of a liquid, more easily storable substrate (for example ethanol), could also be an option in large scale operation.

Other suggestions for improved sulfate reduction are the use of lactate in the beginning to increase the amount of sulfate-reducing biomass, operating the reactors at a higher temperature (approximately 30 °C) to create more favourable conditions for mesophilic sulfate reducers and increasing the sludge bed volume to increase the active area for biological sulfate reduction. The permeability of the sludge bed should be increased as well to prevent the channelling of flow and possible clogging of the reactors by floating sludge.

Quantitative analysis of the *dsr*B gene showed fluctuating SRB concentrations in all reactor effluents. The start of continuous feed had a positive effect on SRB concentrations, whereas the addition of $NaHCO_3$ affected the SRB concentrations negatively. The start of lactate feed did not have a permanent effect on the SRB concentrations in reactors 1 and 2. Despite the fluctuations, the SRB concentrations in all reactor effluents had a slightly rising trend towards the end of the operation.

The DNA analyses showed that a wide range of different bacterial groups were present in the reactor effluents, the most belonging to phyla Proteobacteria and Bacteroidetes, and further to order Bacteroidales and families Campylobacteraceae, Helicobacteraceae and Comamonadaceae, to name a few. The families containing many sulfate reducers, for example Desulfobacteraceae and Desulfovibrionaceae, could not be detected either from the start-up cow manure or any other inocula besides the cow manure enrichment used as an inoculum for reactor 3. The family Desulfobacteraceae was the dominating group of sulfate reducers when for example sulfate reduction and SRB concentration were high.

The frequent microbial analyses from the reactor effluents might offer insight to the biological interactions present inside the sulfate-reducing bioreactors. To acquire more representative results, and perhaps to make new discoveries, sampling from

the sludge bed without disturbing the reactor operation could be developed. To get an even more thorough overview of the biology involved in the reactions, the Archaea and the Eukaryota domains should also be investigated.

Because of low sulfide concentrations in the effluents (Table 7.1), the sulfide oxidation experiments did not produce enough precipitate for further processing and analysis, but the formation of elemental sulfur was assumed to occur based on reaction chemistry. Oxidation without inoculum addition was considered the best alternative, as the growth medium disturbed the measurements and caused the formation of unknown precipitates. Continuous oxidation of sulfate-reducing bioreactor effluents could have produced enough elemental sulfur for proper analyses.

Although the sulfate reduction efficiencies in the experimental part of this study could be improved, the obtained efficiencies are comparable with sulfate-reducing bioreactors in other studies, especially when taking the substrate, the reactor model and the operating temperature into account. Therefore, the sulfate reduction method described in this work could be a viable option for biological sulfate removal from mining waste waters, depending on the required sulfate removal efficiency and rate, among others, and the possibility to recover elemental sulfur from the effluent exists.

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APPENDIX A: OPERATION OF THE UASB REACTORS

Table A.1 *Operative details and the analyses for sulfate, acetate, nutrients and TOC in reactor 1.*

Day	$SO_{4,in}^{2-}$ (mg/l)	$SO_{4,out}^{2-}$ (mg/l)	$SO_{4,red}^{2-}$ $(mg/l*d)$	Acetate (mg/l)	NH_4^+ (mg/l)	$PO_4^{3-}-P$ (mg/l)	TOC (mg/l)	Remarks
0	2125							Reactor in batch mode for 15 days.
15	1140							Start of continuous operation.
21	1140			289	28	12	260	
22	1140							Start of cow manure addition.
24	1140	970	149					0.6 g of $NaHCO_3$ was added for pH control.
28	1140	896	214					0.6 g of $NaHCO_3$ was added for pH control.
30	1140	930	184	218				
32								Addition of inoculum. In batch mode for 3 days.
35	1122	730	360					Start of continuous operation.
38	1122	639	424		25.2	7.2	224	
42	1122	612	447					
45	1122	575	480	52	26.8	8.4		In batch mode for 8 days.
53	1122	312	711					Start of continuous operation.
56	1122	451	589					
59	1122	496	549	BD	24	7.3	52	
63	1122	567	487					

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Table A.1 continued from previous page.

Day	$SO_{4,in}^{2-}$ (mg/l)	$SO_{4,out}^{2-}$ (mg/l)	$SO_{4,red}^{2-}$ $(mg/l * d)$	Acetate (mg/l)	NH_4^+ (mg/l)	$PO_4^{3-}-P$ (mg/l)	TOC (mg/l)	Remarks
66	1092	565	462	BD				
71	1092	568	460					
74	1092	593	438	BD	10.5	6.5	32.4	Start of lactate addition.
77	1092	446	567					
80	1092	397	610	BD				
84	1092	392	614					
87	1070	349	652	BD	4.6	3.9	29.2	
91	1070	451	543					
94	1070	428	563	BD				
98	1070	429	562					
102	1132	448	546	BD	2.7	3.2	22.8	
105	1132	448	546					
112	1132	426	619					
116	1132	491	562	BD	6.1	3.3	19.2	
119	990	433	613					
123	990	493	436	BD				
133	990	485	443					

BD = below detection

Table A.2 *Operative details and the analyses for sulfate, acetate, nutrients and TOC in reactor 2.*

Day	$SO_{4,in}^{2-}$ (mg/l)	$SO_{4,out}^{2-}$ (mg/l)	$SO_{4,red}^{2-}$ $(mg/l*d)$	Acetate (mg/l)	NH_4^+ (mg/l)	$PO_4^{3-}-P$ (mg/l)	TOC (mg/l)	Remarks
0	2125							Reactor in batch mode for 15 days.
15	1140							Start of continuous operation.
21	1140			365	35	11	380	
22	1140							Start of cow manure addition.
24	1140	976	144					0.6 g of $NaHCO_3$ was added for pH control.
28	1140	922	191					0.6 g of $NaHCO_3$ was added for pH control.
30	1140	858	247	230				
32								Addition of inoculum. In batch mode for 3 days.
35	1122	622	454					Start of continuous operation.
38	1122	550	502		35.6	10.4	284	
42	1122	561	492					
45	1122	571	483	112	29	9.9		In batch mode for 8 days.
53	1122	275	743					Start of continuous operation.
56	1122	329	696					
59	1122	422	614	BD	23.6	7.2	52	
63	1122	450	589					
66	1092	598	433	BD				
71	1092	477	539					

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Table A.2 continued from previous page.

Day	$SO_{4,in}^{2-}$ (mg/l)	$SO_{4,out}^{2-}$ (mg/l)	$SO_{4,red}^{2-}$ $(mg/l * d)$	Acetate (mg/l)	NH_4^+ (mg/l)	$PO_4^{3-}-P$ (mg/l)	TOC (mg/l)	Remarks
74	1092	503	517	BD	12.3	7.1	38	Start of lactate addition.
77	1092	390	616					
80	1092	289	704	BD				
84	1092	255	734					
87	1070	317	680	BD	3.3	3.7	28	
91	1070	413	576					
94	1070	376	609	BD				
98	1070	410	579					
102	1132	577	432	BD	1.4	3.5	24.4	
105	1132	565	443					
112	1132	498	556					
116	1132	628	442	BD	2.6	3.9	21.2	
119	990	595	471					
123	990	659	290	BD				
133	990	586	354					

BD = below detection

Table A.3 *Operative details and the analyses for sulfate, acetate, nutrients and TOC in reactor 3.*

Day	$SO_{4,in}^{2-}$ (mg/l)	$SO_{4,out}^{2-}$ (mg/l)	$SO_{4,red}^{2-}$ $(mg/l*d)$	Acetate (mg/l)	NH_4^+ (mg/l)	$PO_4^{3-}-P$ (mg/l)	TOC (mg/l)	Remarks
0	1118							Reactor in batch mode for 27 days.
17								Addition of inoculum.
27	1070							Start of continuous operation.
30	1070	453	583	720	85.8	38.5	520	Start of cow manure addition.
34	1070	509	492					1.2 g of $NaHCO_3$ was added for pH control.
37	1070	577	432	340				
41	1070	630	386					
45	1132	640	377	BD	19.9	14.6	40	
48	1132	637	380					
55	1132	494	560					
59	1132	497	557	16	12.1	6.2	72	
62	990	409	634					
66	990	410	509	BD				
76	990	494	435					
78	990	463	462	BD	12.2	6.2	35.2	
85	1140	510	553					
92	1140	703	383	BD	12.5	6.4	30	
99	1160	559	510					

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Table A.3 continued from previous page.

Day	$SO_{4,in}^{2-}$ (mg/l)	$SO_{4,out}^{2-}$ (mg/l)	$SO_{4,red}^{2-}$ $(mg/l*d)$	Acetate (mg/l)	NH_4^+ (mg/l)	$PO_4^{3-}-P$ (mg/l)	TOC (mg/l)	Remarks
106	1160	772	340	BD	7	6.7	26.4	
113	1160	693	410					
120	1116	682	419	BD	8.0	5.9	30	
127	1116	660	400					

BD = below detection

APPENDIX B: MEDIUM FOR SULFUR-OXIDIZERS

Table B.1 *Thiobacillus thioparus (TK-m) medium, adapted from Leibniz-Institut DSMZ GmbH (2015c).*

Reagent	Amount
KH_2PO_4	2 g/l
K_2HPO_4	2 g/l
NH_4Cl	0.4 g/l
Na_2CO_3	0.4 g/l
$MgCl_2 \cdot 6\,H_2O$	0.2 g/l
Vitamin solution	3 ml/l
Trace element solution	1 ml/l
Bromocresol purple *	2 ml/l
$Na_2S_2O_3 \cdot 5\,H_2O$	5 g/l
Vitamin solution **	
Thiamine$-HCl \cdot 2\,H_2O$	10 mg/l
Nicotinic acid	20 mg/l
Pyridoxine-HCl	20 mg/l
p-Aminobenzoic acid	10 mg/l
Riboflavin	20 mg/l
Ca-pantothenate	20 mg/l
Biotin	1 mg/l
Vitamin B_{12}	1 mg/l
Trace element solution ***	
Na_2-EDTA	50 g/l
$ZnSO_4 \cdot 7\,H_2O$	11 g/l
$CaCl_2 \cdot 2\,H_2O$	7.34 g/l
$MnCl_2 \cdot 4\,H_2O$	2.5 g/l
$CoCl_2 \cdot 6\,H_2O$	0.5 g/l
$(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$	0.5 g/l
$FeSO_4 \cdot 7\,H_2O$	5 g/l
$CuSO_4 \cdot 5\,H_2O$	0.2 g/l
NaOH	ca 11 g/l

* Saturated aqueous solution
** Leibniz-Institut DSMZ GmbH (2015b)
*** Leibniz-Institut DSMZ GmbH (2015a)