Sulfate Reduction under Acidic Conditions for Selective Metals Recovery

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Abstract

Process streams with high concentrations of metals and sulfate are characteristic for the mining and metallurgical industry. Sulfate reducing bacteria convert sulfate to sulfide that can subsequently be used to recover metals as insoluble metal-sulfides. High rate sulfate reduction under acidic conditions opens possibilities for new processes that allow the selective recovery of metals from waste and process water. The thesis aimed at developing processes for selective metal recovery from mixed metal waste and process streams using microbial sulfate reduction under acidic conditions. The development of this process at industrial interesting rates requires knowledge on microbiology, chemistry and process technology. In this thesis, high rate sulfate reduction has been demonstrated at pH 6.0, 5.0, 4.5 and 4.0 in a membrane bioreactor by using formate and hydrogen as electron donor. The specific activity of the sulfate reducing bacteria was relatively constant from pH 6.0 to 4.0, while the volumetric activity increased with the pH. This was due to the biomass yield on sulfate that increased with the pH. The removal of sulfide from the reactor liquor at pH 5.0 had a major impact of the reactor performance and microbial population. Simultaneous sulfate reduction and metal precipitation was investigated with zinc in a single reactor operated at low pH in which zinc precipitated as crystalline sphalerite. A single stage gas-lift bioreactor was fed with a nickel-iron influent to study the selective recovery potential of nickel at pH 5.0. The results show that nickel could be selective recovered from iron, but that a lower pH or sulfide concentration would have resulted in a better separation between nickel and iron. This study shows that selective metal precipitation in a single stage low pH sulfate reducing bioreactor has potential to produce metal-sulfides that can be used by the metallurgical industry as resource for metal production.

Overview of thesis

1

Sulfate reduction for inorganic waste and process water treatment

Abstract

Many inorganic waste and process streams contain high concentrations of sulfate and are frequently accompanied with high metal concentrations, for example in the mining and metallurgical industry. Sulfate reduction is a proven technology for the treatment of these streams, in which products as metal-sulfides and elemental sulfur can be gained. This review discusses the knowledge acquired on sulfate reduction from research to full-scale operation. The main focus is on possible electron donors, process conditions and bioreactors types used for sulfate reduction. Current en future sulfate reducing applications will be described and research questions for future development will be stated.

1. Introduction

Many organisms assimilate sulfur originating from sulfate into their biomass because sulfur is an essential element for all living organisms (Fauque, 1995). Dissimilatory sulfate reduction, on the other hand, is the reduction of sulfate to sulfide to obtain energy for growth and maintenance. This metabolic feature is exclusive for sulfate-reducing bacteria (SRB). Eight mol reduction equivalences are needed for the reduction of one of mol sulfate to one mol of sulfide. These reduction equivalents are obtained by the oxidation of a variety of electron donors (e-donor) to carbon dioxide and water (Eq. 9-12, Table 1). SRB are a diverse group of prokaryotes (Castro et al., 2000), found nearly everywhere in waters, sediments and soils because of their ability to use a wide range of substrates and the ability of many SRB to tolerate extreme conditions (Postgate, 1984). However, sulfate reduction (SR) only occurs when an electron acceptors with a higher redox potential (e.g. oxygen and nitrate) is absent. These sulfate reducing conditions are found in soils (Kusel and Drake, 1995), sediments and stratified waters due to the limited penetration depth of oxygen. Sulfide produced in the anoxic compartment will be partly transported to the aerobic compartment where sulfide is oxidized back to sulfate, and visa versa (Bottrell and Newton, 2006; Holmer and Storkholm, 2001). SR and sulfide oxidation form the main routes of the biological sulfur cycle.

The earth's crust contains large amounts of immobilized sulfides, e.g. pyrite (FeS_2), chalcopyrite ($CuFeS_2$) and sphalerite (ZnS). During mining and processing of ores and fossil fuels (e.g. coal), sulfide minerals are oxidized to sulfuric acid and heavy metals are mobilized, which has resulted in many environmental problems (Lens et al., 2002). Waste streams from the mining industry are called acid mine drainage (AMD) or acid rock drainage, which typically have a pH between 2 and 4. The acidity of AMD is formed by bio-oxidation of sulfide minerals like pyrite (Eq. 13) (Johnson, 2000).

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \qquad (13)$$

This paper focuses on the use of dissimilatory SR by SRB to remove sulfate from waste and process streams and to simultaneously recover sulfur and metals. The following aspects will be discussed: applications, el-donors, process conditions and bioreactor types suitable for SR. Furthermore, possible process schemes and future prospects of SR will be addressed.

Table 1: Stochiometry and Gibbs free energy changes of conversions that play a role in sulfate reducing bioreactors. Gibbs free energy changes were calculated from Thauer *et al.* (1977).

Eq	Reaction equations, in which 8 e-mol are converted	ΔG°' [kJ.mol^{-1}]
	Acetogenesis	
1	$C_2H_5OH + H_2O \rightarrow CH_3COO^- + 2H_2 + H^+$	+9.6
2	$^4/_3CH_3OH + {}^2/_3HCO_3^- + H^+ \rightarrow CH_3COO^- + {}^4/_3H_2O$	-21
3	$4CO + 4H_2O \rightarrow CH_3COO^- + 2HCO_3^- + 3H^+$	-165
4	$4H_2 + 2\ HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$	-105
	Methanogenesis	
5	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31
6	$^4/_3CH_3OH \rightarrow CH_4 + {}^1/_3HCO_3^- + {}^1/_3H_2O + {}^1/_3H^+$	-104
7	$4CO + 5H_2O \rightarrow CH_4 + 3HCO_3^- + 3H^+$	-196
8	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-136
	Sulfate reduction	
9	$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	-48
10	$^4/_3\ CH_3OH + SO_4^{2-} \rightarrow 4/3\ HCO_3^- + HS^- + 4/3H_2O + 1/3H^+$	-121
11	$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	-152
12	$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$	-17

2. Waste and process streams with sulfate

Waste or process streams treated with SR can be divided into three groups: (1) streams with high organic and small sulfate content, (2) inorganic streams with only sulfate, and (3) inorganic streams with sulfate and dissolved metal-ions. Streams with a high organic content can be used to produce biogas in methanogenic reactors. In these reactors, SR is an unwanted side process due to e-donor loss and sulfide inhibition of the methanogenic process by sulfide (Colleran et al., 1995). The effect of SR in methanogenic bioreactors has been well described (Colleran et al., 1995; Oude Elferink et al., 1994) and is not the focus of this paper. In inorganic waste or process streams with a too high sulfate concentration (e.g. because of restrictions in legislation), sulfate can be removed by applying SR. An e-donor is required for SR, which has to be added when the organic content of the waste or process water is insufficient. The produced sulfide can be subsequently removed from the water as insoluble elemental sulfur by applying partial oxidized with oxygen in a second bioreactor (Buisman and Lettinga, 1990; Janssen et al., 1999; Van Den Bosch et al., 2007). When a waste or process water contains both metal-ions and sulfate, metal-ions can be removed from the water by precipitation with the produced sulfide as metal-sulfide (Huisman et al., 2006; Veeken et al., 2003a).

3. Electron donor and carbon source for sulfate reduction

SRB can use a wide range of e-donors, alcohols (Liamleam and Annachhatre, 2007; Widdel et al., 2007), fatty acids (Liamleam and Annachhatre, 2007; Widdel et al., 2007), hydrogen (H_2) (van Houten et al., 1994), carbon monoxide (CO) (Sipma et al., 2007) and methane (CH_4) (Nauhaus et al., 2002). When comparing e-donors for sulfate reducing applications, the following aspects need to be considered: the price of the e-donor, the local availability of the e-donor, the costs of the waste water treatment plant itself, the suitability of the e-donor for a specific waste or process water (depending on its volume, composition, temperature and salinity) and legislation regarding safety and environment. The e-donors can be divided in three groups: organic waste streams, bulk chemicals (relative concentrated, pure liquids or dissolved salts) and gaseous e-donors (Fig. 1).

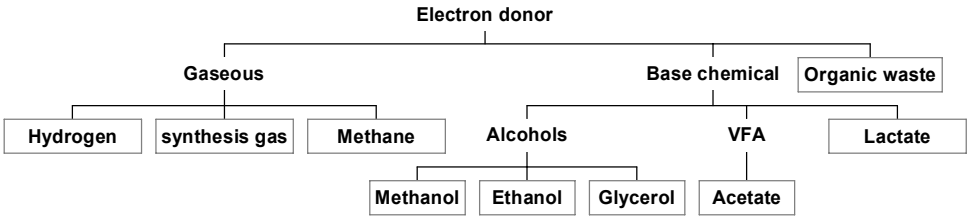

Figure 1. Electron donors used in sulfate reducing applications.

3.1 Organic waste streams. In the food and beverage industry, paper industry, agriculture and households, organic waste streams are produced (De Mes et al., 2003). Currently many producers of organic waste streams pay for the treatment or disposal of these wastes, during which the energy content of the waste stream is lost. Alternatively, these waste streams could be used to produce biobased energy carriers like biogas (Hulshoff Pol et al., 2001), bioethanol (Demirbas, 2007), H_2 (Liu et al., 2005; Nath and Das, 2004) or electricity (Logan et al., 2006). Waste streams high in protein, carbohydrate or crude fat can also be used as e-donor source for SR (Coetser et al., 2006), e.g. the distillage from an ethanol distilleries (Goncalves et al., 2007) or a compost manure mix (Zagury et al., 2006). Although complex organic matter (e.g. plant material) can not be used as e-donor by SRB directly, fermentative bacteria can degrade these compounds to substrates that SRB are able to utilize (Fig. 2). Currently organic waste streams are not used as e-donor for high rate sulfate reducing bioreactors for many reasons. In many cases the quantity and quality of the waste streams is not constant. In addition, waste streams can contain slowly degradable organic matter, which compromises compact bioreactors. Organic waste streams

without slowly degradable solids are often relative diluted. The use of organic waste streams, as e-donor source, would be the cheapest and most sustainable option when the waste is concentrated, easily biodegradable, constant in quality and quantity, available throughout the year and produced in the proximity of the sulfate containing waste stream, which is however rarely the case.

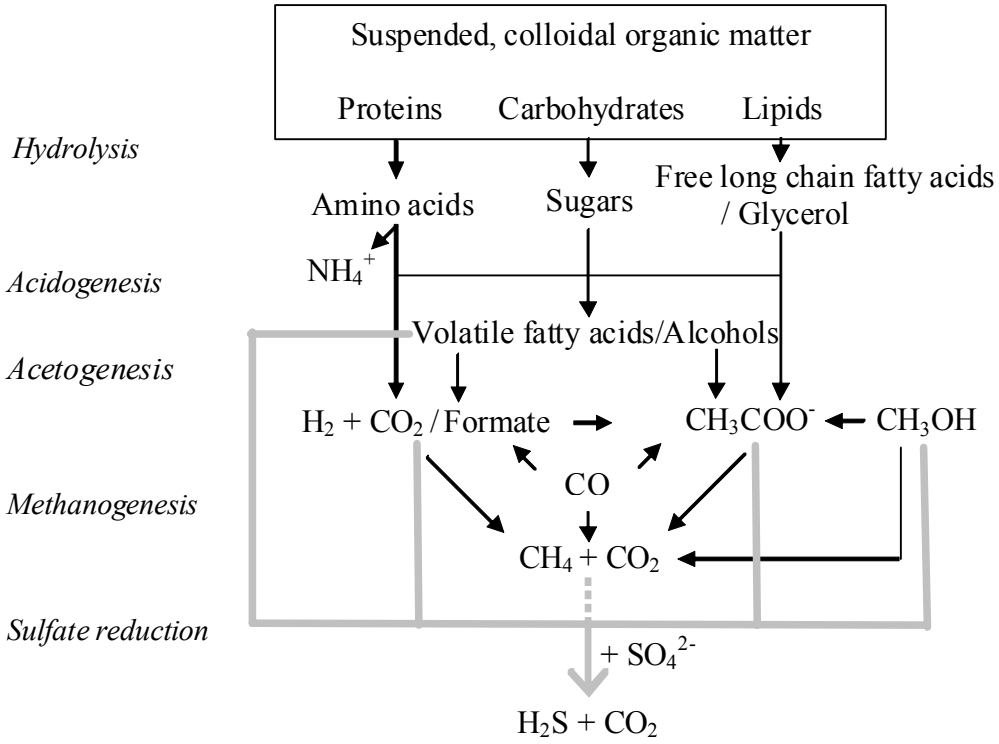

Figure 2. Schematic representation of the anaerobic degradation process (—) in the presence of sulfate (—)

3.2 Bulk chemicals as electron donor. There are numerous bulk chemicals that can be used as e-donor for SR like alcohols and volatile fatty acids (VFA; Fig. 1). The choice which e–donor is most suitable is not based on one sole criterium but a combination of all the factors involved, e.g., transport and storage costs of an e-donor (for instance alcohols are flammable) and the purity of the available e-donor. Methanol sustains SR rates up to 15 g SO_4 L^{-1} d^{-1} but only at 65^0C (Table 2). Ethanol is used as e-donor in full-scale operation (Weijma et al., 2002a). Glycerol is also a suitable e-donor for SR even down to a pH of 3.8 (Kimura et al., 2006). Lactate and VFA's like acetate can be used as e-donor (Kaksonen et al., 2006; Stucki et al., 1993) and as carbon souse in combination with hydrogen as e-donor (Weijma et al., 2002b). Lactate is used in microbial studies as model substrate but becomes too expensive in full-scale operations.

3.3 Gaseous electron donors. Two advantages of gaseous e-donors are that the wastewater is not diluted with a liquid stream (containing the e-donor) and that the e-donor does not wash-out with the effluent. A disadvantage of gaseous e-donors can be that they are voluminous and therefore need to be compressed during transportation. In gas-fed reactors the transfer of the gaseous substrate to the bulk liquid is often rate limiting, because of the low solubility of the gaseous e-donors (Yamamoto et al., 1976). Mass transfer can be improved by better gas-liquid contact (more gas hold up or smaller bubbles) and a higher partial pressure of the gaseous e-donor.

3.3.1. Hydrogen as electron donor. High rate SR with H_2 as e-donor and carbon dioxide (CO_2) as carbon source has been demonstrated at both mesophilic and thermophilic conditions (Table 2). A maximum SR rate of 30 g $SO_4^{2-}.L^{-1}.d^{-1}$ was reached. Van Houten (2006) showed that in a H_2 and CO_2 fed gas-lift reactor, SRB do not take CO_2 as carbon source directly, instead they depend on the acetate produced by homoacetogens (Table 1). Hydrotropic methanogens compete with SRB for the available H_2, using CO_2 as terminal electron acceptor. At well-mixed stable reactor performance the consortium of hetrotrophic SRB and homoacetogens outcompetes methanogens, because of a higher affinity for H_2. At elevated H_2 concentrations (e.g. during startup, in a badly mixed systems or after a disturbance) the methanogens are able to grow, resulting in a loss of e-donor due to methanogenesis (van Houten et al., 2006), which can be prevented by dosing acetate as carbon source in stead of CO_2.

Hydrogen is commonly produced by steam reforming from natural gas or by gasification of oil or coal (Armor, 1999; Bartisch and Drissel, 1978). Steam reforming takes place at high temperatures (750-800°C), the efficiency ranges from 60% to 80%. The gas produced by steam reforming or gasification (synthesis gas) contains, besides hydrogen, between 6 and 60% carbon monoxide (CO) (Bartisch and Drissel, 1978), which is converted by the so called water-gas-shift reaction, in which CO and water react over a chemical catalyst at 360°C to form carbon dioxide and hydrogen. To limited methanogenic and homoacetogenic activity the carbon dioxide can subsequently be removed from the gas as well (e.g. using an alkaline scrubber). More sustainable ways to produce hydrogen are coming up, e.g. by gasification from organic waste or biomass (Van Der Drift et al., 2001); by electrolysis using “green” electricity or biologically using bacteria (Hoekema et al., 2002), dark fermentation (Nath and Das, 2004) or with biocatalysed electrolyses in a biofuelcell (Rozendal et al., 2006).

3.3.2. Synthesis gas as electron donor. The chemical water-gas-shift reaction has two disadvantages. Firstly, the chemical catalysts become polluted by hydrogen sulfide which is present in synthesis gas and secondly, energy is needed to reach the required temperature. Alternatively the untreated synthesis gas, including the CO, could be fed to the SR bioreactor. Van Houten (Van Houten, 1996) found that the SR rate dropped from 12-14 g $SO_4 \cdot L^{-1}$ d^{-1} to 6-8 g $SO_4 \cdot L^{-1}$ d^{-1} when adding 5% CO, while increasing CO to 20% did not further deteriorate the SR rate. Sipma et al. (2004) showed that some SRB were able to tolerate up to 100% CO. At thermophilic conditions, the responsible organisms could convert CO to H_2 and simultaneously use the H_2 for SR. Although CO is inhibitory for methanogenesis, methanogens could only be eliminated in a synthesis gas fed gas-lift bioreactor at a hydraulic retention time of 3 hours (Sipma, 2006).

3.3.3. Methane as electron donor. Another alternative would be the use of natural gas (80 % methane) or biogas (50-75 % methane) as e-donor for SR. Than, both the steam reforming and the CO removal could be skipped. In addition, four times less gas needs to be transferred from the gas to the liquid phase (Table 1). Also substrate losses due to methanogenesis and acetogenesesis can be avoided. SR with methane was shown in batch at a rate of 0.24 g sulfate $L_{sediment}^{-1}$ d^{-1} (Nauhaus et al., 2002). However, to grow the responsible organisms seems to be a bottleneck for application. Nauhaus et al. (Nauhaus et al., 2007) reported a doubling time of 7 months and a maximum activity of 19.2 g SO_4 $L_{sediment}^{-1}$ d^{-1}.

3.4 Economical evaluation of electron donors.

The cost of a sulfate reducing application can be divided into capital costs (CAPEX) and operational costs (OPEX). More than half of the operational costs comes from the electron donor. The choice of e-donor will also influence the capital costs, e.g. the size of the bioreactor, the type of bioreactor and the need for additional facilities (like a steam reformer when H_2 is used as e-donor). The most straightforward way of comparing e-donors is by looking at the cost to reduce one mole of sulfate (Table 3). Prices of e-donors fluctuate over time. By using waste ethanol, the price of a sulfate reducing application can be significantly reduced. Table 3 shows that CH_4 is the cheapest available e-donor to date, but the use of CH_4 in full scale applications is compromised by the low conversion rates reached tot date. H_2 has proven itself in full-scale applications, however H_2 is not an economical feasible e-donor at small applications due to the relative high capital cost for hydrogen production (Van Houten, 1996).

Table 3: Prices of electron donors per amount needed to reduce 1 kg of sulfate.

Electron donor	Industrial market price (January 2008)	Required amount per kg sulfate reduced	Electron donor cost [$.kg$_{sulfate}^{-1}$]
Ethanol	0.60 $.L^{-1} [a, b]	0.40 L	0.24
H_2 [c]	0.21 $.m^{-3} [d]	0.934 m^3	0.20
Synthesis gas [e]	±0.15 $.m^{-3} [f]	0.934 m^3	±0.14
Natural gas [g]	0.24 $.m^{-3} [h]	0.292 m^3	0.07

[a] Ethanol Market, http://ethanolmarket.aghost.net/ accessed January 2008; [b] California Energy Commission, http://www.energy.ca.gov/gasoline/graphs/ethanol_10-year.html accessed January 2008. [c] produced from natural gas. [d] (Mueller-Langer et al., 2007) [e] 76% H_2 and 16% CO (produced from natural gas). [f] the cost will be between the price of hydrogen and natural gas. [g] 80% CH_4. [h] Energy Information Administration, http://tonto.eia.doe.gov/dnav/ng/ng_pri_sum_dcu_nus_m.htm, accessed January 2008

Not all e-donors are suitable for all applications. Factors like pH and temperature of waste or process streams should be taking into account. These factors could also influence the efficiency of the process and therefore the cost. E-donor loss because of methane production or acetate production will add to the total costs of the process, and vary per e-donor. The geographical location of the SR-application will have an influence on the choice of e-donors as well, because of differences in prices throughout the world and the cost related to transport (from e-donor production site to the SR-application) differ.

4 Effect of process conditions on sulfate reduction.

The conditions at which SR takes place influence the efficiency and rate of SR. Some of these conditions can be chosen while others depend on the waste waster stream or on site conditions. Important conditions for sulfate reduction are the temperature and pH of the reactor liquor, the sulfide concentration and the solid retention time. These conditions influence the SRB, but also competing microbial groups like methanogens and acetogens.

4.1 Effect of temperature. Most of the sulfate reducing bioreactors operated today are mesophilic (25-45°C), but also thermophilic (>45°C) (Madigan et al., 2000) sulfate reducing bioreactors are reported (Table 2). Each sulfate reducing species will have an optimum temperature and temperature range, but generally growth and conversion rates are higher at elevated temperatures, however, so are the decay rates. Obviously the energy needed to cool or heat a bioreactor contributes to the costs especially for diluted streams, therefore it is sensible to operate the reactor at a temperature close to the temperature of the sulfate containing waste or process stream.

Depending on the e-donor, thermophilic conditions can favor SR over methanogenesis, e.g. methanogens that use methanol were reported not to grow at or beyond a temperature of 65°C (Zinder et al., 1984). Therefore, methanol is an excellent e-donor for thermophilic SR (Weijma et al., 2000)

Table 2 Effect of the electron donor, pH, temperature, reactor concept on the volumetric sulfate reducing activity.

e-donor	pH [-]	Temp [°C]	Reactor concept	Volumetric activity [gSO_4^{2-} L^{-1} d^{-1}]	Reference
Hydrogen	8.0	30	Gaslift bioreactor	25	(van Houten, 2006)
Hydrogen	7.0	30	Gaslift bioreactor	30	(van Houten et al., 1994)
Hydrogen	7.0	55	Gaslift bioreactor	8	(van Houten et al., 1997)
Hydrogen	6.0	30	Gaslift bioreactor	13	(van Houten et al., 1995a)
Synthesis gas	7.0	30	Gaslift bioreactor	7[a]	(van Houten et al., 1995b)
Synthesis gas	-[b]	35	Anaerobic packet bed reactor	1.2	(du Preez and Maree, 1994)
CO	-[b]	35	Anaerobic packet bed reactor	2.4	(du Preez and Maree, 1994)
CO	6.9	50-55	Gaslift bioreactor	0.2	(Sipma et al., 2007)
Formate	6.0	30	MBR	29	Chapter 2
Formate	5.0	30	MBR	18	Chapter 3
Methanol	7.5	65	EGSB	15	(Weijma et al., 2000)
Ethanol	8	35	FBR	5	(Kaksonen et al., 2004)
Ethanol	7	8	FBR	0.6	(Sahinkaya et al., 2007)
Ethanol	7.2	33	MBR	0.6[c]	(Vallero et al., 2005)
Acetate	8	35	Fixed bed bioreactor	65	(Stucki et al., 1993)
Acetate	8	33	EGSB	10	(Dries et al., 1998)

[a] 80% H_2 20% CO. [b] uncontrolled and varying. [c] bioreactor was operated at high saline conditions with 50 g l^{-1} NaCl

4.2 Effect of pH. The optimum pH for SRB is usually stated as circum neutral. Most sulfidogenic bioreactors are therefore also operated at circum neutral pH. However, SR at other pH's than neutral could be interesting due to higher or lower pH values of a waste streams. In the mining and metallurgical industry large waste and process streams are produced with a low pH that demands for SR at low pH. SR has been described under acidic conditions as low as 3.8 (Kimura et al., 2006). However, SR at industrial interesting rates is only shown until a pH of 5.0 (chapter 3). Sulfide inhibition is usually stated to limit sulfate reduction under acidic conditions. The pH of the waste stream is however also increased during treatment by SRB which shows that the treatment could be performed at pH values higher than that of the waste stream. Next to acidic waste streams there are also streams in industry with a high pH which are often accompanied by high concentrations of salts like for example waste

streams from washing of natural gas. SR has been shown to occur at a pH of 10 (Pikuta et al., 2003), however industrial interesting rates have only been shown until a pH of 8.0 were a volumetric activity of 25 g SO_4 L d^{-1} was reached (van Houten et al., 1995a) .

4.3 Effect of sulfide. Sulfide is inhibiting for microorganisms including SRB. Various data of the effect of the different species of sulfide (S^{2-}, HS^- and H_2S) has been reported, but generally undissociated sulfide (H_2S) is shown to be the most inhibiting species at pH values below 7.5 (Moosa and Harrison, 2006; O'Flaherty et al., 1998). From a pH of 7.5 the fraction of H_2S is very small and it is reported that total sulfide than becomes inhibiting (Moosa and Harrison, 2006; O'Flaherty et al., 1998). There is however no consensus on the mechanism of sulfide inhibition. It is suggested that undissociated sulfide permeates through the cell membrane and once inside the cytoplasm it could complex the iron in cytochromes and other essential iron containing intracellular compounds (Madigan et al., 2000). However, this would mean that sulfide inhibition is not rapidly reversible, which has been shown (Okabe et al., 1992; Reis et al., 1992). A wide range of sulfide concentrations that are partly or completely inhibiting have been reported. In a GLB operated at pH 7 with hydrogen as e-donor, sulfate reduction with 14 mM H_2S (28 mM of total sulfide) was demonstrated (van Houten et al., 1994). Reis *et al.* (1992) showed that a H_2S concentration of 16 mM (547 mg L^{-1}) completely inhibited an acetic acid fed batch reactor at pH 6.2 and 6.7. The effect of sulfide was shown to be both instantaneous and reversible.

4.4 Effect of solid retention time. In sulfate reducing bioreactors, SRB compete for the available e-donor with methanogens and homoacetogens (Fig.1; (Stams et al., 2005)). SRB can obtain more energy from the utilization of the e-donor than methanogens, therefore, SRB have a higher affinity for the e-donor. However, methanogens are very versatile and manage to survive in predominant sulfate-reducing bioreactors. The competition between SRB and methanogens can be affected by the solid retention time (SRT).

In a H_2/CO_2 fed gas-lift bioreactor for example, the outcompetition of methanogens by SRB was delayed by elevated SRT (Esposito et al., 2003). Sipma et al. (2007) was able to sustain a pre-dominant sulfate reducing gas-lift bioreactor with CO as e-donor by applying a HRT of 3h. At longer retention times however, methanogens become more dominant over time. Another mechanism that can effect the outcome of the competition can be the difference in retention of methanogenic and sulfidogenic sludge, due to differences in attachment propperties and settling velocity. Weijma et

al. (2002b) ascribed the rapid and fast outcopetition of methanogens by heterotrophic SRB in a H_2/CO_2 fed gas-lift bioreactor to the superior settling velocity of sulfidogenic acetogenic sludge compared to that of methanogenic sludge.

5. Bioreactor types used for sulfate reduction.

The activity of a bioreactor is determined by the activity of the biomass and the biomass concentration. Biomass retention is applied to increase biomass concentrations, which is especially important in sulfidogenic bioreactors because of the low growth rate of anaerobic microorganisms. Most biomass retention systems rely on difference in density between the sludge and the reactor liquor, resulting in settling or floatation of the sludge. The most common biomass retention systems for anaerobic bioreactors are three-phase separators (internal settlers), which separate gas, solid and liquid. External settlers can be used in combination with an internal settler or as only retention system. Centrifugation is not often used to retain biomass due to high operational cost. Biofilm formation onto other biomass, called granulation, or on carrier material results in larger sludge particles, which settle faster. The discovery of granulation caused a breakthrough in environmental technology, because methanogenic reactors became a serious alternative for conventional wastewater treatment systems. These granules consist of micro-organism, matrix material of inert particles, *Methanothrix* filaments and extra-cellular polymers (Hulshoff Pol et al., 2004). Granuals are best formed in high organic strength waste waters and low shear bioreactors. The initial stages of granulation follow the same principles as biofilm formation onto a solid surface (Hulshoff Pol et al., 2004). Next to gravitation, filtration of the effluent could be applied to retain biomass. Membranes can be submerged in the reactor (submerged membrane bioreactor) or placed externally. Numerous bioreactor systems have been used for sulfate reduction (Fig. 3). The description of some of these bioreactor systems is given below.

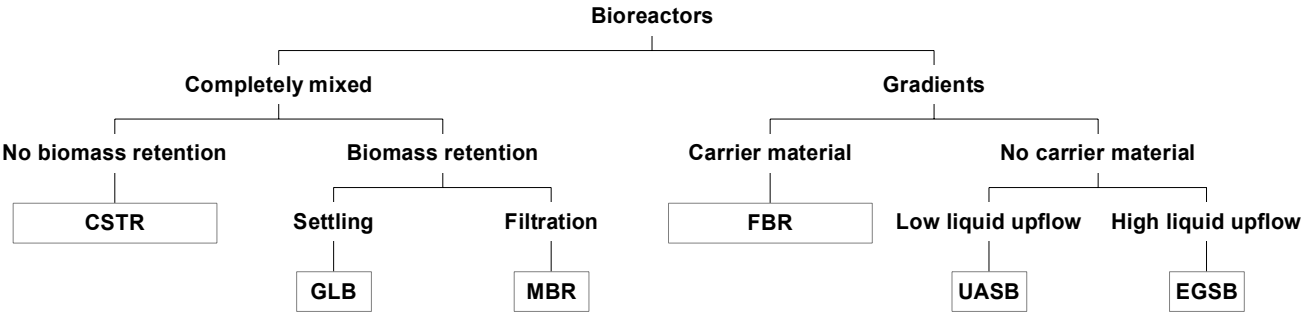

Figure 3. Reactor types used in sulfate reducing applications.

5.1 Continuous stirred tank reactor (CSTR). The CSTR reactor liquor is mixed by a mechanical stirrer (Fig. 4a), resulting in a completely mixed system. CSTR's are usually used in fundamental studies on SR processes, while the lack of biomass retention and high energy requirements limits its industrial application. A CSTR has for instance been used to study sulfate reduction at low pH by Kimura et al. (2006), who reduced sulfate at pH as low as 3.8 in batch by addition of zinc which precipitated with the formed sulfide to form zinc-sulfide and therefore removing the inhibiting sulfide.

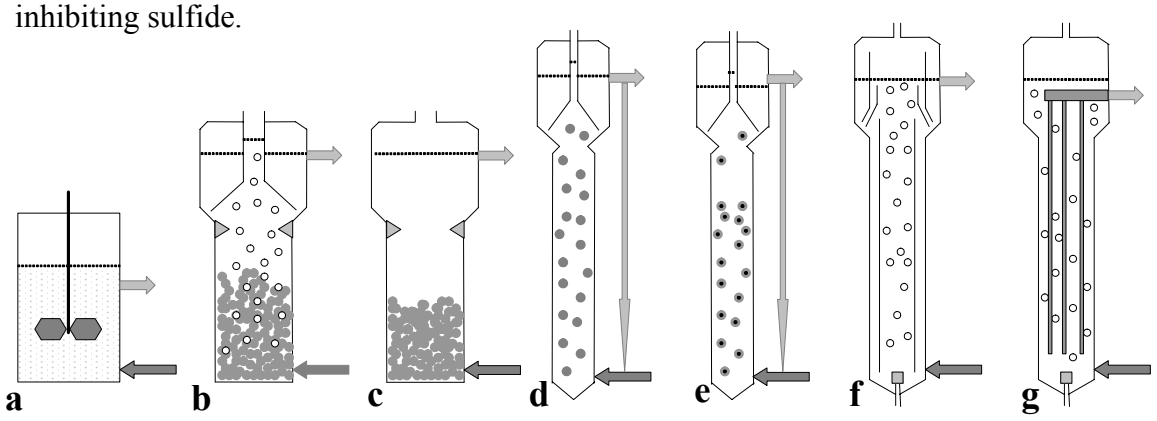

Figure 4. Reactor types used for sulfate reduction with: a) continues stirred tank reactor (CSTR), b) upflow anaerobic granular sludge bed (UASB) bioreactor with gas production, c) UASB without gas production, d) expended granular sludge bed (EGSB) reactor, e) fluidized bed reactor (FBR), f) gas-lift bioreactor (GLB) with internal loop, g) submerged membrane bioreactor (MBR)

5.2 Upflow Anaerobic Sludge Bed (UASB) reactor. The UASB reactor was developed for methane production from highly concentrated organic wastewater (Hulshoff Pol et al., 2001). It is a robust system in which the produced methane gas provides the mixing of the reactor liquor (Fig. 4b). However, in sulfate reducing reactors mixing dependents solely on the upflow of the waste stream (Fig. 4b), since the gasses produced during SR stay mainly in solution. A three phase separator is therefore also not required. Biomass retention depends on granulation, however, SRB do not granulate as well as methanogenic microorganisms (Weijma et al., 2002b). The UASB reactor is therefore more suitable as methanogenic reactor then sulfidogenic reactor. UASB's have for instance been used to study the effect of SR in methanogenic reactors treating synthetic paper mill waste water (Lens et al., 2003; Sipma et al., 1999).

5.3 Expanded Granular Sludge Bed (EGSB) reactor. The EGSB-reactor is an improved version of the UASB reactor in which additional mixing is provided by a high liquid upflow rate achieved by recycling water using the upflow of the produced gas (Fig. 4d). The EGSB-reactor is favorable for low strength organic wastewater

because they produce low amounts of gas for mixing, but not for SR in which no gas is produced. SR with methanol at thermophilic conditions has been studied in a EGSB reaching SR rates of 15 g L^{-1} d^{-1} at 65°C by applying a high liquid upflow due to recycling of the effluent to the bottom of the reactor (Weijma et al., 2000). A BEST reactor is an EGSB reactor designed for sulfate reduction (Mierzejewski et al., 2004), in which liquid is recycled from the top of the reactor to the bottom by pumping and which has a plate separator instead of a three phase separator.

5.4 Fluidized Bed Reactor (FBR). In a FBR, carrier material is used to obtain well settable biomass by biofilm formation on the carier material in contrast to granulation in a UASB and EGSB (Fig. 4e). Fluidization of the carrier material is achieved by effluent recycling similar to the EGSB. FBR's have been used for SR for treatment of acidic metal containing wastewaters with sand as carrier material (Kaksonen et al., 2006; Kaksonen et al., 2003) and to study SR at low (8°C) and high (65°C) temperature with ethanol as e-donor (Sahinkaya et al., 2007).

5.5 Gas-lift bioreactor (GLB). For gaseous e-donors a GLB is the most common bioreactor due to the enhanced gas mass transfer rates. A GLB consists of two compartments of which in one gas is sparged at the bottom (riser) which causes an upflow of gas, liquid, and biomass while in the other compartment (downcomer) the liquid and biomass (with or without a small amount of gas) will flow down (Fig. 4f). A GLB usually has a three-phase separator (Esposito et al., 2003; van Houten et al., 1994; Weijma et al., 2002b) or an external settler (Sipma et al., 2007) to retain the biomass in the system. GLB's can be operated with and without carrier material. The carrier materials pumice and basalt have also been used in a hydrogen fed laboratory GLB (van Houten et al., 1994), while in a CO fed laboratory GLB natural granulation has been used for biomass separation (Sipma et al., 2007). Also metal-sulfides can be produced in GLBs that can be used as carrier material.

5.6 Membrane bioreactor (MBR). MBR's are relatively new in the field of SR. The advantage is that almost complete biomass retention can be obtained which is especially useful in slow growing processes. MBR's has been applied in research on SR under high saline conditions (Vallero et al., 2005) and SR at low pH (chapter 2 and 3) were at pH 5.0 an volumetric activity of 18 g SO_4 L^{-1} d^{-1} was reached with formate as e-donor.

6 Sulfate reducing applications and metal recovery.

Sulfate reduction has been demonstrated in bench-scale, pilot-scale and full-scale applications. The size of full scale applications have increased from 1.2 ton-S d^{-1} in

1992 to 15 ton-S d^{-1} in 2005, showing the maturity of the process (Table 4; Fig. 5). Process schemes can be tailor made depending on the aim of the process e.g. sulfate removal or metal recovery.

Table 4. Technology characteristics of sulfate reduction technology (Buisman et al., 2007).

Project	Application	Sulfate form	Electron donor	Product
Nystar Budel Zink I	groundwater	$ZnSO_4/FeSO_4$	Ethanol	S^0, ZnS, FeS
Emmtec	Chemical industry	Na_2SO_4	Ethanol	S^0
Nystar Budel Zink II	Metal refining	$ZnSO_4$	H_2	ZnS
Landau collery	Mining	$CaSO_4$	Concentrated organic waste	$CaCO_3$, S^0
Lenzing	Chemical industry	Na_2SO_4	Cellulose wastewater	ZnS, S^0
BioDeSOx	Flue gas treatment	SO_x	Fermentation effluent	S^0

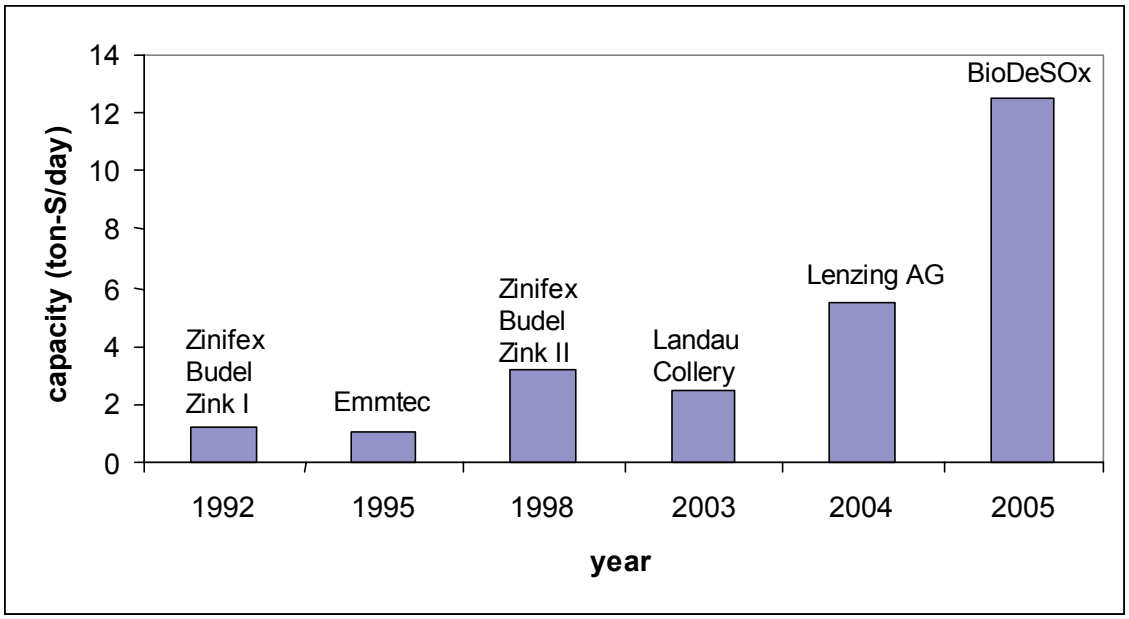

Figure 5. The capacity of full scale sulfate reducing plants (projects with a load < 1 ton-S d^{-1} are not shown; adapted from (Buisman et al., 2007). Nearstar budel Zink I (formally called Zinifex) is a ethanol fed UASB treating zinc containing groundwater (Budel, The netherlands), Emmtec an ethanol fed UASB treating sulfate containing wastewater (Emmen, The netherlands), Nearstar Budel Zink II a hydrogen fed gas-lift bioreactor treating a zinc containing process stream (Budel, The netherlands), Landau Collery a ethanol fed reactor treating acidic mining wastewater (Anglo Coal, South Afrika), Lenzing AG a BEST reactor treating viscose fiber production wastewater (Lenzing, Austria), $BioDeSO_x$ treating flue gas in China using wastewater from a fermentation industry.

6.1 Full scale sulfate reduction and zinc recovery from zinc refinery waste water. At the Nyrstar zinc refinery in Budel (the Netherlands), a single stage 500 m^3 bioreactor is used for SR and zinc-sulfide precipitation to recover zinc from the wastewater (Boonstra et al., 1999; Weijma et al., 2002a) following Eq. 14-16:

$$SO_4 + 4H_2 \rightarrow 4H_2O + S^{2-} \quad (14)$$

$$S^{2-} + Zn \rightarrow 4H^+ + ZnS\downarrow \quad (15)$$

Net reaction:

$$ZnSO_4 + 4H_2 \rightarrow 4H_2O + ZnS\downarrow \quad (16)$$

The sulfate concentration is in one step reduced from 5 - 15 g L^{-1} to 0.05 g L^{-1}, while the zinc concentration is reduced from 100 mg L^{-1} to <0.3 mg L^{-1}, recovering about 8.5 tons of zinc-sulfide a day (Boonstra et al., 1999; Weijma et al., 2002a). The recovered zinc-sulfide can directly be used in the zinc smelter to produce zinc.

6.2 Pilot scale metal removal from acid mine drainage. AMD is not only a waste stream but by recovering metals it becomes a resource for valuable resources e.g. metals. This was demonstrated in Bringham Canyon (Utah, USA) were Kennecot Utah Copper operated a mine, mill, smelter and refinery complex. The process flow sheet (Fig. 6a) demonstrates the many possibilities of metal recovery with sulfide (Boonstra et al., 1999; Weijma et al., 2002a). The sulfate concentration from the contaminated groundwater was reduced from 30 g L^{-1} to <0.5 g L^{-1} by a hydrogen fed sulfidogenic bioreactor (Weijma et al., 2002a). The sulfide present in the liquid phase was partly used to precipitate a mix of metals from the influent. The pH was simultaneously increased from 2.5 to 8.5 (Boonstra et al., 1999; Weijma et al., 2002a). The sulfide present in the gas-phase was put thought a liquid/gas contactor in which copper containing diluted leach water was added with small concentrations of zinc and iron. Copper-sulfide could selectively be recovered and directly added to the smelter. The excess sulfide was converted to elemental sulfur by partial oxidation (Fig. 6a).

6.3 Flow schemes for selective metal precipitation.

Various flow sheets can be designed for the selective recovery of metals from mixed metal containing streams because the formation of metal-sulfide depends on the type of metal, the sulfide concentration and the pH (Huisman et al., 2006; Tabak et al., 2003; Veeken et al., 2003b). Metal-ions can be recovered by use of biological produced sulfide in a single stage reactor or by transporting the sulfide through the liquid or gas phase in a separate reactor (Fig. 6b). Sulfide can also be produced by

bio-oxidation of sulfur (Huisman et al., 2006). To allow for selective recovery of metals, each metal has to react with sulfide in a separate reactor and has to be recovered in a separate settler (Fig. 6b). This will increase the investment cost, but produce pure metal-sulfides that could directly be added to a smelter process to produce metals.

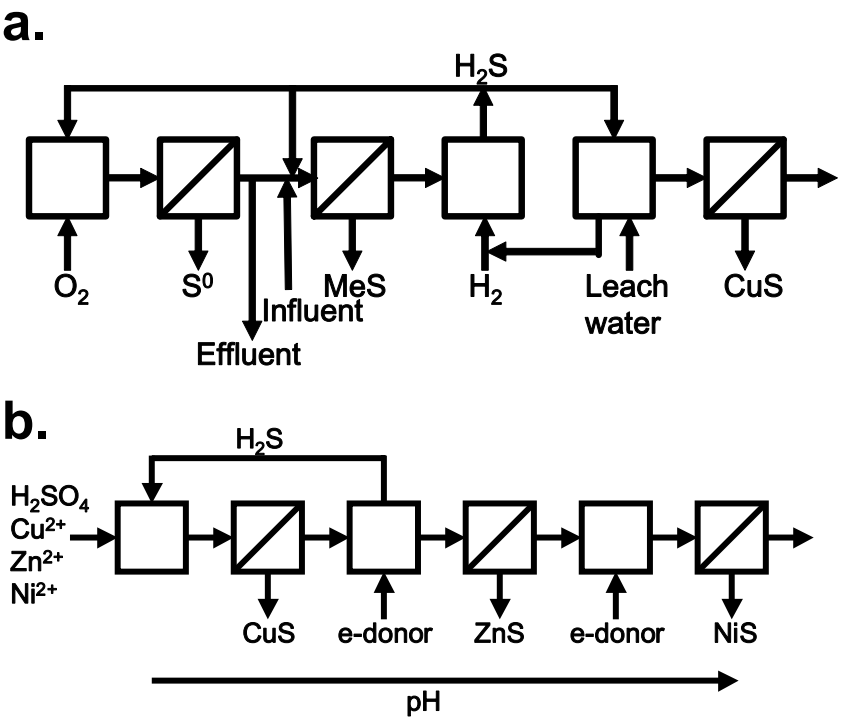

Figure 6. Flow schemes of sulfate reducing applications with a) The Kennecot process scheme, and b) Process scheme for selective metal recovery

7 Future prospect of sulfate reduction.

Three trends that can be identified which improve the future interest in sulfate reducing applications: (1) The metal prices have significantly increased over the last couple of years, (2) large quantities of cheaper e-donors have became available due to the development of biofuells, and (3) full scale sulfate reducing applications have significantly increased in size of the last couple of years.

The current status of the knowledge on SR has been discussed in this paper. However, research is expected to further increase the possibilities to apply SR and to make SR even more economical interesting. For SR to have a brother application range it will be important to expand the range of knowledge and to test possible new process flow schemes which rely on SR. Two areas's which will most likely be focused on are the search for new and cheaper e-donors and the ability to selectively recover metals from

mixed metal containing waste streams. Future focus should also shift more in the formation of products from industrial wastewaters and thus making wastewaters, resources instead of waste. Sulfate reducing systems could be used as end of pipe for waste water treatment but also as an integrated process treating process water which would significantly help the sustainability of a process.

The e-donor forms the major operational cost for SR applications, cheap electron donors like natural gas may therefore have great potential. SR with methane in a bioreactor was only demonstrated recently, more research is required to find the possibilities and limitations of this process. A major challenge is to increase the growth rate and conversion rate.

A second future focus could be on selective recovery of metals from high metal concentrated streams from the mining and metallurgical industry. These streams could be waste streams like AMD or the wastewater of a metallurgical operation, but also bioleaching liquors which are produced by the bioleaching of ores. Knowledge on selective metal recovery is limited, especially for single stage bioreactors. There is limited knowledge on the effect of metal-ions and metal-sulfide on SRB and the effect of SRB on metal-sulfides.

8 Scope of the thesis.

This chapter shows the many possibilities of sulfate reduction and interesting areas for future research. One of these areas is the development of selective metal precipitation processes. This thesis will focus on the all the aspects of this process. Chapter 2, 3 and 4 will focus on the effect of low pH (6.0 – 4.0) on sulfate reduction. Chapter 5 will study the effect of sulfide on the reactor performance and microbial population at a pH of 5.0. While in chapter 6 the effect of sulfide on the bio-precipitation and bio-crystallization of zinc will be studied. In chapter 7 the selective recovery of nickel from a nickel-iron solution will be studied. In chapter 8 the concluding remarks will be made and an outlook will be given.

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High rate sulfate reduction at pH 6 in a pH-auxostat submerged membrane bioreactor fed with formate

Abstract

Many industrial waste and process waters contain high concentrations of sulfate, which can be removed by sulfate reducing bacteria (SRB). This paper reports on mesophilic (30^{o}C) sulfate reduction at pH 6 with formate as electron donor in a membrane bioreactor with a pH-auxostat dosing system. A mixed microbial community from full-scale industrial wastewater treatment bioreactors operated at pH 7 was used as inoculum. The pH-auxostat enabled the bacteria to convert sulfate at a volumetric activity of 302 mmol sulfate reduced per liter per day and a specific activity of 110 mmol sulfate reduced per gram volatile suspended solids per day. Biomass grew in 15 days from 0.2 to 4 gram volatile suspended solids per liter. This study shows that it is possible to reduce sulfate at pH 6 with formate as electron donor at a high volumetric and specific activity with inocula from full-scale industrial wastewater treatment bioreactors operated at neutral pH. The combination of a membrane bioreactor and a pH-auxostat is a useful research tool to study processes with unknown growth rates at maximum activities.

Introduction

Many industrial waste and process waters contain high concentrations of sulfate. Sulfate can be removed from the wastewater by sulfate reducing bacteria (SRB), which reduce sulfate to sulfide. The produced sulfide can be partially oxidized biologically with oxygen to elemental sulfur (Buisman and Lettinga, 1990; Janssen et al., 1999). SRB require an electron donor for sulfate reduction, which has to be supplied externally if it is not present in the wastewater. Hydrogen is an efficient electron donor for mesophilic sulfate reduction in laboratory (van Houten et al., 1994; Weijma et al., 2002b; Widdel, 1988) and full scale (Weijma et al., 2002a) applications. In laboratory scale experiments, formate is a safe alternative for hydrogen (Paulo et al., 2005). Reducing sulfate at lower pH than neutral saves costs for neutralizing acidic wastewaters. However, sulfate reduction at lower than neutral pH makes the process more vulnerable to undissociated sulfide inhibition (Colleran et al., 1995; Moosa and Harrison, 2006; O'Flaherty et al., 1998; Reis et al., 1992). The pKa of sulfide is 6.96 at 30^0C (Kawazuishi and Prausnitz, 1987), and therefore 89 % of the total sulfide is in the undissociated form at pH 6.

An auxostat is a system in which the feeding rate depends on a culture variable. The pH-auxostat is the most applied type of auxostat and doses medium when the pH of the culture liquor drifts beyond the pH set-points (Gostomski et al., 1994). Compared to a chemostat, a pH-auxostat is more efficient in retrieving the maximum volumetric activity by minimizing the change of substrate limitation or substrate inhibition (Gostomski et al., 1994).

The present study focused on mesophilic sulfate reduction in a submerged membrane bioreactor (MBR) to study the feasibility of expanding the pH range of SRB to pH 6 for treatment of sulfate containing wastewater. A pH-auxostat was used to dose formic acid (electron donor and carbon source) and sulfuric acid (electron acceptor). A pH-auxostat has been previously applied for mesophilic sulfate reduction at neutral pH with formate as the electron donor (Paulo et al., 2005), however, cell retention was found to be the limiting factor. Therefore, a submerged MBR was integrated in the pH-auxostat set-up in the present study to assure complete biomass retention. The combination of a MBR and pH-auxostat is novel and the functionallity as research tool will be evaluated. The inhibition of undissociated sulfide to SRB is prevented by removing sulfide from the gas-phase. The performance of the system was investigated by measuring components in the gas and liquid phase and by following the evolution of the sulfate reducing volumetric and specific activity in time.

2.1 Experimental set-up.

A submerged membrane bioreactor (MBR) with a working volume of 6 liter was used in this study (Fig. 1). The reactor consisted of a gas-lift bioreactor in which a modified polyethylene membrane (Triqua, Wageningen, The Netherlands) was submerged in the reactor liquid. The membrane pore size of 0.2 μm enables the separation of biomass from the effluent. The temperature of the reactor liquor was controlled at 30^0C with a water jacket, heated by a Tamson T1000 (Tamson, Zoetermeer, The Netherlands). The pH was measured with a Schott H63 electrode (Schott A.G., Mainz, Germany) and controlled by an Endress+Hauser Liquisys P control unit (Endress+Hauser Holding A.G., Reinach, Germany). The tubing and connectors were made from PTFE (Schott A.G., Mainz, Germany and Serto A.G., Fuldabrück, Germany). The effluent gas of the reactor was recirculated though a teflon sparger (with 0.4 mm holes) to prevent loss of electron donor. A KNF-Verder N840.3FT.18 vacuum pump was used to recirculate the gas at a rate of 4 l min^{-1}, which was measured with a 100-9 McMillan (McMillan, Georgetown, USA).

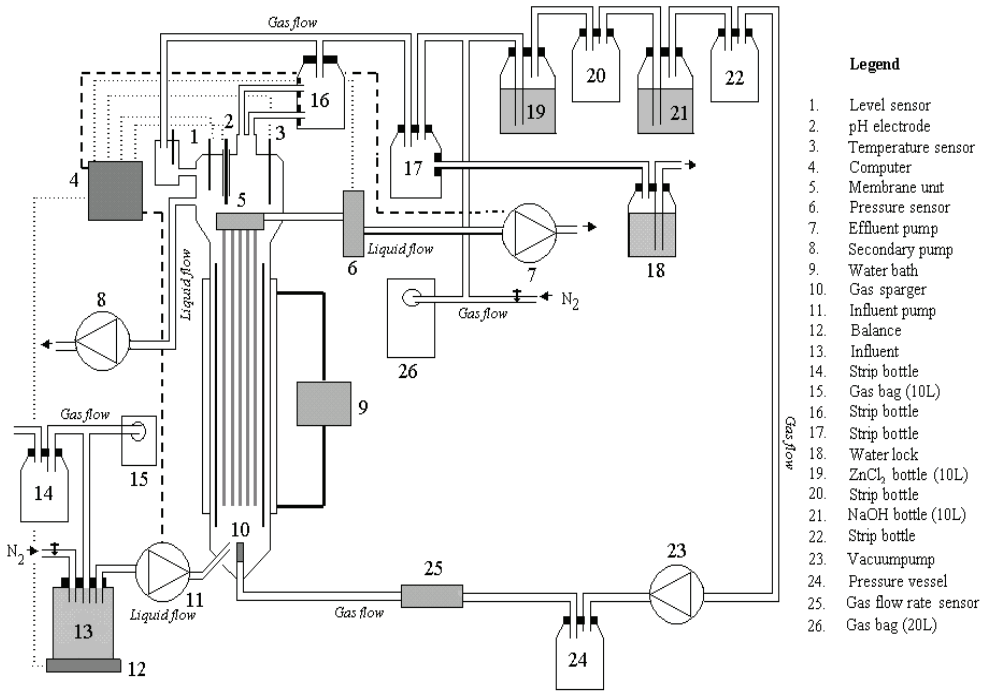

Figure 1: Scheme of the gas and liquid circuit in the submerged membrane reactor

2.2 pH-auxostat.

A pH-auxostat similar to Paula et al. (2005) was used in this study. This system is based on a proton equilibrium in which new influent was added when the $[H^+]$ decreases through neutralizing processes like sulfate reduction. The build-up of sodium was prevented by using sulfuric and formic acid instead of sodium sulfate and

sodium formate. The decrease of the [H^+] was measured via a pH electrode. To be able to operate below the inhibiting concentration of sulfide for sulfate reducing bacteria (SRB), sulfide was removed from the gas phase, which was recycled over the reactor, by passing the recycle gas through a $ZnCl_2$ solution in which sulfide was precipitated as zinc sulfide (Fig. 1, nr. 19). CO_2 was removed from the gas phase by bubbling the gas flow though a NaOH solution (Fig. 1, nr. 21).

2.3 Inoculum.

The inoculum was a mix of wet Eerbeek sludge (Industrie water Eerbeek, Eerbeek, The Netherlands), wet Nedalco sludge (Royal Nedalco, Bergen op Zoom, The Netherlands) and supernatant of Zinifex sludge (Zinifex Budel Zinc, Budel, The Netherlands). The amounts of wet sludge used is given in Table 3. The inoculum was crushed using a household blender for three minutes.

2.4 Medium.

The experiments were performed with artificial wastewater (Table 1). At start-up, the reactor was filled with 6 liter start-up medium, and than only run medium was dosed to the reactor. The start-up medium had a lower sulfate and formate concentration than the run medium (Table 2) to prevent formate inhibition. Acid and alkaline trace element solutions were prepared according to (Stams et al., 1993). All chemicals were of analytical grade and supplied by Merck (Darmstad, Germany).

Table 1: Chemical composition of start-up and run medium

	Start-up medium	Run medium	Unit
Na_2SO_4	1.48	- [a]	g l^{-1}
$NaHCO_2$	2.83	- [b]	g l^{-1}
KH_2PO_4	0.41	2.05	g l^{-1}
NH_4Cl	0.3	1.5	g l^{-1}
KCl	0.37	1.85	g l^{-1}
$MgCl_2 \cdot 6H_2O$	0.1	0.5	g l^{-1}
$CaCl_2 \cdot 2H_2O$	0.11	0.5	g l^{-1}
$NaHCO_3$	1	1	g l^{-1}
Yeast extract	0.1	0.5	g l^{-1}
Acid trace elements[c]	1	5	ml l^{-1}
Alkaline trace elements[c]	1	5	ml l^{-1}

[a] Sulfate present as sulfuric acid of which the concentration is varying per run medium and shown in table 2. [b] Formate present as formic acid of which the concentration is varying per run medium and shown in table 2. [c] As shown in (Stams et al., 1993)

Table 2: Concentration of sulfate and formate, and the ratio of formate to sulfate in the run media

Medium	Sulfate [M]	Formate [M]	Ratio Formate/Sulfate
RM1	1	6	6
RM2	0.5	2.81	5.61
RM3	0.75	2.81	3.75

2.5 Experimental design.

Two runs in a sulfate reducing MBR were performed at a pH of 6 and with formate as electron donor. The operational parameters of the two runs are given in Table 3. The focus was to study sulfate reduction at pH 6 with sludge from full-scale wastewater treatment bioreactors operated at neutral pH. Oxygen was also measured during the experiment but not detected. There was an excess of electron donor, based on the stoichiometry of sulfate reduction, in run medium RM1 and RM2 (Table 2). This excess in electron donor was necessary to compensate for possible methanogenesis, acetogenesis or production of other VFA's, because the pH-auxostat stops dosing if insufficient electron donor is available for sulfate reduction. In RM3, there was not enough formate to reduce all the sulfate present in the medium, based on the reaction stoichiometry (Table 2).

Table 3: Operational parameters of run 1 and 2

	Run 1	Run 2
pH	6.0	6.0
Temperature	30°C	30°C
Medium	RM1 (day 0-20)	RM2 (day 0-13)
		RM3 (day 13-19)
Inoculum: - Eerbeek	12 g	60 g
- Nedalco	6 g	30 g
- Budelco	100 ml	100 ml
Start-up period	0-9	0-7
Period I	9-13	7-9
Inactive period	13-14	9-12
Period II	14-20	12-14

2.6 Analyses.

Total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed following standard methods (Clesceri et al., 1998). Sulfate and formate were analysed with ion-chromatography as described by Sipma et al. (2004). Gas composition in the headspace was measured with gas-chromatography, where H_2, N_2, O_2 and CH_4 were analyzed on a Hewlett Packard 5890 and CO_2 on a Fisons Instruments GC8000

according to Weijma et al. (2000). Volatile fatty acids were analyzed on a Hewlett Packard series II GC according to Weijma et al. (2000). Sulfide was analyzed using Dr. Lange sulfide kit LCK-653 and a Xion 500 spectrophotometer (Hach Lange GMBH, Düsseldorf, Germany). The particle size distribution (PSD) was analysed with laser scattering image analysis (Coulter laser LS 230, Beckman Coulter, USA).

2.6 Calculations

2.6.1 Gibbs free energy calculations.

Gibbs free energies of the reactions were calculated using thermodynamic data from Amend et al. (2001). At the start of the experiment 0.1% was used as hydrogen concentration to make thermodynamic calculations possible. The same concentration was used for CO_2 of which the concentration was low due to stripping with a NaOH solution. For sulfide, a constant concentration of 0.4 mM was used which was found to be an average value in the reactor liquor.

2.6.2 Mass balance sulfate calculations.

Assumptions:

1. The liquid volume of the reactor is considered constant during the experiment, which also means that the liquid flow going into the reactor is equal to the liquid flow going out the reactor ($\phi_{l,in} = \phi_{l,out}$) and is called ϕ_l. Evaporation was found to be negligible.
2. The liquid flow is assumed to be the difference in weight of the medium divided by the density of the medium (determined by weighing 1 L medium) multiplied by the difference in time ($\phi_l = \frac{\Delta M}{(\rho_m \cdot \Delta t)}$). The time interval between the data points was 15 minutes of which an average of 19 data points was used.
3. The sulfate accumulated in the reactor and the sulfate present in the effluent were neglected, which was possible due to the high concentration of sulfate in the influent compared to the reactor concentration and due to the small hydraulic retention time.
4. To prevent false high specific activities, the highest $C_{x,r}$ value of the interval was taken. The biomass concentration in the liquid phase was used because there was no visible biofilm formation on the membrane or bioreactor wall. The biofilm activity can thus be neglected compared to the high biomass concentration present in the in the mixed reactor liquor.

With assumption 1-4, the sulfate mass balance ($\frac{dS}{dt}$) is:

$$\frac{dS}{dt} = \phi_l \cdot C_{s,in} - r_v \cdot V_r \qquad (1$$

For steady state conditions, the volumetric (r_v) and specific (r_s) sulfate reducing activity becomes:

$$r_v = \frac{\phi_l \cdot C_{s,in}}{V_r} \qquad (2$$

$$r_s = \frac{\phi_l \cdot C_{s,in}}{C_{x,r} \cdot V_r} \qquad (3$$

Nomenclature

Φ	Flow rate	[l d^{-1}]
ρ_m	Density of run medium	[kg l^{-1}]
C_s	Sulfate concentration	[mmol l^{-1}]
C_x	Biomass concentration	[gVSS l^{-1}]
M	Mass of influent	[kg]
r_v	Volumetric activity	[mmol L^{-1} d^{-1}]
V_r	Reactor volume	[L]
S	Sulfate	[-]
r_s	Specific activity	[mmol $gVSS^{-1}$ d^{-1}]

Subscript

in	going in the reactor
l	liquid phase
out	going out the reactor
r	in the reactor

Results

3.1 Reactor performance during the start-up period.

Adaptation of the inoculum to the applied conditions resulted in a lag phase of 9 days in run 1 (Fig. 2a-2b) and 7 days in run 2 (Fig. 3a-3b). In this phase, the activity was

low and therefore little medium was dosed to the bioreactor by the pH-auxostat (Fig. 2a-3a). However, in run 1 there was a short period of fast addition of medium on day 5 (Fig. 2a), resulting in a peak in the volumetric activity (Fig. 2b). After correction for the sulfate which accumulated in the reactor in the first 5 days (Fig. 2c), the volumetric activity was nearly zero. Formate was depleted at day 5 in both runs and remained depleted for the duration of the runs, which indicates that the supplied formate was immediately converted (Fig. 2c). The acetate concentration increased rapidly in run 1 from day 5 onward to 28 mM (day 9, Fig. 2e), while other volatile fatty acids were only present in minor amounts (< 0.3 mM). The sulfide concentration was on average 0.4 mM and was never more than 1 mM during run 1. The VSS concentration increased in run 1 from 0.2 gVSS l^{-1} at day 5 to 1.0 gVSS l^{-1} at day 9 (Fig. 2f). The amount of hydrogen increased during the start-up phase from 0% at start-up to, respectively, 50 % and 60 % (Fig. 2d) for run 1 and 2, while only minor quantities of methane were present (2 % run 1; 6 % run 2). The remaining part of the headspace consisted of nitrogen gas.

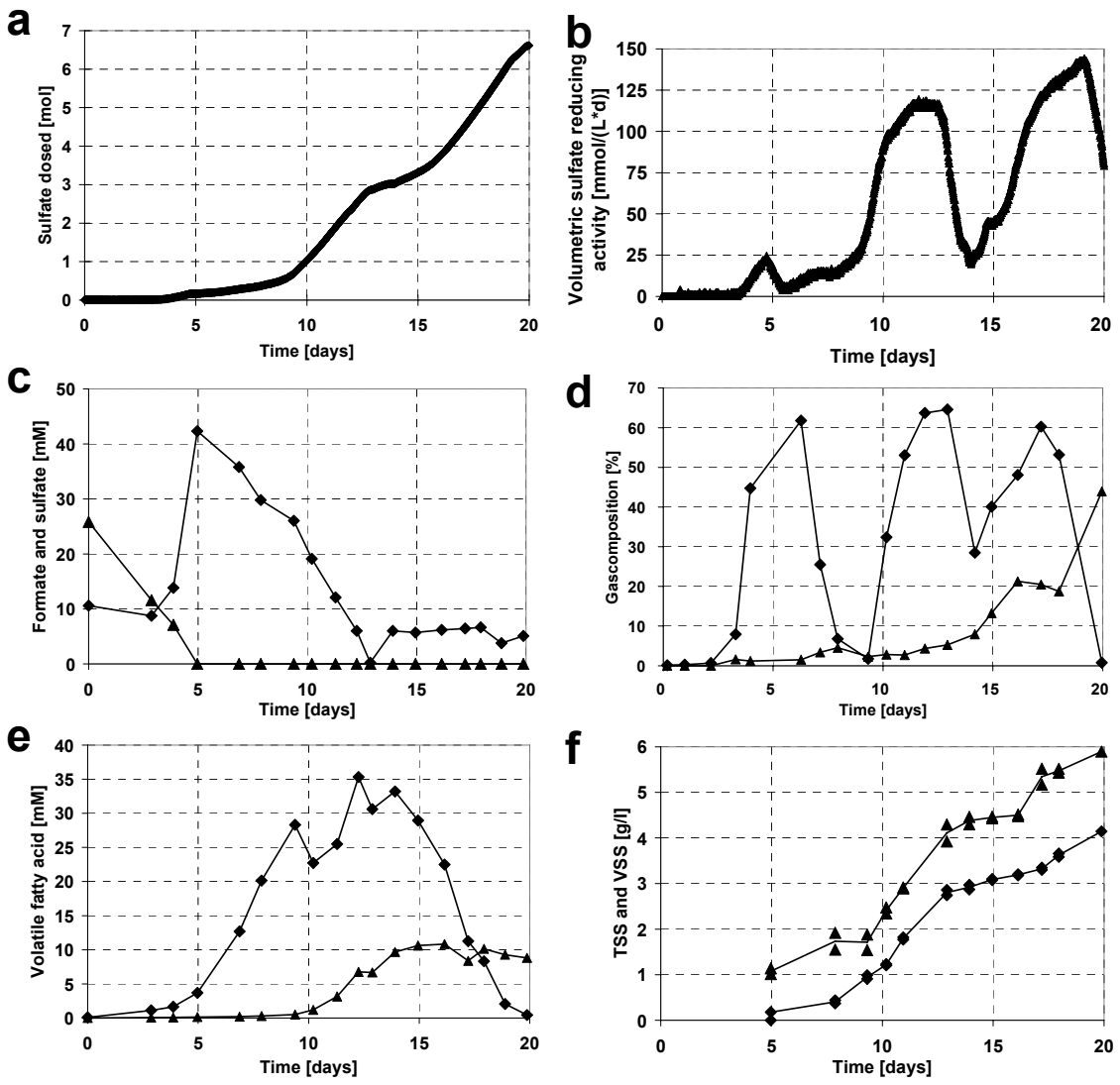

Figure 2: Results from run 1 in a membrane bioreactor at pH 6 with; (a) Amount of sulfate dosed to the reactor by the pH-auxostat in mol sulfate, (b) Volumetric activity of the reactor expressed as mM sulfate reduced per liter of reactor liquor per day, (c) Formate (▲) and sulfate (♦) concentration in the reactor expressed as mM sulfate/formate per liter, (d) Gas phase composition with hydrogen (♦) and methane (▲) in percentage of total gas phase, (e) Acetate (♦) and n-butyrate (▲) concentration in the reactor expressed as mM acetate/n-butyrate per liter of reactor liquor, (f) Total suspended solids (TSS, ♦) and volatile suspended solids (VSS, ▲) of the reactor expressed as gram TSS/VSS per liter of reactor liquor

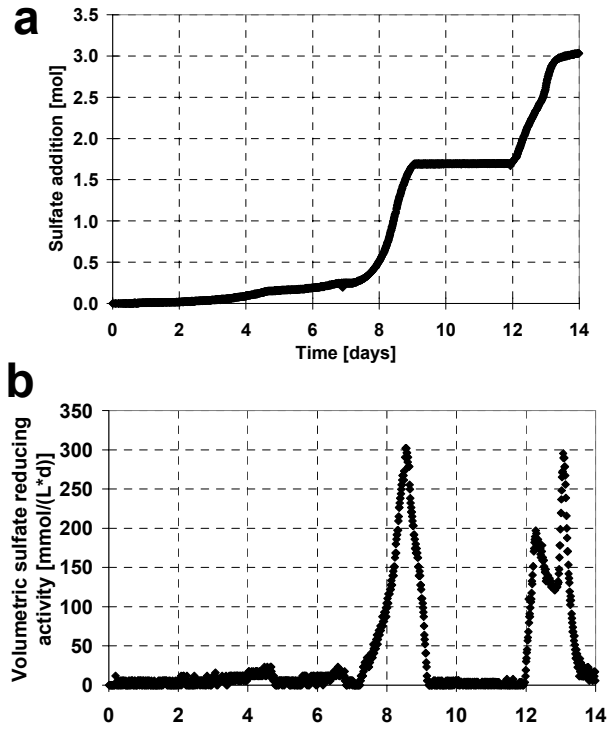

Figure 3: Results from run 2 in a membrane bioreactor at pH 6 with; (a) Amount of sulfate dosed to the reactor by the pH-auxostat in mol sulfate, (b) Volumetric activity of the reactor expressed as mM sulfate reduced per liter of reactor liquor per day

3.2 Reactor performance during period I.

After the start-up period, the volumetric activity increased to 119 mmol SO_4^{2-} reduced L^{-1} d^{-1} in run 1 (Table 4) and 302 mM d^{-1} in run 2 (Table 4). The VSS concentration in run 1 increased rapidly from 0.95 to 2.8 gVSS l^{-1} from day 9 to day 13 (Fig. 2f). At day 10, this resulted in a specific activity of 80 mmol SO_4^{2-} reduced $gVSS^{-1}$ d^{-1} for run 1 and 106 mmol SO_4^{2-} reduced $gVSS^{-1}$ d^{-1} for run 2 (Table 4). The sulfate concentration in the reactor liquor decreased in run 1 from 26 mM at day 9 to 0.2 mM at day 13, while sulfate was continuously added by the pH-auxostat, indicating that the incoming sulfate was immediately converted to sulfide. The acetate concentration reached a peak concentration in this phase of 35 mM in run 1 and 28 mM in run 2.

Propionate (data not shown) and n-butyrate (Fig. 2e) reached concentrations of 2 mM and 7 mM in run 1, respectively. Hydrogen increased in this period from 2% to 65% in run 1 (Fig .2d).

Table 4: Overview of volumetric and specific activities reached in run 1 and 2

	Volumetric activity [mM SO_4 d^{-1}] [a]		Specific activity [mmol SO_4 $gVSS^{-1}$ d^{-1}] [b]	
	Run 1	Run 2	Run 1	Run 2
Active phase I	119	302	80	110
Active phase II	143	295	35	106

[a] Calculated using Eq. 2, [b] Calculated using Eq. 3.

3.3 Reactor performance during the inactive period.

The active period of run 1 was interrupted for one day (day 13), which was probably caused by saturation of the sodium hydroxide solution, which removes the CO_2 from the gas phase. An accumulation of CO_2 in the gas phase stops the neutralization of the pH and therefore stops the influent feed by the pH-auxostat. During this day there was no growth (Fig. 2f) and no significant change in gas and liquid phase composition (Fig. 2d and 2c, respectively). In run 2, the inactive phase lasted for 3 days and was caused by excess foam production in which half of the medium was lost and replaced by new start-up medium.

3.4 Reactor performance during period II.

During period II, the rate of influent addition was comparable to period I (Fig. 2a) and reached a peak volumetric activity of 143 mM SO_4^{2-} d^{-1} in run 1 and 295 mM SO_4^{2-} d^{-1} in run 2 (Table 4). The specific activity, however, decreased in run 1 to 35 mmol SO_4^{2-} $gVSS^{-1}$ d^{-1} and was stable with 106 mmol SO_4^{2-} $gVSS^{-1}$ d^{-1} in run 2 (Table 4). The acetate concentration decreased in run 1 from 29 mM at day 15 to 0.4 mM at day 20. Formate remained depleted while sulfate remained around 5 mM, which indicated that all dosed sulfate and formate were converted. During active phase II, the VSS concentration increased significantly in run 1 from 2.9 g VSS l^{-1} at day 14 to 4.2 g VSS l^{-1} at day 20. Hydrogen accumulated in run 1 in the beginning of active phase II to 60% at day 17, but was almost depleted by day 20 while methane reached a maximum concentration of 44 % on day 20 in the first run (Fig. 2d) and 36% at day 13 in the second run. This phase suddenly stopped and medium addition stagnated at day 20 for run 1 and day 14 for run 2. Formate, hydrogen and acetate were depleted, while in run 1, n-butyrate was still present. The sulfate concentration remained around 5 mM while the pH-auxostat stopped dosing. Thus, both runs came to a hold.

3.5 Biomass characteristics.

The TSS/VSS graph of the first run (Fig. 2f) shows an increase in biomass from 0.18 gVSS l^{-1} to 4.1 gVSS l^{-1} in 15 days with a yield on sulfate of 38 gVSS kg^{-1} sulfate. The crushed inoculum sludge had a wide variety of particles sizes. Most of the particles (83%) were smaller than 100 μm with a mean diameter of 29 μm (Table 5), of which the size shows a decrease over time from 30 μm at day 11, to 22 μm on day 14, and to 21 μm on day 20 in run 1. The decrease in particle size could be due to the large shear forces in the reactor caused by gas sparging.

Table 5: Particle size distribution of the sludge from run 1

	0 – 1 [μm]		1 – 10 [μm]		10 – 100 [μm]		100 – 250 [μm]		250 – 2000 [μm]	
Day	gVSS l^{-1}	%	gVSS l^{-1}	%	gVSS l^{-1}	%	gVSS l^{-1}	%	gVSS l^{-1}	%
11	0.15	8	0.26	15	1.09	61	0.29	16	>0.01	0
14	0.36	12	0.41	14	1.68	58	0.47	16	>0.01	0
20	0.83	20	1.68	16	2.12	51	0.51	12	>0.01	0

3.6 Electron transfer characteristics.

The electron transfer was calculated over time by using the electrons entering the system through formate and the collected data from the reactor liquor and gas phase. During both runs, electrons were transferred from formate, the only electron donor in the influent, to the end products sulfide, methane and biomass, as well as the intermediates hydrogen and VFA (table 6). At the end of run 1, the majority of the electrons were transferred to sulfide (69%), in contrast to the first 5 days where no sulfate was reduced to sulfide (Table 7). In the first 5 days, the major process was production of hydrogen from formate accounting for 49% of the total electron flow (Table 7). Methane was detected in the gas phase from the beginning of the experiment and the concentration increased gradually in the headspace (Fig. 2d), but methane accounted never for more than 2-6 % of the electrons transferred. During the experiment a variety of volatile fatty acids were produced and consumed of which acetate was the major VFA, but also n-butyrate and propionate accumulated in the medium. The electrons transferred to VFA reached a maximum of 9 % of the total electron flow on day 10 (Table 7), after which the flow decreased until all VFA except n-butyrate were depleted at the end of the run (Fig. 2e). There was a significant amount of growth during the experiment so biomass became a significant part of the electron flow which accounted for 10 % of the electrons transferred at the end of the experiment (Table 7). There was also an amount of electrons unaccounted for. This fraction was most likely hydrogen and methane, which left the system via a water lock after exceeding the capacity of the headspace (Fig 1, nr. 18).

Table 6: Overview of possible reactions in reactor liquor

$4HCO_{2(aq)}^{-} + SO_{4(aq)}^{2-} + 6H_{(aq)}^{+} \rightarrow H_2S_{(aq)} + 4CO_{2(g)} + 4H_2O_{(l)}$	1
$4HCO_{2(aq)}^{-} + SO_{4(aq)}^{2-} + 5H_{(aq)}^{+} \rightarrow HS_{(aq)}^{-} + 4CO_{2(g)} + 4H_2O_{(l)}$	2
$4HCO_{2(aq)}^{-} + 3H_{(aq)}^{+} \rightarrow Ac_{(aq)}^{-} + 2CO_{2(g)} + 2H_2O_{(l)}$	3
$4HCO_{2(aq)}^{-} + 4H_{(aq)}^{+} \rightarrow CH_{4(aq)} + 3CO_{2(g)} + 2H_2O_{(l)}$	4
$HCO_{2(l)}^{-} + H_{(aq)}^{+} \rightarrow H_{2(g)} + CO_{2(g)}$	5
$4H_{2(g)} + SO_{4(aq)}^{2-} + 2H_{(aq)}^{+} \rightarrow H_2S_{(aq)} + 4H_2O_{(l)}$	6
$4H_{2(g)} + SO_{4(aq)}^{2-} + H_{(aq)}^{+} \rightarrow HS_{(aq)}^{-} + 4H_2O_{(l)}$	7
$4H_{2(g)} + 2CO_{2(g)} \rightarrow Ac_{(aq)}^{-} + H_{(aq)}^{+} + 2H_2O_{(aq)}$	8
$4H_{2(g)} + CO_{2(g)} \rightarrow CH_{4(g)} + 2H_2O_{(l)}$	9

Table 7: Electrons transferred during run 1 over time expressed in mole of transferred electrons and percentage of the total electron flow

	Formate	Sulfide		Hydrogen		Methane		VFA		Biomass		Unaccounted	
Day	e-mol [a]	e-mol	%	e-mol	%	e-mol	%	e-mol	%	e-mol	%	e-mol	%
5	-2.4	-0.16	-7	1.2	49	0.16	6	0.16	6	0.87	37	0.35	15
10	-12.8	7.4	58	0.76	6	0.24	2	1.17	9	2.8	22	1.5	12
15	-40.0	26.6	66	0.92	2	1.2	3	1.66	4	2.8	7	8.3	21
20	-79.7	53.0	69	0.02	0	4.1	5	0.25	0	7.7	10	14.6	19

[a] Mole of electrons transferred in reaction

Discussion

4.1 Reactor performance and culture characteristics.

This study shows that it is feasible to reach high rate mesophilic sulfate reduction at pH 6 with an inoculum from pH neutral full-scale bioreactors. High volumetric and specific activities as well as high sulfate conversion efficiencies could be achieved by using a pH-auxostat. To the best of our knowledge, the volumetric and specific activities achieved in this study have not been reported at pH 6 so far (Table 8). In this study, volumetric activities of 143 and 302 mM sulfate reduced per liter per day were reached in run 1 and 2, respectively (Table 4). The highest reported volumetric activity at pH 6 so far is 130 mM d^{-1}, reached in a gas-lift bioreactor with hydrogen as electron donor, pumice as carrier material and cell retention via a three-phase separator (Table 8). This experiment had a sulfate conversion efficiency of 49 % at the maximum volumetric activity, whereas in this study a conversion efficiency of 100% at the maximum volumetric activity was reached. Specific activities of 80 and

110 mmol sulfate reduced per gram VSS per day were reached in this study for run 1 and 2, respectively (Table 4). These rates are substantially higher than those obtained in UASB reactors operated at pH 6, where a specific activity of only 2 mmol $gVSS^{-1}$ day^{-1} was reached (Table 8), possibly due to mass transfer limitation by granule formation.

Table 8: Overview of the performance of sulfate reducing bioreactors from literature and this study

Reactor concept	pH	Temp	Substrate	Volumetric activity	Specific activity	Conversion efficiency	Reference
-	-	^oC	-	mM SO_4 d^{-1}	mmol SO_4 $gVSS^{-1}$ d^{-1}	%	-
MBR[a]	7.2	33	Ethanol	69	69	62	(Vallero et al., 2005)
Gaslift[b]	7.0	30	Hydrogen	312	-[c]	59	(van Houten et al., 1994)
UASB	6.0	55	Mix[d]	52	2[e]	90	(Lens et al., 2003)
UASB	6.0	55	Mix[f]	77	2[g]	50	(Sipma et al., 1999)
Gaslift[2]	6.0	30	Hydrogen	130	-[h]	49	(van Houten et al., 1995)
MBR	6.0	30	Formate	143	80	100	This study
MBR	6.0	30	Formate	302	110	100	This study

[a] bioreactor was operated at high saline conditions with 50 g l^{-1} NaCl. [b]With pumice as carrier material. [c]No biomass concentration measured. [d]Mix contains starch 6 g l^{-1}, sucrose 5.35 g l^{-1}, lactate 11.21 g l^{-1}, propionate 4.3 g l^{-1} and acetate 9.35 g l^{-1}. [e]Calculated using 165 g VSS at start-up in a 6.5 L reactor [f]Mix contains sucrose, propionate and butyrate in a ratio of 2:1:1 on COD basis. [g]Calculated using 240 g VSS at start-up in a 6.5 L reactor [h]biomass was not measured as VSS.

4.2 Metabolic characteristics.

The conversion of formate into hydrogen was the dominant electron flow at the beginning of the run (Table 7), even though the production of acetate, methane and sulfide with formate was thermodynamically more feasible (Fig. 4a - 4b). When the hydrogen concentration increased, sulfate reduction, acetogenesis and methanogenesis became thermodynamically feasible with hydrogen and started to become a dominant electron flow (Fig. 4b). This suggested that in this study formate was an energy carrier for hydrogen production and that actually hydrogen was the major electron donor for sulfate reduction, acetogenesis and methanogenesis. However, without further research on the metabolic pathway, formate cannot be excluded as direct electron donor for sulfate reduction, acetogenesis or methanogenesis. Formate could have been used as carbon source, however, compounds produced from formate like acetate are also likely options.

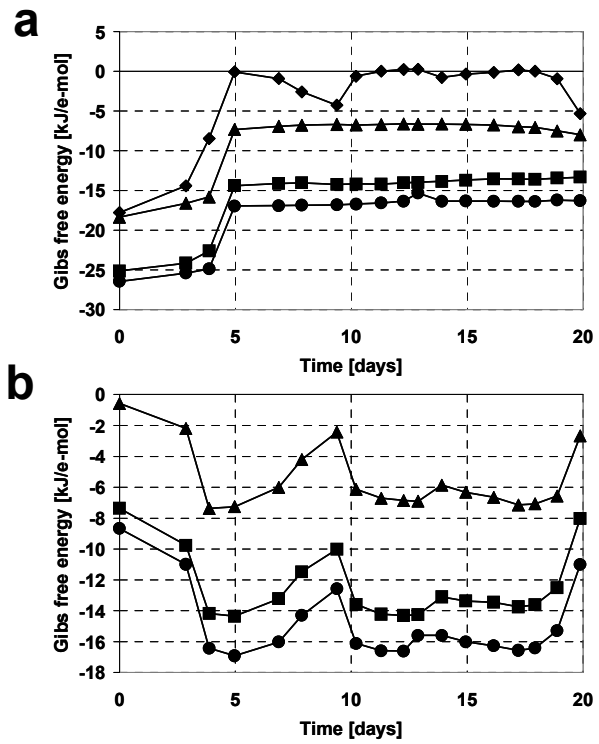

Figure 4: Thermodynamic calculations using the reactor data from Fig. 2 and thermodynamic data from (Amend and Shock, 2001) with; (a) reactions with formate as electron donor for sulfate reduction Eq. 1-2 (♦),acetate production Eq. 3 (•), methane production Eq. 4 (▲) and hydrogen production Eq. 5 (■) from Table 6, expressed in kJ per mole of transferred electron, and (b) reactions with hydrogen as electron donor for sulfate reduction Eq. 6-7 (♦), acetate production Eq. 8 (•) and methane production Eq.9 (▲) from Table 6, expressed in kJ per mole of transferred electron

The difference in Gibbs free energy (ΔG) between 25ºC and 30 ºC are small compared to the difference between pH 7 and pH 6 (Table 9). All reactions in Table 6 except for the production of acetate from hydrogen are more favorable in an acidic environment (Table 9). Reactions with formate were thermodynamically the most favorable at the start of the experiment, however, in the first 5 days the ΔG increased rapidly (Fig. 4a), which was caused by the decrease in formate concentration in the first 5 days (Fig. 2c). After day 5 the Gibbs free energy stabilized at values where all reactions were still thermodynamically feasible. The conversion of formate into hydrogen had only a slightly negative ΔG (Fig. 4a), but this reaction still took place during the entire experiment. Interactions between hydrogen and formate in anaerobic systems have been well described (Boone et al., 1989; Peters et al., 1999; Thiele and Zeikus, 1988). Widdel (1988) reported that many of the known hydrogenotrophic SRB can also use formate as electron donor. Thiele et al. (1988) reported that in an ethanol fed anaerobic digester, formate was the most important electron shuttle between

acetogenic and methanogenic organisms. However, the bicarbonate concentration was higher which is thermodynamically in favor of formate as electron donor.

Table 9: Gibs free energy (ΔG) values of reaction 1-9 under various conditions

	Gibs free energy (Δ G) for reaction nr. [a] [kJ e-mol^{-1}]						
	1 - 2[b]	3	4	5	6 - 7	8	9
$\Delta G_r^{0'}$ [c]	-17	-14	-18	-2	-15	-12	-16
ΔG_{pH7} [d]	-17	-14	-18	-2	-15	-11	-16
ΔG_{pH6} [e]	-21	-16	-21	-5	-16	-11	-16

[a] reactions can be found in Table 6 and were calculated in kJ per mole of transferred electron, [b]based on reaction 1 and 2 table 6 and pKa of 6.96 (Kawazuishi and Prausnitz, 1987) ,[c] 25 ºC, pH 7 and standard conditions of 1M and 1 bar, d 30 ºC, pH 7 and concentrations of 1M and 1 bar, [d] 30 ºC, pH 6 and concentrations of 1M and 1 bar, [e] 30 ºC, pH 6 and concentration as measured in the reactor

The Gibbs free energy of the reactions with hydrogen as electron donor followed the pattern of the hydrogen concentration in a mirror image (Fig. 2d and 4b). The volumetric activity also followed the hydrogen concentration except for the peak at day 5 (Fig 2b. and 2d). Sulfate reduction was the most energetically favorable reaction at all times, closely followed by methanogenesis (Fig. 4b). However, only a small percentage of the electrons were transferred to methane (Table 7), which indicated that the bacteria were not thermodynamically, but kinetically limited. Acetogenesis represented, however, a significant electron flow (Table 7) even though the ΔG for this reaction was relatively low (Fig. 4b). After day 10, the amount of obtainable energy for acetogenesis increased while the concentration decreased, which indicated that acetate was consumed faster than it was produced (Fig. 2e).

4.3 pH-auxostat performance.

The pH-auxostat was an attractive system for this experiment because the bacteria were not limited in sulfate or inhibited by formate (Fig. 2c). In the first five days of run 1, formate was rapidly converted into hydrogen, which caused the formate concentration to drop in the reactor. As a response, the pH-auxostat dosed new influent of which the formate was converted to hydrogen, however, sulfate was not converted and accumulated as sulfuric acid in the reactor to compensate for the loss of protons of the converted formic acid. This effect is not common for a pH-auxostat which is normally controlled with only one acidic or alkaline compound (Gostomski et al., 1994). The sulfate concentration decreased even further and was depleted on day 13. The acetate concentration continued to decrease in run 1 until it was depleted at day 20. The pH remained nevertheless at 6 during the entire run 1. On completion of run 1, it was discovered that the pH of the run medium without sulfuric and formic

acid was below 6, due to the high concentration of KH_2PO_4 (2.05 g L^{-1}; Table 1) in the rum medium. This indicated that the alkaline start-up medium with lower concentrations of KH_2PO_4 (0.41 g L^{-1}; Table 1) was slowly washed out by the more acidic run medium and therefore the run came to a hold. This problem can be solved by the addition of an alkaline compound to the run medium like NaOH. Due to this dilution effect, the maximum achievable volumetric activity of this system was not reached. The pH-auxostat system has shown to be a good system to rapidly establish the reachable sulfate reducing rates, making it an interesting tool for studying sulfate reducing processes under thus far unexplored conditions.

Conclusion

This study shows that:

1. It is possible to reduce sulfate at pH 6 with a high volumetric and specific activity with sludge from full-scale waste water treatment bioreactors operated at neutral pH as inoculum
2. Formate was rapidly converted into hydrogen, which was the most likely electron donor used for sulfate reduction, acetogenesis and methanogenis
3. Sulfate reduction was responsible for the majority (69 %) of the electrons transferred
4. The combination of a MBR and pH-auxostat is a useful research tool to study processes with unknown growth rates at a maximum activity.

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3

Sulfate reduction at pH 5 in a high-rate membrane bioreactor: reactor performance and microbial community analyses

Abstract

High rate sulfate reduction under acidic conditions opens possibilities for new flow sheets that allow the selective recovery of metals from mining and metallurgical waste and process water. Knowledge of high rate sulfate reduction under acidic conditions is however limited. This paper therefore investigates sulfate reduction in a membrane bioreactor at a controlled pH of 5. Sulfate and formate were dosed using a pH-auxostat system while formate was converted into hydrogen, which was used for sulfate reduction. Sulfide was removed from the gas phase to prevent sulfide inhibition. This study shows a high-rate sulfate reducing bioreactor system for the first time at pH 5, with a volumetric activity of 188 mmol SO_4^{2-} L^{-1} d^{-1} and a specific activity of 81 mmol SO_4^{2-} g volatile suspended $solids^{-1}$ d^{-1}. The microbial community that developed at pH 5 still consisted at the end of the study of a diverse mixed population.

Introduction

Waste and process water from the mining and metallurgical industry typically contains dissolved metal-ions and sulfuric acid (Johnson and Hallberg, 2003). Although these acidic waste streams are a major environmental problem, valuable metals can be recovered by microbiological reduction of the sulfate to sulfide, followed by metal-sulfide precipitation (Huisman et al., 2006; Tabak et al., 2003; Veeken et al., 2003). Sulfate reduction at neutral pH has been well studied (van Houten et al., 2006; van Houten et al., 1995; van Houten et al., 1994; Weijma et al., 2002), but by expanding the pH range of high rate sulfate reduction to acidic conditions, the potential of metal-sulfide recovery would be increased because metals can be selectively precipitated and recovered as metal-sulfides by varying the pH and sulfide concentration (Huisman et al., 2006; Tabak et al., 2003). Another advantage of sulfate reduction under acidic conditions is the reduced requirement for a neutralizing agent to increase the pH of the acidic waste stream. In addition, sulfide could be recovered more easily from the waste stream as more of the sulfide is in the gaseous form (H_2S) at low pH and consequently, decreases the cost of sulfide conversion to elemental sulfur by partial oxidization with oxygen (Buisman and Lettinga, 1990; Janssen et al., 1999).

Several reports described sulfate reduction in column experiments with acidic influent (Elliott et al., 1998; Jong and Parry, 2006); however, the sulfate reduction process itself occurs at non-acidic conditions as indicated by the neutral effluent pH. Little is known about sulfate reduction under acidic conditions and to our knowledge no literature exists on bioreactor runs under a controlled pH below 5.5. Sulfate reduction at a pH as low as 3.8 is possible (Kimura et al., 2006), but its use in industrial applications seems limited due to the low conversion rate of 0.2 mmol L^{-1} SO_4^{2-} d^{-1} achieved.

In sulfidogenic systems, the major biological conversion processes are sulfate reduction, acetogenesis, and methanogenesis (Lens et al., 2002). Even though these processes are well understood, the microbial populations responsible for these processes are not well described (Roest et al., 2005). Combining molecular microbial community analyses with reactor performance data can substantially enhance understanding of sulfidogenic systems. One method of to characterise microbial communities is using denaturing gradient gel electrophoresis (DGGE) that separates DNA fragments based on the sequence rather than size, followed by sequence analysis of the DNA fragment. This approach had been used to analyze microbial communities in a full scale sulfidogenic system at neutral pH treating wastewater from a zinc

refinery (van Houten, 2006), an Upflow Anaerobic Sludge Bed (UASB) treating papermill wastewater (Roest et al., 2005), and a laboratory scale sulfidogenic fluidized bed reactor treating acidic metal rich influent (Kaksonen et al., 2004). One disadvantage of DGGE is that it does not identify microorganisms that are less than 1 % of the total population (Muyzer, 1999).

This paper studies the performance of a formate fed sulfate reducing high rate membrane bioreactor (MBR) at a controlled pH of 5. Sulfate (electron acceptor) and formate (electron donor and carbon source) were dosed using a pH-auxostat that has been applied earlier for sulfate reducing systems at pH 7 (Paulo et al., 2005) and pH 6 (chapter 2). This system doses influent based on the activity of the microorganisms and thus prevents formic acid inhibition and sulfate limitation. Sulfide was removed from the gas phase to prevent potential sulfide inhibition, while biomass washout was prevented using membrane separation. The microbial community was characterized with DGGE followed by sequencing of the isolated bands.

Material and methods

Experimental design.

A 6 l MBR as described in chapter 2 was operated at pH 5.0 and a temperature of 30^{o}C. Formate was used as electron donor which can be a good replacement for H_2 in laboratory experiments because the redox couple formate/H_2+CO_2 is nearly the same (Table 4). The experiment was divided in 2 periods; where period I provided the inoculum for period II. In period I, the reactor was filled with mineral medium A and operated in batch (Table 1), followed by dosing of medium B with higher concentrations of sulfate and formate. From day 29-33 (period II) medium C was dosed with double concentrations of sulfate and formate. Medium B and C were dosed via a pH-auxostat system as described previously in chapter 2. In a pH auxostat, medium is dosed to maintain the reactor liquor within the pH set-points (Gostomski et al., 1994). Sulfuric acid and formic acid were dosed to compensate for the pH increase due to sulfate reduction.

Table 1: Chemical composition of media A, B, and C

Chemical	medium A	medium B	medium C
Na_2SO_4 [g l^{-1}]	1.48	-	-
$NaHCO_2$ [g l^{-1}]	2.83	-	-
H_2SO_4 [M]	-	0.5	1.0
H_2CO_2 [M]	-	2.81	5.61
KH_2PO_4 [g l^{-1}]	0.41	2.05	2.05
NH_4Cl [g l^{-1}]	0.3	1.5	1.5
KCl [g l^{-1}]	0.37	1.85	1.85
$MgCl_2 \cdot 6H_2O$ [g l^{-1}]	0.1	0.5	0.5
$CaCl_2 \cdot 2H_2O$ [g l^{-1}]	0.11	0.5	0.5
$NaHCO_3$ [g l^{-1}]	1	1	1
Yeast extract [g l^{-1}]	0.1	0.5	0.5
Acid trace elements[a] [ml l^{-1}]	1	5	5
Alkaline trace elements[a] [ml l^{-1}]	1	5	5

[a] Described in (Stams et al., 1993)

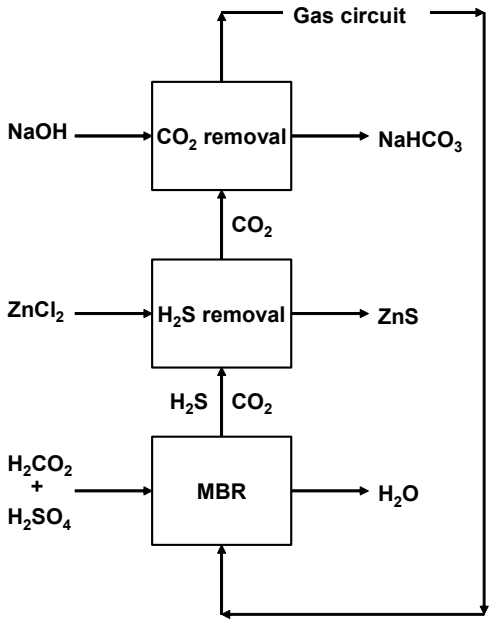

Figure 1: Flow sheet of the 6 liter membrane bioreactor and H_2S and CO_2 removal units.

The inoculum contained 60 g wet Eerbeek sludge (Industrie water Eerbeek, Eerbeek, The Netherlands), 30 g wet Nedalco sludge (Royal Nedalco, Bergen op Zoom, The Netherlands), and 100 ml supernatant of Zinifex sludge (Zinifex Budel Zinc, Budel, The Netherlands). The inoculum was crushed with a household blender for three minutes. Period II was started with 0.5 l reactor liquid from period I.

Physico-chemical analyses.

Sulfate and formate were analyzed with ion-chromatography as described by Sipma et al. (2004). Gas composition in the headspace was measured by gas chromatography, whereby H_2, N_2, O_2, and CH_4 were analyzed on a Hewlett Packard 5890 and CO_2 on a Fisons Instruments GC8000 according to Weijma et al. (2000). Volatile fatty acids (VFA) were analyzed on a Hewlett Packard series II GC (Weijma et al., 2000). Sulfide was analyzed using the Dr. Lange sulfide kit LCK-653 and a Xion 500 spectrophotometer (Hach Lange GMBH, Düsseldorf, Germany). Total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed following standard methods (Clesceri et al., 1998). The particle size distribution of the sludge was analyzed with laser scattering image analysis (Coulter laser LS 230, Beckman Coulter, USA).

Molecular phylogenetic analysis of the microbial community.

The microbial community present at the end of period II was identified by DGGE and DNA sequencing. Duplicate samples (10 ml) from the bioreactor sludge were taken at the end of the experiment. DNA was isolated from one of the duplicate samples by bead beating followed by purification using the Wizard DNA Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions (Dopson and Lindström, 2004). DNA was isolated from the second duplicate sample by pelleting the cells (10 000 × g for 10 min) and re-suspending in 500 µl TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 7) containing 0.12 % (wt/vol) sodium dodecyl sulfate and 0.06 mg/ml proteinase K. The mixture was incubated for 1 h at 37°C and then DNA isolated by phenol chloroform extraction (Bond et al., 2000). Both DNA preparations were PCR amplified (Dopson and Lindström, 2004) using the archaeal (ARC344F-GC and ARC915R) and bacterial (GM5F-GC and DS907R) specific primers (Table 2). The amplified DNA fragments were analyzed by DGGE using a denaturing gradient of 30-70 % denaturant (Dopson and Lindström, 2004). The DGGE bands were excised, cloned into the pGEM-T Easy Vector System (Promega, Madison, WI, USA), transformed into *Escherichia coli*, and sequenced (primers M13 Universal and M13R; Table 2) as described in Dopson and Lindström (2004). The obtained DNA sequences were checked for chimeras at the Ribosome Database Project II site (http://rdp.cme.msu.edu/html (Cole et al., 2003)) and aligned in the ARB program package (Ludwig et al., 2004) as described in (Morales et al., 2005). One chimeric artefact (chimera) was identified which was not included in the phylogenetic analyses. The number of base pairs used for alignment and phylogenetic analysis for each of the clones is given in Table 3.

Table 2:16S rRNA gene and sequencing primers used in this study

Primer	Position[a]	Sequences (5′-3′)	Reference
ARC344F-GC[b]	344-363	ACG GGG YGC AGC AGG CGC GA	(Raskin et al., 1995)
ARC915R	915-934	GTG CTC CCC CGC CAA TTC CT	(Stahl and Amann, 1991)
GM5F-GC	341-357	CCT ACG GGA GGC AGC AG	(Muyzer et al., 1995)
DS907R	907-927	CCG TCA ATT CCT TTR AGT TT	(Muyzer et al., 1995)
M13 Universal	NA[c]	GTAAAACGACGGAGT	(Messing, 1983)
M13R	NA[c]	CAGGAAACAGCTATGACCATG	(Messing, 1983)

[a]Base position numbers correspond to *Escherichia coli* 16S rRNA gene.
[b]GC-clamp (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′) is attached to the 5' end of the primer (Muyzer and Smalla, 1998).
[c]NA, not applicable.

Gibbs free energy calculations.

Gibbs free energies of the reactions were calculated using thermodynamic data from Amend and Shock . At the start of the experiment 0.1 % was used as hydrogen concentration to make thermodynamic calculations possible. The same concentration was used for CO_2 of which the concentration was low due to stripping with a NaOH solution. For sulfide, a constant concentration of 0.4 mM was used which was found to be an average value in the reactor liquor.

Mass balance sulfate calculations.

The mass balance calculations are done as described in chapter 2.

Results and discussion

Period I reactor performance.

Sulfate reduction did not occur during the first 14 days despite an initial decrease in formate concentration (electron donor) during the first 6 days of the experiment. This initial decrease in formate was followed by a rapid decrease in formate concentration resulting in formate depletion at day 12 (Fig. 2a). Formate was converted into hydrogen (Table 4, Eq. 5) and acetate (Table 4, Eq. 3 and 8) as indicated by the rapid increase in the hydrogen and acetate concentrations from day 6 onwards (Fig. 2b and

2c). The hydrogen concentration further increased and reached a maximum concentration of 72 % of the gas-phase on day 17 (Fig. 2b) and acetate reached a maximum concentration of 17 mM on day 13 (Fig. 2c).

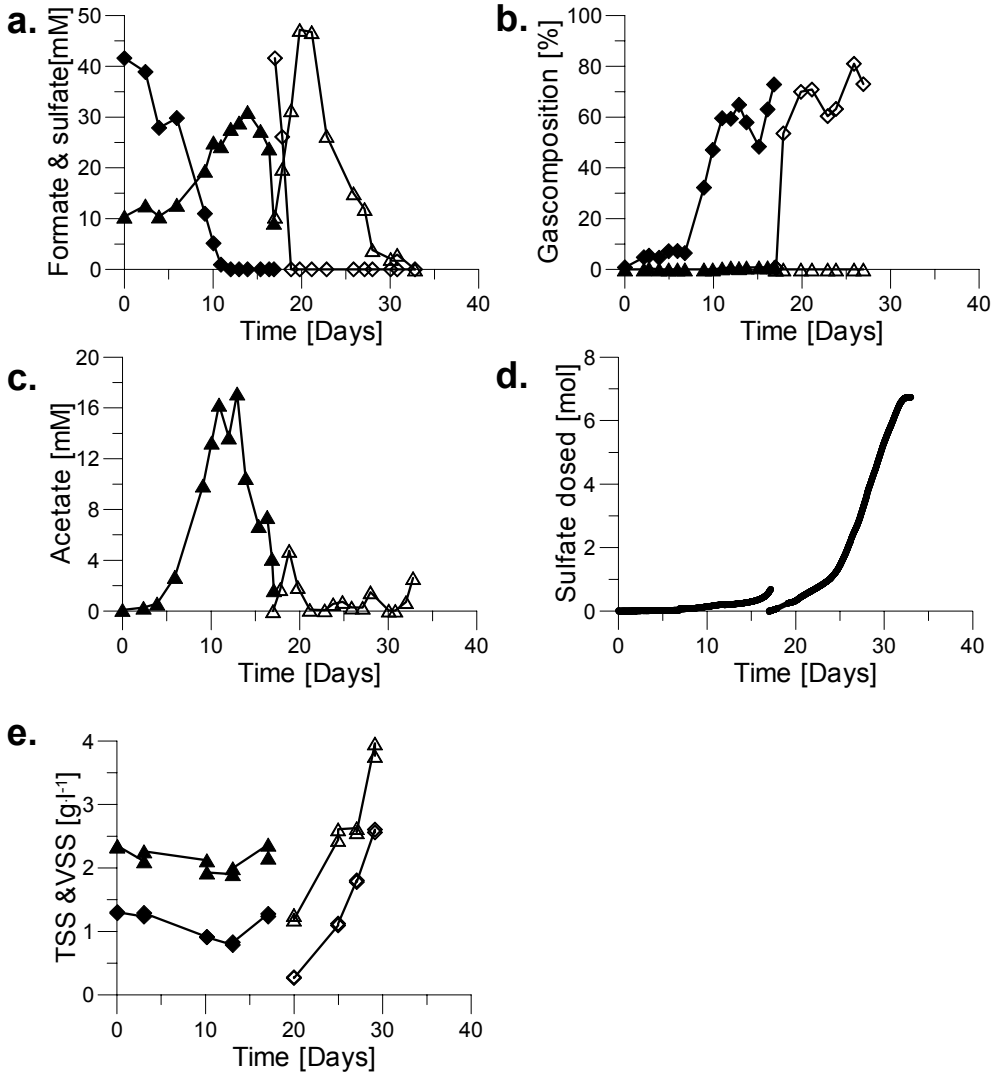

Figure 2: Performance of the sulfate reducing membrane bioreactor at pH 5 with closed triangles and diamonds for period I and open closed triangles and diamonds for period II: (a) formate (♦) and sulfate (▲) concentration in the reactor expressed as mM sulfate/formate; (b) gas phase composition with hydrogen (♦) and methane (▲) in percentage of total gas phase; and (c) acetate (▲) concentration in the reactor expressed as mM acetate; (d) accumulative sulfate dosed to the reactor by the pH-auxostat; and (e) total suspended solids (TSS, ▲) and volatile suspended solids (VSS, ♦) of the reactor expressed as gram TSS/VSS per liter of reactor liquor.

Interactions between formate and hydrogen in anaerobic systems have been well described and the preferred compound depends on the concentrations of the chemical species in the thermodynamic equation (Chapter 2; (Boone et al., 1989; Peters et al.,

1999; Thiele and Zeikus, 1988). The removal of CO_2 from the headspace of the present study resulted in the equilibrium shifting to the production of hydrogen. Hydrogen production from formate is a proton consuming reaction (Table 4, Eq. 5) and thus pH increasing. The pH increase was compensated by dosage of sulfuric acid and formic acid containing medium by the pH-auxostat (Fig. 2d). Until day 14 only formate was converted from the medium, resulting in sulfate accumulation (Fig. 2a), indicating that no sulfate was reduced during the first 14 days (Table 4, Eq. 1 and 2). Sulfate reduction started after day 14 as indicated by the decrease in sulfate concentration (Fig. 2a). In similar experiments at pH 6, sulfate reduction started within 7 to 9 days (chapter 2), suggesting that development of an active population at pH 5 demands more time. A small amount of methane was produced but did not rise above 1 % of the gas phase (Fig. 2b). No significant biomass growth was observed (Fig. 2e), as expected when only a small amount of electron donor was converted. Period I ended because of technical problems, therefore run II was started in the same bioreactor with 0.5 l reactor liquor of period I.

Table 4: Overview of possible reactions and their Gibbs free energy (ΔG) values calculated in kJ per mole of transferred electron under various conditions

Nr.	Formula	$\Delta G_r^{0'}$ [a] [kJ e-mol^{-1}]	ΔG_{pH7} [b] [kJ e-mol^{-1}]	ΔG_{pH5} [c] [kJ e-mol^{-1}]
1	$4HCO_{2(aq)}^{-} + SO_{4(aq)}^{2-} + 6H_{(aq)}^{+} \rightarrow H_2S_{(aq)} + 4CO_{2(g)} + 4H_2O_{(l)}$	-17[d]	-17[d]	-25[d]
2	$4HCO_{2(aq)}^{-} + SO_{4(aq)}^{2-} + 5H_{(aq)}^{+} \rightarrow HS_{(aq)}^{-} + 4CO_{2(g)} + 4H_2O_{(l)}$			
3	$4HCO_{2(aq)}^{-} + 3H_{(aq)}^{+} \rightarrow Ac_{(aq)}^{-} + 2CO_{2(g)} + 2H_2O_{(l)}$	-14	-14	-18
4	$4HCO_{2(aq)}^{-} + 4H_{(aq)}^{+} \rightarrow CH_{4(aq)} + 3CO_{2(g)} + 2H_2O_{(l)}$	-18	-18	-24
5	$HCO_{2(l)}^{-} + H_{(aq)}^{+} \rightarrow H_{2(g)} + CO_{2(g)}$	-2	-2	-8
6	$4H_{2(g)} + SO_{4(aq)}^{2-} + 2H_{(aq)}^{+} \rightarrow H_2S_{(aq)} + 4H_2O_{(l)}$	-15[e]	-15[e]	-18[e]
7	$4H_{2(g)} + SO_{4(aq)}^{2-} + H_{(aq)}^{+} \rightarrow HS_{(aq)}^{-} + 4H_2O_{(l)}$			
8	$4H_{2(g)} + 2CO_{2(g)} \rightarrow Ac_{(aq)}^{-} + H_{(aq)}^{+} + 2H_2O_{(aq)}$	-12	-11	-10
9	$4H_{2(g)} + CO_{2(g)} \rightarrow CH_{4(g)} + 2H_2O_{(l)}$	-16	-16	-16

[a] 25 ºC, pH 7 and standard conditions of 1M and 1 bar

[b] 30 ºC, pH 7 and concentrations of 1M and 1 bar

[c] 30 ºC, pH 6 and concentrations of 1M and 1 bar

[d] Based on reaction 1 and 2 and pKa of 6.96 (Kawazuishi and Prausnitz, 1987)

[e] Based on reaction 6 and 7 and pKa of 6.96 (Kawazuishi and Prausnitz, 1987)

Period II reactor performance.

After the restart on day 17, formate was rapidly converted to hydrogen and was depleted in two days (day 19; Fig. 2a). Hydrogen accumulated rapidly to 50 % of the gas phase after 1 day (day 18) and 70 % by day 20 (Fig. 2b). Despite the conversion of formate into hydrogen in the beginning of period II, sulfate accumulated suggesting that sulfate reduction was absent, as was observed the first 14 days of period I. Acetate was the only VFA that exceeded 1 mM, with a peak concentration of 5 mM on day 19, followed by a rapid decrease and full depletion on day 21 (Fig. 2c). Unlike experiments at pH 6 (chapter 2), methane was absent during period II (Fig. 2b).

Sulfate reduction took place from day 21 onwards, leading to medium dosage by the pH auxostat. The rate of medium dosage increased from day 24 onwards (Fig. 2d), suggesting an increase in volumetric activity (Fig. 3a) that fluctuated between 99 mmol L^{-1} SO_4^{2-} d^{-1} and 188 mmol L^{-1} d^{-1} between day 26 and 32, with a maximum specific activity of 81 mmol SO_4^{2-} $gVSS^{-1}$ d^{-1} (Fig. 3b). These rates were considerably higher than from other reported bioreactors operated close to pH 5 (Table 5). Van Houten et al. (1995) reported a volumetric activity of 52 mM SO_4^{2-} d^{-1} at pH 5.5 in a hydrogen fed gas-lift bioreactor, while at pH 5.0 no activity was obtained. The sulfate conversion efficiency at the maximum volumetric activity in the present experiment was 97 %, which was higher than most previously reported high-rate experiments even at neutral pH (Table 5). The specific activity of the sludge in this study shows that the biomass retained by the membrane was very active and only comparable to results from other MBR (chapter 2 and gas-lift bioreactors (van Houten et al., 1995; van Houten et al., 1994) operated at a higher pH.

Table 5: The reactor concept, pH, temperature, substrate, volumetric activity, specific activity and conversion rate from this study and literature

Reactor concept	pH	Temp [^oC]	Substrate	Volumetric activity [mmol L^{-1} SO_4 d^{-1}]	Specific activity [mmol SO_4 $gVSS^{-1}$ d^{-1}]	Conversion efficiency [%]	References
MBR	5.0	30	Formate	188	81	97	This study
Gaslift[a]	7.0	30	Hydrogen	288	-[b]	59	(van Houten et al., 1994)
Gaslift[a]	7.0	30	Hydrogen	156	-[b]	59	(van Houten et al., 1995)
MBR	6.0	30	Formate	302	110	100	Chapter 2
Gaslift[a]	6.0	30	Hydrogen	130	-[b]	49	(van Houten et al., 1995)
Gaslift[a]	5.5	30	Hydrogen	52	-[b]	19	(van Houten et al., 1995)

[a] With pumice as carrier material.

[b] No VSS concentration measured.

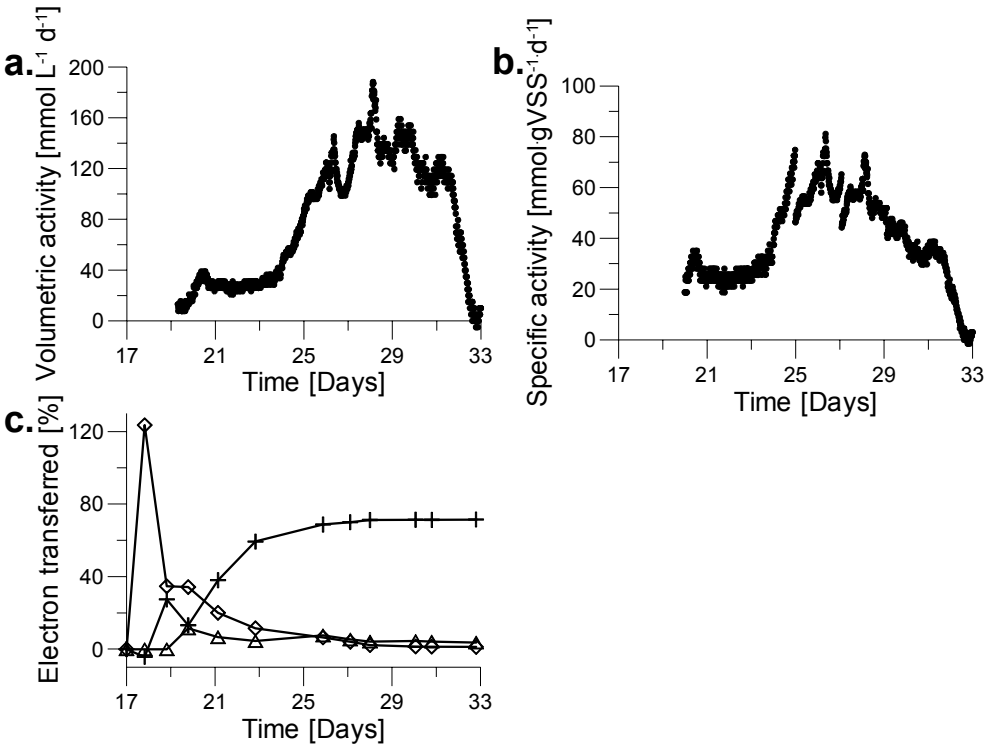

Figure 3: Performance of the sulfate reducing membrane bioreactor at pH 5 during period II with: (a) volumetric activity of the reactor (mmol sulfate reduced per liter of reactor liquor per day); (b) specific activity of the reactor (mmol sulfate reduced per gram of VSS per day); and (c) accumulative electrons transferred from formate to hydrogen (♦), sulfide (+) and biomass (▲) (% of total electrons transferred from formate).

Despite increased medium dosage from day 21 onwards, formate remained depleted in the reactor liquor, indicating that it was directly converted and all remaining and incoming sulfate had been reduced (Fig. 2a). The hydrogen concentration in the gas phase also increased to a maximum of 81 % at day 26, from the remaining formate after all sulfate had been reduced, while methane was still absent in the gas phase (Fig. 2b). During period II, biomass gradually increased from 0.3 g VSS l^{-1} at day 20, to 2.5 g VSS l^{-1} at day 29 (Fig. 2e). The culture had a mean particle size of 29 μm at start-up, which decreased to 21 μm at day 27, which was similar at pH 6 (chapter 2).

After day 32, the volumetric activity suddenly dropped and sulfate reduction stopped at day 33 (Fig 2a). At this point, sulfate and formate were depleted while the reactor liquor remained at pH 5, indicating that the acidity of the bioreactor liquor was not longer based on formic or sulfuric acid. As the pH of the medium without formic and sulfuric acid was below the set-point, the alkalinity originally present in the start-up

medium was washed out during reactor operation. This resulted in depletion of both formic and sulfuric acid causing the pH-auxostat to stop dosing and thus for the run to terminate. Addition of an alkaline compound to medium B and C would prevent depletion of formic and sulfuric acid by the pH auxostat. Even though a MBR with a pH-auxostat has advantages in fundamental studies, applications in full scale systems could be unstable due to the delicate chemical balance needed to operate a pH-auxostat, especially in the case when multiple acids are present.

Molecular microbial phylogeny of bioreactor in period II.

The 16S rRNA gene similarities of the microorganisms present at the end of the bioreactor run in period II shows the presence of a diverse population of sulfate reducers (*Proteobacteria*), acetogens (*Firmicutes*), and methanogens (*Eukarchaeota*) (Table 3; Fig. 4) as expected from a sulfidogenic bioreactor (Kaksonen et al., 2004; Kaksonen et al., 2006; Roest et al., 2005; van Houten, 2006). Bacteria from the phyla *Actinobacteria*, *Spirochaetes,* and *Bacteroidetes* were also present, which are not uncommon in laboratory (Kaksonen et al., 2004; Kaksonen et al., 2006; Roest et al., 2005) and full-scale (van Houten, 2006) sulfidogenic bioreactors. These bacteria most likely live on organic matter from decayed biomass retained in the system by the membrane.

Table 3. Cloned 16S rRNA gene fragments from the bioreactors and their closest related named gene sequences in the NCBI database.

Clone	Closest relative named species in database	Accession nr.[a]	% similarity[b]	Nr. of bases[c]
MBR5-1	*Methanosaeta concilii* H-3	AB212065	97	423
MBR5-2	No named species in first 100 identified		<93	422
MBR5-3	*Petrimonas sulfuriphila* BN3	AY570690	99	577
MBR5-4	*Clostridium bowmanii* DSM 14206	AJ506120	99	555
MBR5-5	*Parabacteroides goldsteinii*	AY974070	94	563
MBR5-6	*Enterococcus durans* CECT411^T	AJ420801	98	433
MBR5-7	*Desulfovibrio fructosovorans*	AF050101	95	309
MBR5-8	*Desulfovibrio aerotolerans* DvO5	AY746987	93	583
MBR5-9	*Spirochaeta bajacaliforniensis* DSM 16054^T	AJ698859	92	580
MBR5-10	*Eggerthella lenta*	AF292375	91	550
MBR5-11	*Eggerthella lenta*	AF292375	90	511

[a]Accession number of the closest related named gene sequence obtained by BLAST comparison in the NCBI database (http://www.ncbi.nlm.nih.gov/).

[b]Percentage sequence similarity to the closest relative in the NCBI database.

[c]Number of base pairs used for the BLAST comparison and alignment in the ARB program.

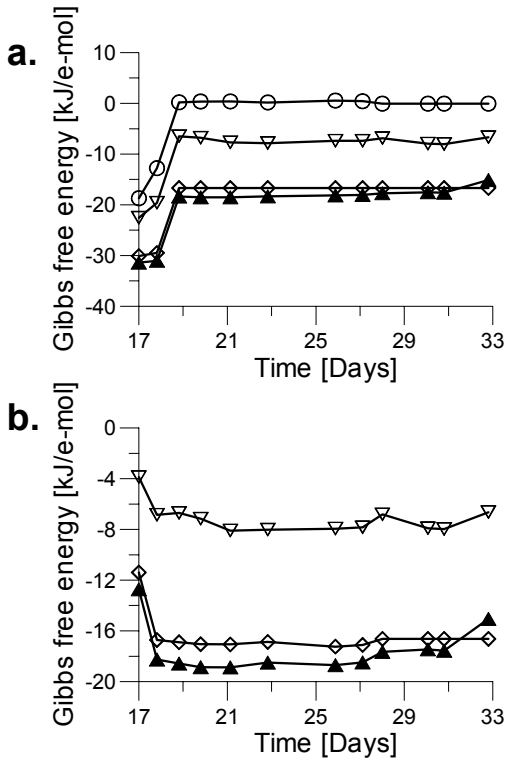

Figure 4: The Gibbs free energy of reaction expressed in kJ e-mol^{-1} for: (a) reactions with formate as electron donor and as product sulfide (▲, Table 4, Eq. 1-2), acetate (Δ, Table 4, Eq. 3), methane (♦, Table 4, Eq. 4) and hydrogen (○, Table 4, Eq. 5), and (b) reactions with hydrogen as electron donor and as product sulfide (▲, Table 4, Eq. 6-7), acetate (Δ, Table 4, Eq. 8) and methane (♦, Table 4, Eq. 9).

In total 11 clones were identified by DGGE (Fig. 3) with between 90 % and 99 % 16S rRNA gene similarities with known species, indicating that there were both novel and previously characterized species present in the sludge. Of these 11 clones, MBR5-1 aligned within the archaeal domain and the other clones with the bacteria (Table 3 and Fig. 5). Two clones were found in the bacterial δ-proteobateria clade (Fig. 5), belonging to the sulfate reducing *Desulfovibrio* family (Table 3). According to the NCBI database, the named species most closely related to MBR5-7 was *D. fructosovorans* (95 % 16S rRNA gene similarity; Table 3), while MBR5-8 was most closely related to *D. aerotolerans* (93 % 16S rRNA gene similarity; Table 3) suggesting that they might be novel sulfate reducing species. In addition, MBR5-4 and MBR5-6 aligned within the *Firmicutes* clade (Fig. 6). MBR5-4 was most closely related to the named acetogenic species *Clostridium Bowmanii* (99 % similarity) and MBR5-6 to *Enterococcus durans* (98 % similarity). The most closely related named species to the archaeon (MBR5-1) was the acetate utilizing methane producer *Methanosaeta concilii* H-3 (97 % similarity). MBR5-2, MBR5-3, and MBR5-5 all aligned within the *Bacteroidetes* clade. MBR5-9 was most closely related to the

named species *Spirochaeta bajacaliforniensis*. MBR5-10 and 11 were both closely related to the named species *Eggerthella lenta* however, with different alignments in the phylogenetic tree and 91 % similarity for MBR5-10 and 90 % similarity for MBR5-11.

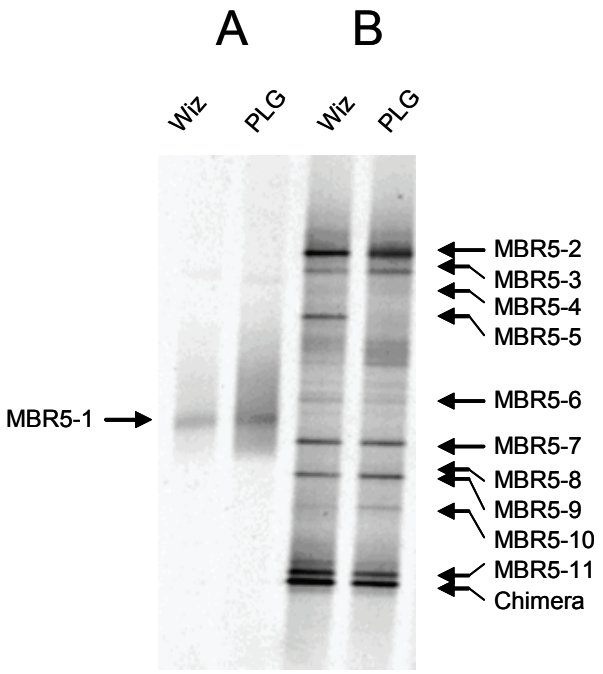

Figure 5: DGGE gel of archaea (A) and bacteria (B) amplified from the pH 5 membrane bioreactor mixed culture. Duplicate DNA samples were prepared by the Wizard DNA Clean Up System (Wiz) and phenol chloroform extraction with phase lock gel tubes (PLG). One chimeric artefact was identified that was not included in the phylogenetic analysis.

The archaeon and sulfate reducers found in the present study were closely related to uncultured archaea (strains 1B7, 1A3, 1E4, 1H10, and 1A7) and bacteria (strain E14 and E21) present in the papermill wastewater treatment sludge used in the inoculum ((Roest et al., 2005); Fig. 6). Although no exact match was found it is likely that these strains originate from the papermill sludge, indicating that the identified microorganisms were more closely related to microorganisms found in bioreactors than in acidic waste streams. This is in agreement with the fact that the inoculum originated from three near neutral pH full-scale bioreactors. Lowering the reactor liquid pH to 5 still resulted in a diverse population of probably acid tolerant microorganisms during the bioreactor operation.

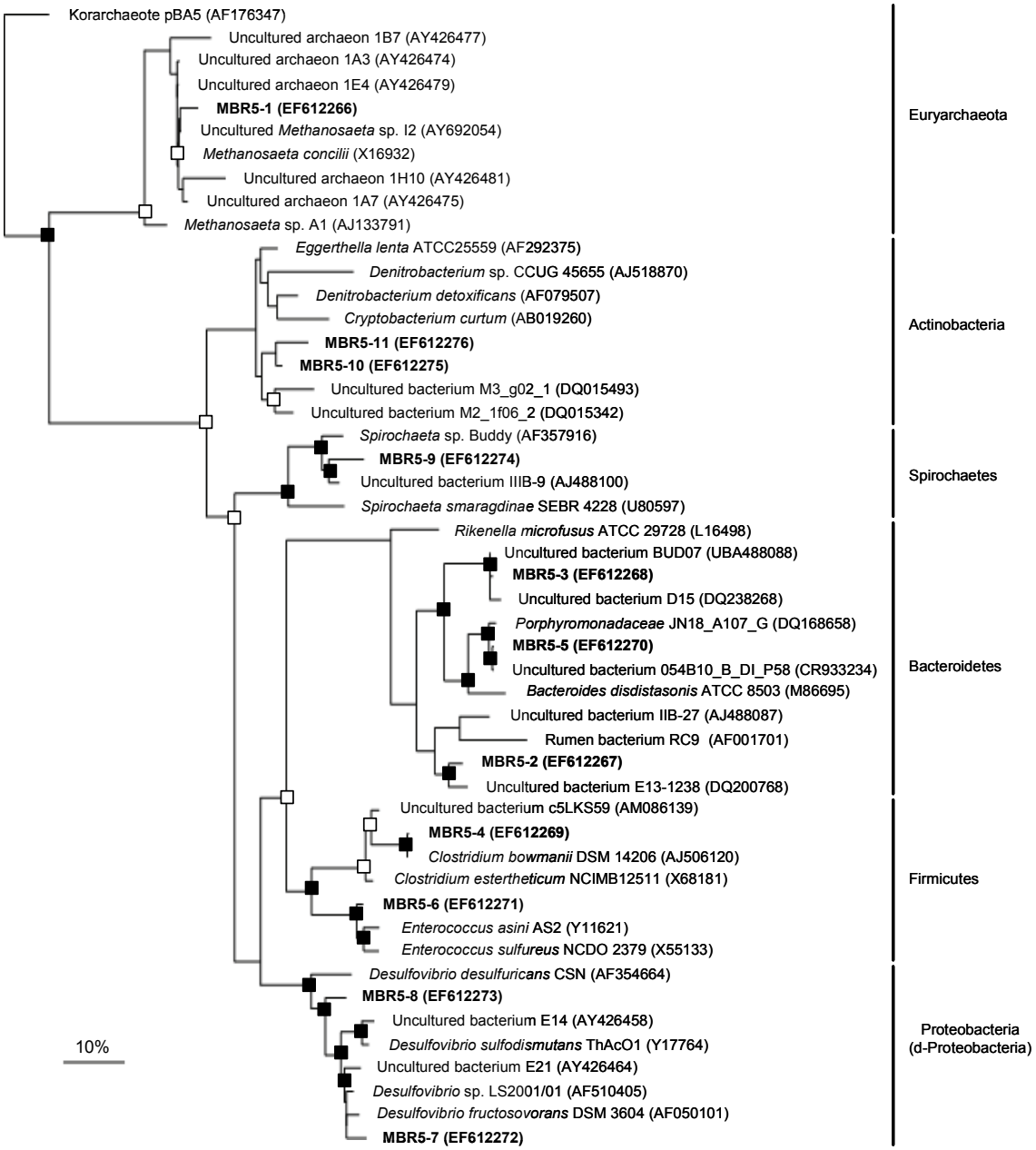

Figure. 6: Maximum likelihood phylogentic tree based on partial 16S rRNA gene sequences of clones isolated from the pH 5 membrane bioreactor (in bold). Phylogenetic analysis was carried out by the maximum likelihood, distance neighbour joining, and DNA parsimony methods and the nodes supported by all 3 trees (■) and 2 trees (□) have been marked. Accession numbers are given in parenthesis. The scale bar corresponds to 10% sequence similarity.

Electron transfer characteristics. During the experiment, formate was rapidly converted into hydrogen (Table 4, Eq. 5), which was the most likely electron donor for sulfate reduction (Table 4, Eq. 6-7). The Gibbs free energy of formation (ΔG) of H_2 production from formate at pH 7 was –2 kJ e-mol^{-1} compared to -8 kJ e-mol^{-1} at pH 5 (Table 4, Eq. 5), indicating that a lower pH the production of hydrogen from formate generates more energy. In the first 2 days of period II (day 19), hydrogen production accounted for the major electron flow while sulfate reduction was absent (Fig. 3c). At the end of period II the major electron flow was to sulfide (71 %) while no other major electron flows could be identified (Fig 3c). The ΔG of hydrogen from formate (Table 4, Eq. 5) increased from -19 kJ e-mol^{-1} to around 0 kJ e-mol^{-1} on day 19 and then stabilized (Fig. 4a).

The ΔG of sulfate reduction with hydrogen decreased to -18 kJ e-mol^{-1} on day 1 (Fig. 4b), which made this reaction more thermodynamically favorable. The ΔG of methane production was close to that of sulfide production (Fig. 4), while methane was not produced in period II. Even though methane was not found in the headspace, archaeal specific primers amplified a single 16S rRNA gene sequence that aligned with the methanogenic archaea. This indicates that the archaeon was transferred with the inoculum and persisted in the MBR without significant activity. Although these methanogenic archaea were not thermodynamically limited, their activity did not proliferate due to other factors such as non-optimal pH.

Conclusion. This study shows for the first time that a high-rate sulfate reducing bioreactor system with high volumetric and specific activities can be reached at a controlled pH of 5. This opens possibilities in new process flow schemes that require sulfate reduction at low pH, e.g. selective recovery of metals from waste and process water from mining and metallurgical industries.

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4

Sulfate reduction at pH 4.0 for treatment of process and wastewater from mining and metallurgical industries

Abstract

A large number of industrial process and wastewaters are acidic and contain high sulfate concentrations. Sulfate reducing bacteria convert sulfate to sulfide that can subsequently be used to recover metals as insoluble metal-sulfides. This study reports on high rate sulfate reduction with a mixed microbial population at pH 4.0 and 4.5 with hydrogen and formate as electron donors. The maximum sulfate reducing activity at pH 4.0 was 151 mmol sulfate reduced per litre of reactor liquid per day with an upper specific activity of 84 mmol sulfate per gram of volatile suspended solid per day. The biomass yield gradually decreased between pH 6 to 4 from 38 to 0.4 gram volatile suspended solid per kilogram of sulfate, respectively, showing the population was acidotolerant. The population diversity in the bioreactor decreased in the pH 4.0 reactor over time, while the sulfate reducing population increased over time.

Introduction

A large number of industrial process and wastewaters contain high sulfate concentrations many of which have a low pH and contain little organic matter (Johnson, 2000a). The mining and metallurgical industry produces large quantities of these acidic wastewaters that also contain high concentrations of dissolved metal-ions (Olson et al., 2003). The most common treatment process for these waste streams is chemical precipitation of both sulfate and metal by lime or limestone (Weijma et al., 2002) that yields large quantities of gypsum contaminated with heavy metals with a limited re-use potential. Microbial treatment has also been commercially applied (Johnson and Hallberg, 2005) based on sulfate reducing bacteria (SRB) which reduce sulfate to sulfide. This treatment option allows for recovery of metals as metal-sulfides (Tabak et al., 2003; Weijma et al., 2002) that can be directly used in hydrometallurgical plants (Weijma et al., 2002). Reducing sulfate at low pH decrease costs associated with caustic addition to increase the influent pH as well as opening possibilities to selectively recover metals from multi metal streams by varying the pH and sulfide concentration. For instance by operating a reactor at pH 4 to 4.5 metals as copper, nickel and zinc will precipitate while iron does not thus enabling these metals to be separated from iron. This has been demonstrated with both chemically (Veeken et al., 2003) and biologically produced sulfide by sulfate reduction (Tabak et al., 2003) and sulfur reduction (Huisman et al., 2006).

A shortcoming of most studies on sulfate reduction under acidic conditions is the short duration and the lack of pH control during the experiment. Studies performed in batch bottles usually rely on phosphate buffering which is weak at low pH, resulting in a rapid increase in pH when microbial activity is present. Hard et al. (1997) reported an isolate from a waste water pond that reduces sulfate between pH 4.0 and 9.0, but the pH at the end of the experiment was not reported. Sulfate reduction has also been studied in column experiments with acidic influent and neutral pH effluent. Jong & Parry (2006) reported an upflow anaerobic packed bed bioreactor increasing the synthetic wastewater at pH 4 to an effluent pH of 7 reaching sulfate reduction rates of 1 mmol SO_4 L^{-1} d^{-1}.

Sulfate reduction at pH 3.8 has been reported in a batch reactor fed with glycerol(Kimura et al., 2006) and a pH 4.0 sucrose fed upflow anaerobic sludge bed (UASB) reactor (Lopes et al., 2007). However, the achieved rates limit industrial application (Lopes et al., 2007) and growth has not been demonstrated in these cultures. A potential cause for the lack of growth is that the organic electron donor was partly converted to acetate which has been shown to be toxic under acidic

conditions(Reis et al., 1990). Therefore, H_2 is a more suitable electron donor for sulfate reduction under acidic conditions. The Gibbs free energy for sulfate reduction increases with the pH, suggesting there is no thermodynamic limitation to sulfate reduction under acidic conditions (chapter 3).

This study reports on sulfate reduction at pH 4.0 and 4.5 with H_2 and formate as electron donor in the absence of high metals concentrations to be able to focus on sulfate reduction. A submerged membrane bioreactor was used to prevent biomass loss, while sulfate was dosed using a pH-auxostat in which medium was dosed to maintain the reactor liquor within the pH set-points (Gostomski et al., 1994). This system has previously reached high rate sulfate reduction at pH 6.0 (chapter 2) and 5.0 (chapter 3). The complete microbial population (using archaeal and bacterial 16S rRNA gene primers) and the sulfate reducing population (using *dsrB* gene primers) was followed through time using denaturing gradient gel electrophoreses (DGGE) followed by sequencing of the isolated bands.

Material and Methods

Experimental design.

A 6.0 L membrane bioreactor (chapter 2) was operated at 30^{o}C. Sulfate was added by a pH auxostat in which sulfuric acid containing medium was dosed to maintain the reactor liquor within the pH set-points (Gostomski et al., 1994). Three runs were performed; Run 1 was started at pH 5.0 and at day 30 the reactor pH was decreased to pH 4.5 for 1 day (day 30-31) followed by another decrease in pH to 4.0 (day 31-39). In run 1,formate was used as electron donor and carbon source. Run 2 was operated at pH 4.0 for 35 days after which the pH was raised to 4.5 (day 35-81). H_2 was dosed as electron donor (70%, day 0- 36; 95%, day 36-81) and CO_2 as carbon source (30%, day 0- 36; 5%, day 36-81) at a total flow rate of 100 mL min^{-1}. Run 3 was operated at pH 4.0 for 54 days with H_2 as electron donor (98.75%, day 0-19; 95%, day 19-54) and CO_2 as carbon source (1.25%, day 0- 19; 5%, day 19-54) at a total flow rate of 100 mL min^{-1}.

Inocula.

Run 1 was inoculated with 100 ml sludge from a previous reactor run at pH 5.0 (chapter 3). Run 2 was inoculated with 60 g wet Eerbeek sludge (Industrie water Eerbeek, Eerbeek, The Netherlands), 30 g wet Nedalco sludge (Royal Nedalco, Bergen op Zoom, The Netherlands), and 100 ml supernatant of Nyrstar sludge (Nyrstar Budel Zinc, Budel, The Netherlands). The inoculum was crushed with a

household blender for three minutes. Run 3 was inoculated with 500 ml of reactor liquor from run 1.

Media.
A defined medium dissolved in demineralized water (all chemicals were of analytical grade; Merck, Darmstad, Germany) was used containing at startup (g L^{-1}): Na_2SO_4, 1.48; KH_2PO_4, 0.41; NH_4Cl, 0.3; KCl, 0.37; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.11; $NaHCO_3$, 1; yeast extract, 0.1; and 1 mL L^{-1} of an acid and alkaline trace element solution (Stams et al., 1993). The start-up medium in run 1 and 3 also contained 2.3 g L^{-1} $NaHCO_2$. After start-up in run 1 the medium contained (g L^{-1}): KH_2PO_4, 2.05; NH_4Cl, 1.5; KCl, 1.85; $MgCl_2 \cdot 6H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.55; $NaHCO_3$, 1; yeast extract, 0.5; and 5 mL L^{-1} of an acid and alkaline trace element solution (Stams et al., 1993) and from day 0 – 33 also 1 M H_2SO_4 and 1 M H_2CO_2 and from day 33 also 2 M H_2SO_4 and 2 M H_2CO_2. After start-up in run 2 the medium contained (g L^{-1}): KH_2PO_4, 2.05; $(NH_4)_2SO_4$, 1.5; K_2SO_4, 1.85; $MgSO_4 \cdot 6H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.55; $NaHCO_3$, 1; yeast extract, 0.5; and 5 mL L^{-1} of an acid and alkaline trace element solution (Stams et al., 1993) and 1 M H_2SO_4. After start-up in run 3 the medium contained the same concentrations as the start-up medium in addition to 1 M H_2SO_4.

Molecular phylogentic analysis of the microbial community.
The pH 4.5 16S rRNA molecular phylogenetic characterization (DGGE denaturing gradient from 0 to 80%) was carried out according to Rzhepishevska et al.(Rzhepishevska et al., 2005). The pH 4.0 MBR 16S rRNA characterization and both *dsrB* gene molecular phylogenetic analyses (DGGE denaturing gradient from 20 to 80%) were carried out according to Dar (2007). DNA sequences were compared to GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and tested for chimeras at the RDP II site (http://rdp.cme.msu.edu/html/). Phylogenetic trees were constructed according to Morales et al. (2005) using the ARB program package (Ludwig et al., 2004) for the 16S rRNA gene sequences and the *dsrAB* ARB database.

Physico-chemical analyses. Sulfate, formate, sulfide, VFA, H_2, N_2, O_2, CH_4, CO_2, VSS and particle size distribution (PSD) of the sludge was analyzed according to chapter 3.

Results

Reactor performance of run 1 from pH 5.0 to pH 4.0. The biomass was adapted to acidic conditions by operating the bioreactor at pH 5.0 before changing the pH to 4.5 on day 30 and to pH 4.0 on day 31. The electron donor formate depleted at the

beginning of the experiment and remained depleted throughout the run (Fig. 1a). Formate was converted to hydrogen (Fig. 1b), which could be used as an electron donor for sulfate reduction. The sulfate concentration in the reactor liquor rapidly decreased between day 25 and 33 (Fig. 2), indicating that both the sulfate present in the reactor liquor and the dosed sulfate were converted. The sulfate reducing activity caused the reactor pH to increase, resulting in addition of medium by the pH-auxostat (Fig. 2). The volumetric sulfate reducing activity reach a maximum of 151 mmol L^{-1} d^{-1} on day 32 (Fig. 1c. The sulfate concentration remained at approximately 6 mM despite medium dosage to the reactor, indicating that the supplied sulfate was reduced to sulfide (Fig. 2). The acetate concentration increased from day 30 and reached 6 mM on day 33, before being consumed by day 35 (Fig. 1a). Methane was absent until day 25, after which it gradually increased until it dominated the headspace from day 37 onwards. The increase in methane in the headspace and thus decreasing of the hydrogen concentration (Fig. 1b). The biomass concentration increased from a VSS concentration of 0.9 gVSS L^{-1} at day 31 to 2.1 gVSS on day 36 (Fig. 1d).

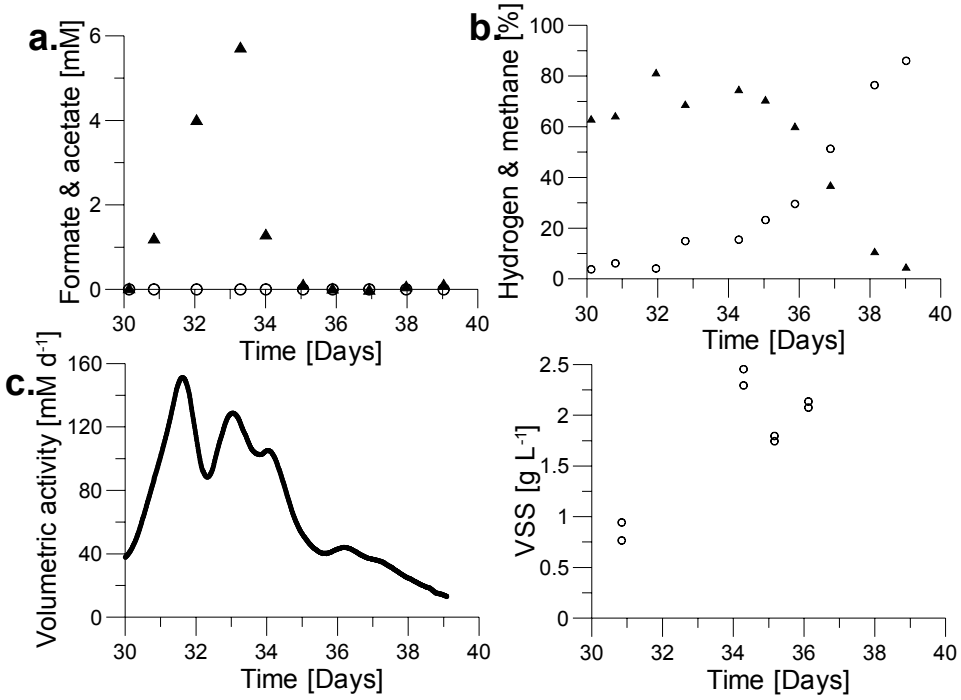

Figure 1: Reactor performance data of run 1 from pH 5.0 - 4.0 with, a) formate and acetate concentration in the reactor liquor, b) the hydrogen (▲) and methane (o) concentration in the headspace, c) the volumetric sulfate reducing activity, and d) the volatile suspended solids (VSS) in the reactor liquor.

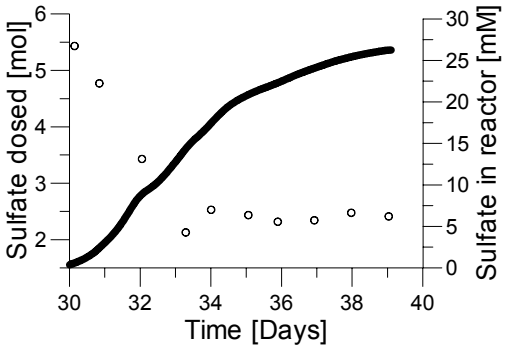

Figure 2: Amount of sulfate dosed (●) and sulfate present (o) in the reactor liquor in run 1 from pH 5.0 to 4.0.

Reactor performance of run 2 from pH 4.0 to 4.5. No sulfate reduction had been observed in the first 35 days at pH 4.0 (Fig. 3 and Fig. 4a). The pH was increased to 4.5 on day 35 and the sulfate reduction rate started to increase from day 50, reaching a maximum volumetric activity of 111 mmol SO_4 L^{-1} d^{-1} on day 58 (Fig. 4a). Sulfate was dosed to maintain a constant 17 mM sulfate concentration in the reactor liquor (Fig. 4b). Acetate was produced from the start of the run, reaching a concentration of 18 mM on day 20 and remained high until day 36 (Fig. 3). The decrease in acetate concentration from day 36 onwards could have been triggered by lowering the CO_2 concentration in the influent gas from 30 to 5 % on day 36 or by the increase in pH from 4.0 to 4.5 on day 35. The decreased CO_2 concentration in the gas influent resulted in a near depletion of CO_2 in the headspace (Fig. 4c). Almost no methane was found in the headspace in the first 34 days, but methane accumulated from day 35 onwards reaching 8 % of the headspace on day 58 (Fig. 4d online). The biomass concentration decreased from 1.1 gVSS L^{-1} to 0.5 gVSS L^{-1} on day 36, but after the pH was set to 4.5, the biomass concentration rapidly increased to 2.2 gVSS L^{-1} at day 55 (Fig. 4e). The biomass concentration dropped to 1.3 gVSS L^{-1} on day 59 which was most likely due to biomass loss by the backup system which was installed in case the dosing rate exceeded the membrane flow capacity. From day 59, the biomass concentration increased again and reached 2.1 gVSS L^{-1} on day 86 (Fig. 4e).

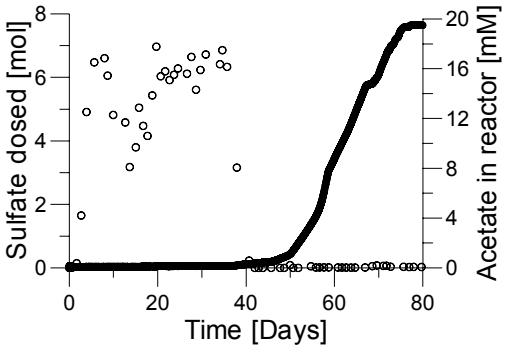

Figure 3: Amount of sulfate dosed (●) and acetate (o) in the reactor liquor in run 2 from pH 4.0 to 4.5.

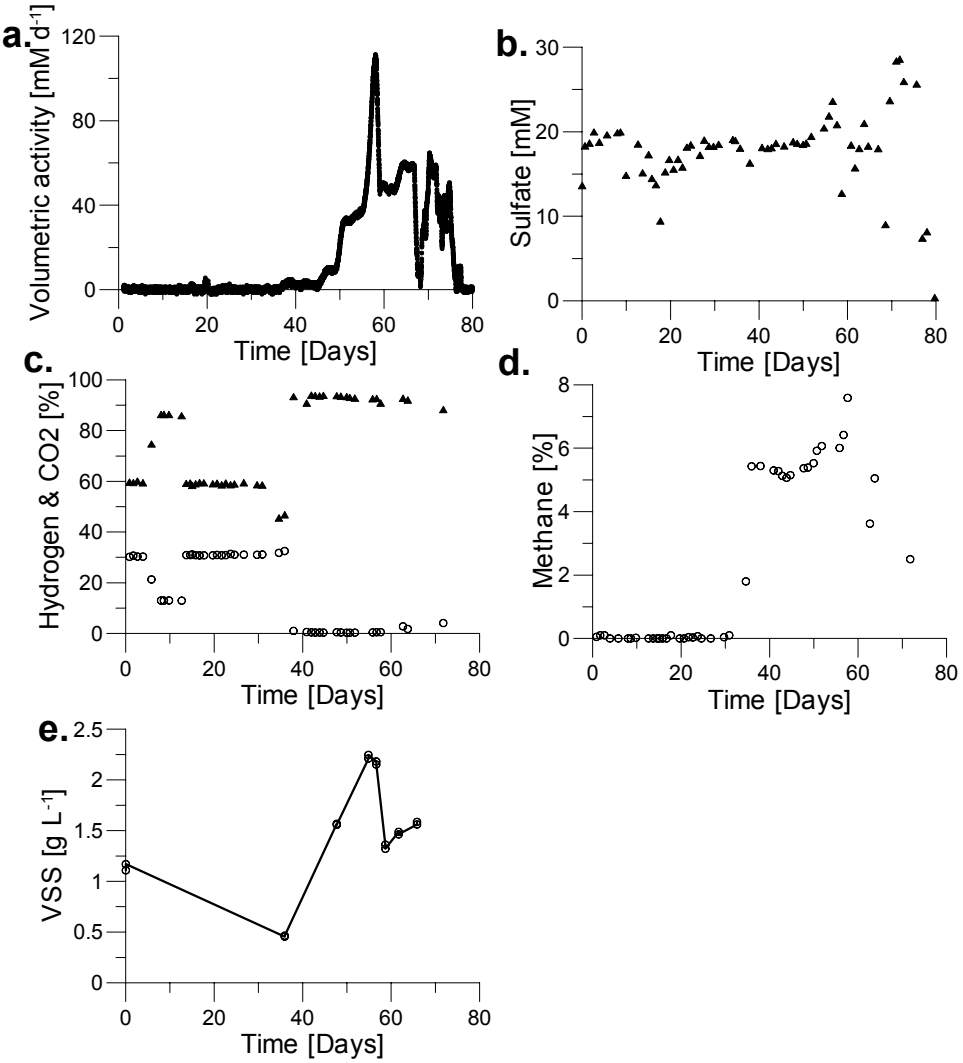

Figure 4: Reactor performance data of run 2 from pH 4.0 to 4.5, with a) the volumetric sulfate reducing activity, b) sulfate concentration in the reactor liquor, c) hydrogen (▲) and CO_2 (o) concentration in the headspace, d) methane concentration in the headspace and, e) volatile suspended solids (VSS) in the reactor liquor.

Reactor performance of run 3 at pH 4.0. Sulfate reduction was maintained at pH 4.0 for over 54 days (Fig. 5a). The volumetric sulfate reducing activity gradually increased from 1 mmol L^{-1} d^{-1} to 5 mmol L^{-1} d^{-1} between day 10 till day 50 (Fig. 5b). Hydrogen was used as electron donor, however, formate was added in the start-up medium and was depleted by day 10. The CO_2 was set to 1.25 % for the first 19 days and an average concentration of 1.0 % was found (Fig. 6b). During this period the acetate concentration was relatively low with an average of 0.1 mM (Fig. 5a). On day 19, the CO_2 concentration was set to 5 % resulting in an increase of the CO_2 concentration in the headspace with an average value of 4.2 % (Fig. 6b) and the acetate concentration increased to 3 mM (day 49; Fig. 3a). The rest of the gas-phase consisted of hydrogen (Fig. 6b). The biomass dropped from 0.21 g L^{-1} at start-up to 0.11 g L^{-1} at day 20, after which the concentration gradually increased to 0.23 at day 50 (Fig. 5b).

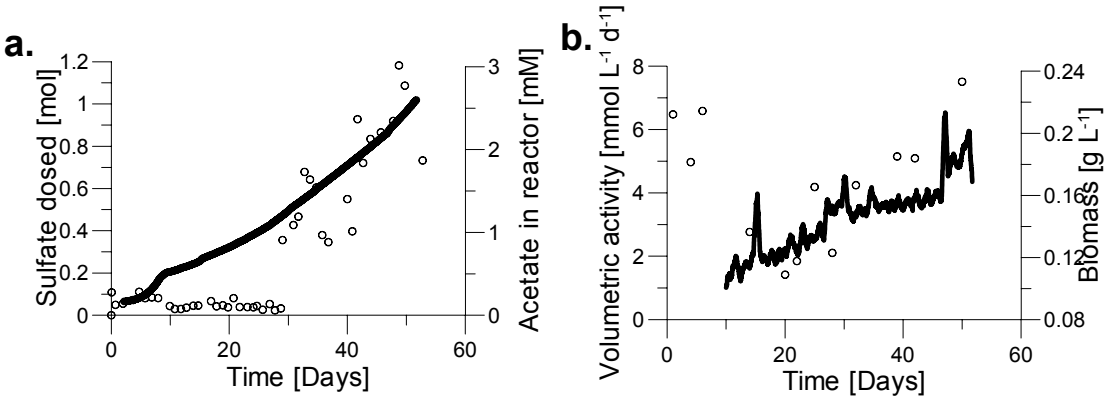

Figure 5: Reactor performance data of run 3 at pH 4.0, with a) the amount of sulfate dosed (●) and acetate (o) in the reactor liquor and b) the volumetric sulfate reducing activity (●) and biomass concentration (o).

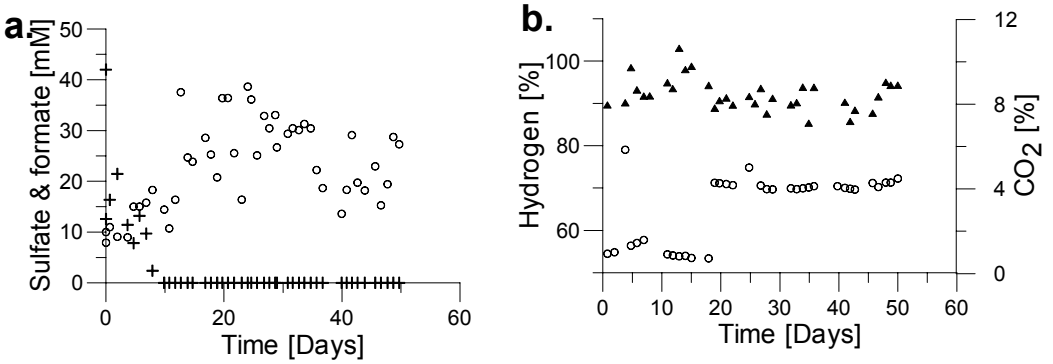

Figure 6: Reactor performance data of run 2 at pH 4.0 with a) the sulfate (o) and formate (+) concentration in the reactor liquor and b) the hydrogen (▲) and CO_2 (o) concentration in the headspace.

Microbial population of the bioreactors. The biomass present in the bioreactor at the end of run 2 (pH 4.5) and during run 3 (pH 4.0) contained a diverse microbial population as determined with 16S RNA gene and sulfate reducing population determined with *dsrB* (Table 1).

Table 1. Cloned 16S rRNA gene fragments from the bioreactors and their closest related named gene sequences in the NCBI database.

Clone	Closest named specie relative in database	Accession.[a]	% similarity[b]	Bases[c]
MBR pH 4.5 16S rRNA gene				
MBR4.5-1	*Methanobacterium congolense* C (AF233586)	AF233586	96.9	548
MBR4.5-2	*Paludibacter propionicigenes* WB4 (AB078842)	AB078842	89.9	580
MBR4.5-3	*Paludibacter propionicigenes* WB4 (AB078842)	AB078842	90.0	579
MBR4.5-4	*Acetobacter peroxydans* IFO 13755 (AB032352)	AB032352	99.5	559
MBR4.5-5	*Sporobacter termitidis* SYR (Z49863)	Z49863	94.9	563
MBR4.5-6	*Acetobacter peroxydans* IFO 13755 (AB032352)	AB032352	97.9	558
MBR4.5-7	*Desulfovibrio alcoholovorans* DSM 5433 (AF053751)	AF053751	96.1	558
MBR4.5-8	*Desulfovibrio marrakechensis* EMSSDQ4 (EF514217)	EF514217	97.7	566
MBR pH 4.5 *dsrB* gene				
Dsr4.5-1	*Desulfovibrio vulgaris* DP4 (CP000527)	CP000527	92.3	374
Dsr4.5-2	*Desulfovibrio vulgaris* DP4 (CP000527)	CP000527	99.2	375
Dsr4.5-3	*Desulfovibrio desulfuricans* SRDQC (DQ450464)	DQ450464	95.2	374
Dsr4.5-4	*Desulfovibrio vulgaris* DP4 (CP000527)	CP000527	99.2	374
Dsr4.5-5	*Desulfovibrio desulfuricans* SRDQC (DQ450464)	DQ450464	88.9	374
MBR pH 4.0 16S rRNA gene				
MBR4.0-1	*Methanobacterium congolense* C (AF233586)	AF233586	95.7	541
MBR4.0-2	*Methanobacterium palustre* 21 (DQ649333)	DQ649333	98.2	537
MBR4.0-3	No named species in the top 100 hits		NA[d]	540
MBR4.0-4	No named species in the top 100 hits		NA	522
MBR4.0-5	*Paludibacter propionicigenes* WB4 (AB078842)	AB078842	90.2	542
MBR4.0-6	*Desulfovibrio desulfuricans* 0104 959 (AJ415573)	AJ415573	99.1	568
MBR4.0-7	*Desulfovibrio desulfuricans* DM18 (DQ417602)	DQ417602	95.7	542
MBR4.0-8	*Desulfosporosinus orientis* DSM 765 (Y11570)	Y11570	94.2	554
MBR4.0-9	*Rhodobacter vinaykumaraii* JA249 (AM600642)	AM600642	97.6	533
MBR4.0-10	*Thiomonas intermedia* ATCC 15466 (AY455809)	AY455809	96.1	514
MBR4.0-11	*Desulfotomaculum orientis* DSM 765 (Y11570)	Y11570	91.4	546
MBR4.0-12	*Desulfovibrio marrakechensis* EMSSDQ4 (EF514217)	EF514217	95.5	557
MBR4.0-13	*Desulfovibrio burkinensis* HDv (AF053752)	AF053752	97.6	540
MBR4.0-14	*Desulfovibrio alcoholovorans* DSM 5433 (AF053751)	AF053751	95.0	534
MBR4.0-15	*Eggerthella hongkongensis* HKU10 (AY288517)	AY288517	91.8	520
MBR4.0-16	*Eggerthella hongkongensis* HKU10 (AY288517)	AY288517	89.6	522

MBR pH 4.0 *dsrB* gene

Dsr4.0-1	*Desulfovibrio desulfuricans* SRDQC (DQ450464)	DQ450464	92.8	317
Dsr4.0-2	*Desulfovibrio desulfuricans* SRDQC (DQ450464)	DQ450464	88.1	362
Dsr4.0-3	*Desulfovibrio desulfuricans* DSM 642 (AF273034)	AF273034	90.0	356
Dsr4.0-4	*Desulfovibrio desulfuricans* F28-1 (DQ092635)	DQ092635	92.8	341
Dsr4.0-5	*Desulfovibrio desulfuricans* F28-1 (DQ092635)	DQ092635	93.0	353
Dsr4.0-6	*Desulfovibrio desulfuricans* DSM 642 (AF273034)	AF273034	95.9	354
Dsr4.0-7	*Desulfovibrio desulfuricans* DSM 642 (AF273034)	AF273034	95.2	360
Dsr4.0-8	*Desulfovibrio desulfuricans* DSM 642 (AF273034)	AF273034	90.0	359
Dsr4.0-9	*Desulfovibrio desulfuricans* DSM 642 (AF273034)	AF273034	95.9	339
Dsr4.0-10	*Desulfovibrio desulfuricans* DSM 642 (AF273034)	AF273034	94.9	355
Dsr4.0-11	*Desulfovibrio fructosovorans* DSM 3604 (AF418187)	AF418187	93.3	353
Dsr4.0-12	*Desulfovibrio fructosovorans* DSM 3604 (AF418187)	AF418187	96.2	350
Dsr4.0-13	*Desulfovibrio fructosovorans* DSM 3604 (AF418187)	AF418187	92.9	353
Dsr4.0-14	*Desulfovibrio fructosovorans* DSM 3604 (AF418187)	AF418187	96.5	341
Dsr4.0-15	*Desulfovibrio fructosovorans* DSM 3604 (AF418187)	AF418187	95.8	331

[a]Accession number of the closest related named gene sequence obtained by BLAST comparison in the NCBI database (http://www.ncbi.nlm.nih.gov/).

[b]Percentage sequence similarity to the closest named relative in the NCBI database.

[c]Number of base pairs used for the BLAST comparison and alignment in the ARB program.

[d]NA., not available as no named species in top 100 hits

At the end of run 2 (pH 4.5), 8 separate 16S rRNA gene DGGE bands were sequenced of which 1 (MBR4.5-1) was most similar to the methanogenic archaeon *Methanobacterium congolensi* which was inactive until day 31 after which methane was present in the headspace (Figs. 4, 7 and 8 and Table 1). The other 7 16S rRNA DGGE bands were most related to the known SRB species *Desulfovibrio alcoholovorans* (MBR4.5-7) and *Desulfovibrio marrakechensis* (MBR4.5-8), and acetogenic bacteria including *Paludibacter propionicigenes* (MBR4.5-2 and -3), *Acetobacter peroxydans* (MBR4.5-6) and *Sporobacter termitidis* (MBR4.5-5; Fig. 7 and Table 1). The pH 4.5 *dsrB* gene DGGE identified 5 bands all of which were most similar to the named species *D. vulgaris* (Dsr4.5-1, -2, and -4) and *D. desulfuricans* (Dsr4.5-3 and -5; Fig. 9 and 10 and Table 1). All the *drsB* gene sequences from the MBR4.5 reactor formed a clade most similar to *D. vulgaris* although the gene similarities ranged from 88.9 to 99.2 % (Table 1).

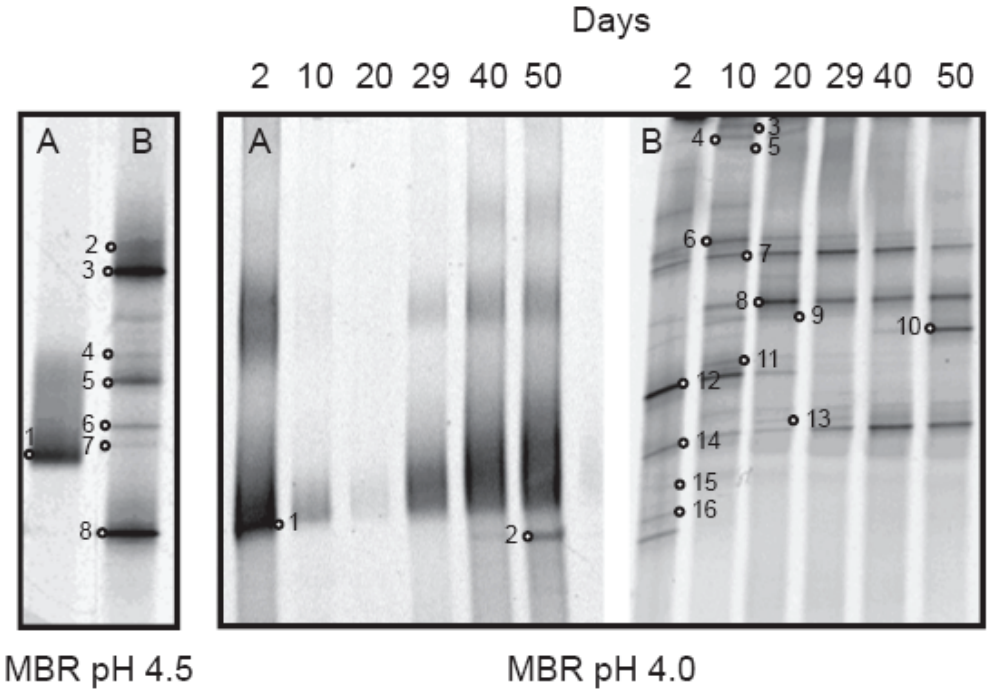

Figure 7: DGGE profiles of partial archaeal (A) and bacterial (B) 16S rRNA gene fragments from a single analysis carried out at the end of the MBR pH 4.5 (left hand panel) and a time course assay of the MBR pH 4.0 bioreactor (right hand panel; sample times are specified). Band labels correspond to MBR 4.5 and MBR 4.0 bands, respectively. Separate gels have been cropped at different denaturing levels to produce a single figure.

During run 3 (pH 4.0) the population was followed over time and in total 16 separate 16S RNA gene bands were identified. Thirteen of the identified 16 bands were present at day 2 that was reduced to 9 identified bands at day 50, of which 5 were SRB (Table 1). This showed that although the population decreased over time and became more stable at the end of the run the percentage of SRB increased corresponding to the increased sulfate reducing volumetric activity and selection for acidotolerant sulfate reducers. The SRB species were most closely related to *Desulfovibrio desulfuricans*, *Desulfosporosinus orientis, Desulfovibrio marrakechensis, Desulfovibrio burkinensis* and *Desulfovibrio alcoholovorans* with similarities ranging from 91.4 to 99.1 %. Furthermore, 2 archaeon bands were found most closely related to *Methanobacterium congolense* and *Methanobacterium palustre*. A larger number of DGGE bands were detected with the *dsrB* primers than SRB identified with the 16S RNA primers. This was probably due to the greater specificity of the *dsrB* primer for the SRB than the 16S primers that targeted all known microorganisms. The *drsB* DGGE showed 18 separate bands of which 10 were most closely related to *D. desulfuricans* with gene similarities from 88.1 to 95.9 % (Table 1) and 8 bands most closely related to *Desulfovibrio fructosovorans* with similarities from 92.9 to 96.5 % (Table 1).

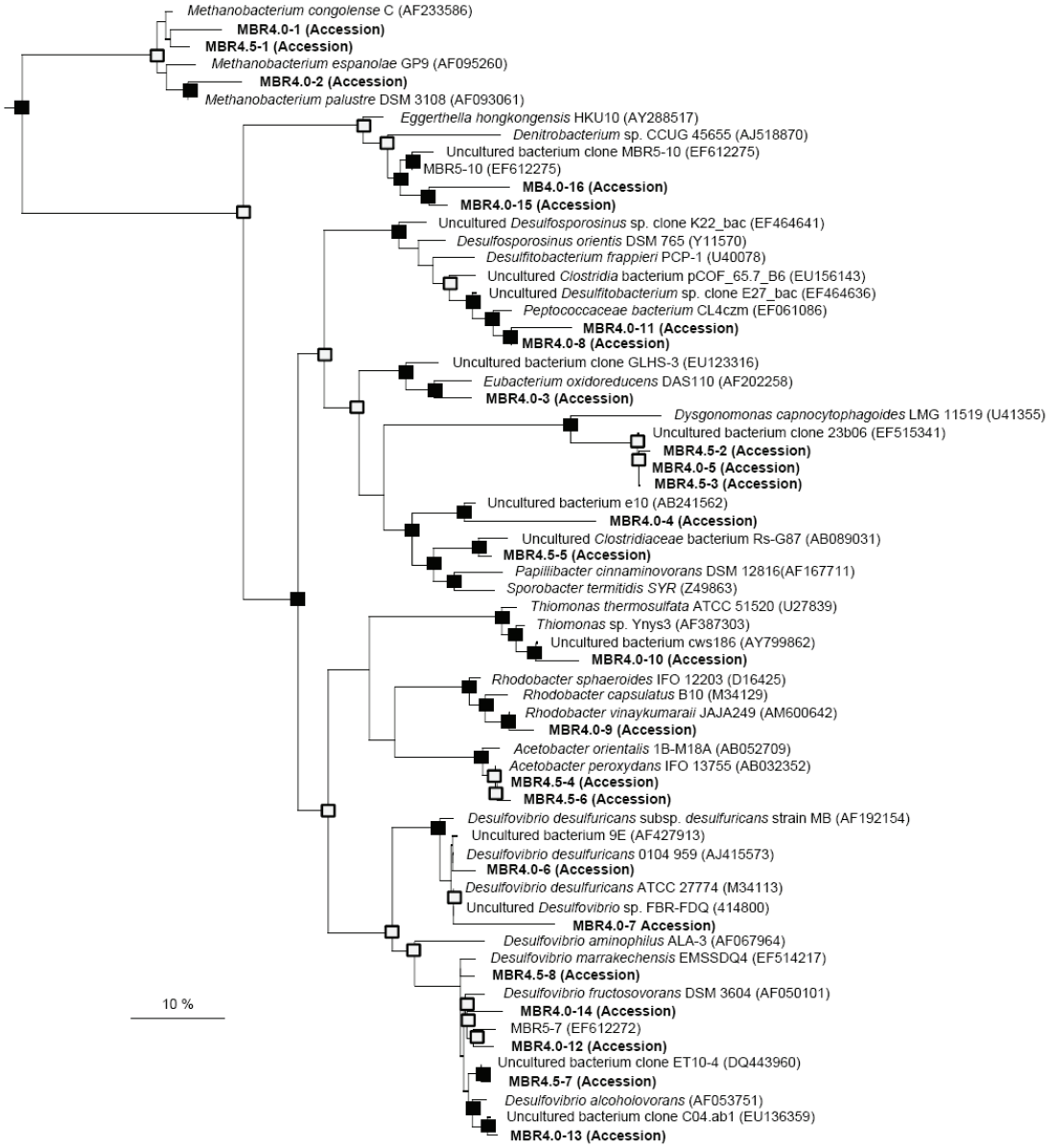

Figure 8: Maximum likelihood phylogenetic tree based on partial 16S rRNA gene sequences isolated from the MBR pH 4.5 and 4.0 (both in bold) aligned with sequences from the database (accession numbers are in parenthesis). Phylogenetic analysis was carried out by the maximum likelihood, distance neighbor joining, and DNA parsimony methods in ARB and the nodes supported by all three trees (■) and two trees (□) have been marked. The tree was rooted with uncultured *Korarchaeote* sp. pBA5 (AF176347; not shown). The scale bar corresponds to 10% sequence similarity.

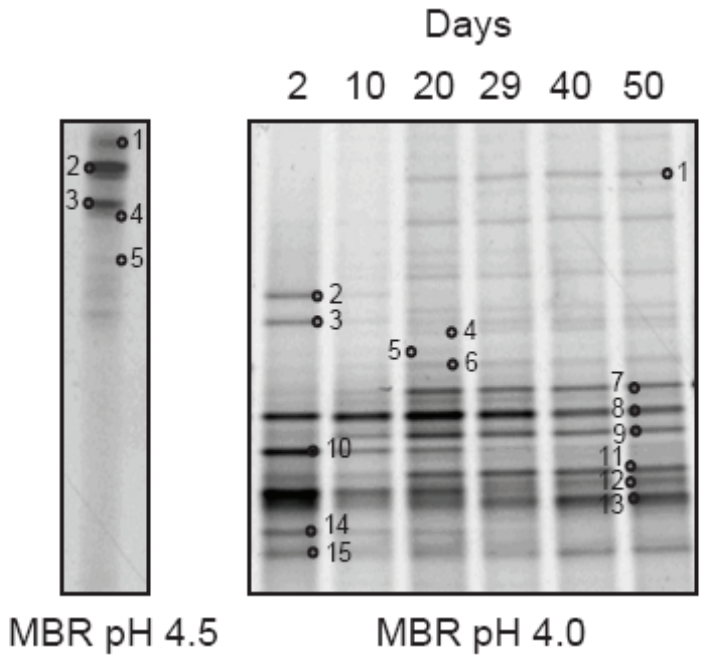

Figure 9: DGGE profiles of partial *dsrB* genes from the MBR pH 4.5 from a single analysis carried out at the end of the leaching (A) and a time course assay of the MBR pH 4.0 bioreactor (B). Band labels correspond to MBR 4.5 and MBR 4.0 bands, respectively. Separate gels have been cropped at different denaturing levels to produce a single figure.

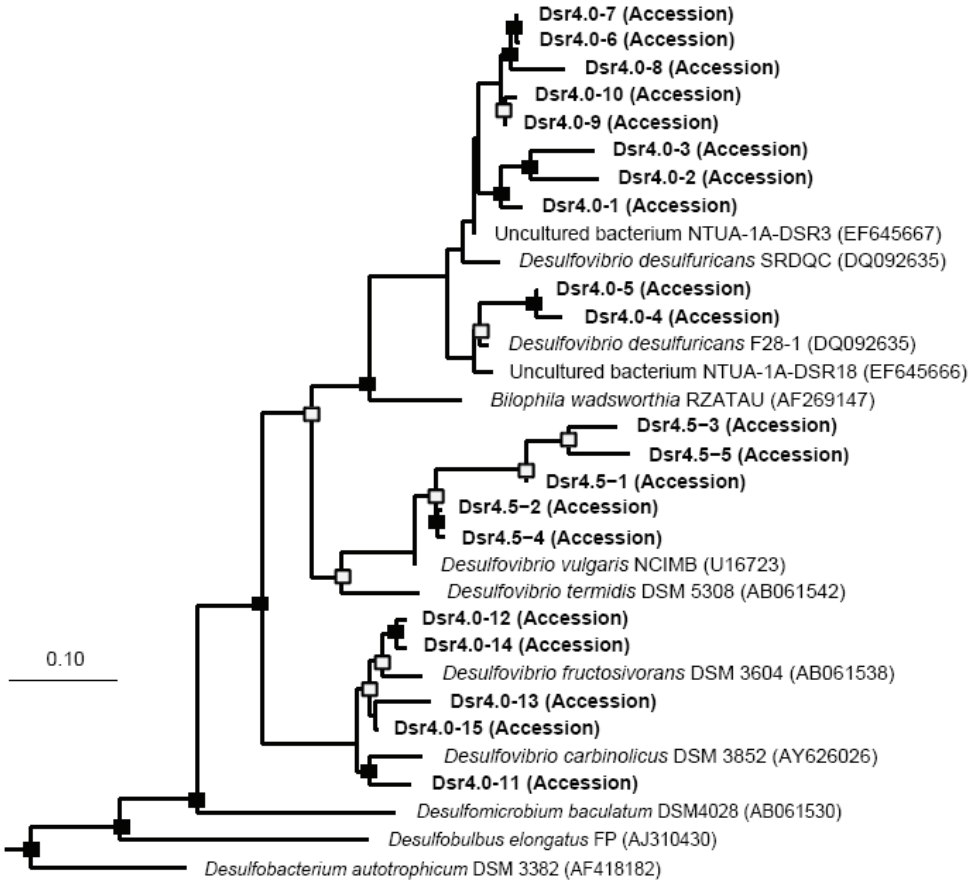

Figure 10: Maximum likelihood *dsrB* phylogenetic tree (partial sequences) from the MBR pH 4.5 and 4.0 (both in bold) aligned with sequences from the database (accession numbers are in parenthesis). Phylogenetic analysis was carried out by the maximum likelihood, distance neighbor joining, and DNA parsimony methods in ARB and the nodes supported by all three trees (■) and two trees (□) have been marked. The tree was rooted with *Thermodesulfovibrio yellowstonii* (U518122; not shown). The scale bar corresponds to 10% sequence similarity.

Discussion

The volumetric sulfate reducing activity and biomass yield in this and previous studies at pH 5.0 (chapter 3) and 6.0 (chapter 2) increased with the pH although the specific sulfate reducing activity was relatively constant at 89 ± 14 mmol $gVSS^{-1}$ d^{-1} between pH 4.0 and 6.0 (chapter 2 and 3; Fig. 11 and Table 2). This increase in volumetric sulfate reducing activity with the pH was also observed for a H_2 fed gas lift bioreactor between pH 5.5 and 8.0 where a specific sulfate reducing activity of 33 ± 6 mmol $gVSS^{-1}$ d^{-1} (Fig. 11 and Table 2). Low volumetric sulfate reducing activities at pH 4.0 have also been reported in a pH controlled batch continuous stirred tank

reactor for 9 days at pH 3.8 with a rate of 0.3 mmol L^{-1} d^{-1} and for 6 days at pH 4.0 at a rate of 2 mmol L^{-1} d^{-1} (Kimura et al., 2006). Also, Lopes et al.(Lopes et al., 2007) reported on a pH controlled UASB fed with sucrose at pH 4.0 at 55°C which was inoculated with 326 g L^{-1} wet sludge (26 g VSS L^{-1}) from an active full-scale bioreactor. The volumetric sulfate reducing activity was 4 mmol L^{-1} d^{-1} and did not increase over time (Lopes et al., 2007), suggesting that a small part of the sludge originally grown at pH 6.9 (Oude Elferink et al., 1998) was still active (Table 1). To achieve increased volumetric sulfate reduction rates the solid retention time should be increased. This can be achieved by immobilizing the bacteria on a solid support material, in granuals with other microorganism, or by growing the cells at a slightly higher pH and lowering the pH when needed. The later was demonstrated in run 1 where the biomass was grown at pH 5.0 and were able to achieve a volumetric sulfate reducing active of 155 mmol L^{-1} d^{-1} at pH 4.0.

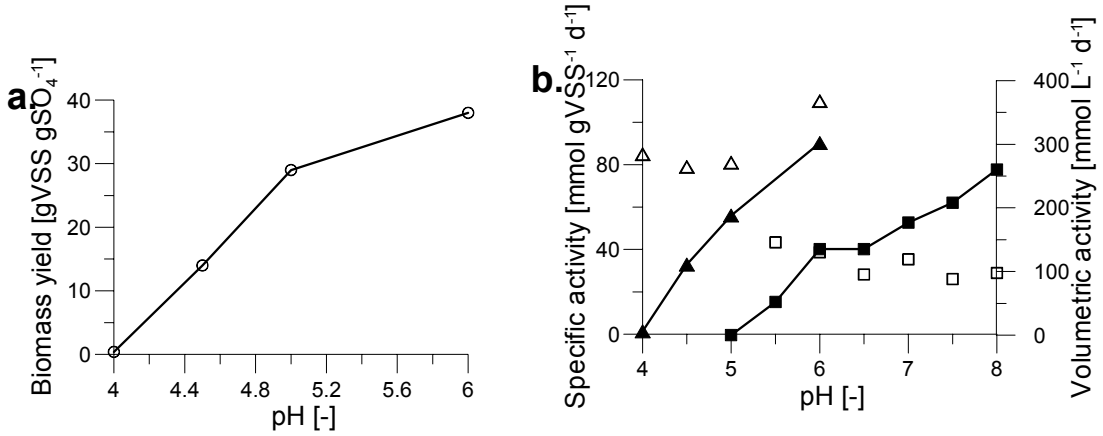

Figure 11: a) Biomass yield over pH 4.0 – 6.0, and b) volumetric sulfate reducing activity of current and previous obtained results (▲) and those of Van Houten et al.(van Houten et al., 1995) (■) and specific sulfate reducing activity of current and previous obtained results (Δ) and those of Van Houten et al.(1995) (□).

Table 2. The reactor concept, pH, temperature, substrate, volumetric activity, specific activity and conversion efficiency at the maximum conversion rate from this study and literature

Reactor concept	pH	Temp [°C]	Electron donor	Volumetric activity [mmol SO_4 L^{-1} d^{-1}]	Specific activity [mmol SO_4 $gVSS^{-1}$ d^{-1}]	Biomass yield [gVSS kg^{-1} SO_4]	References
MBR	4.0	30	Formate	151	64	-[a]	This study
MBR	4.0	30	Hydrogen	6	85[b]	0.4	This study
MBR	4.5	30	Hydrogen	111	79	14	This study
CSTR	3.8	30	Glycerol	0.3	-[c]	-[d]	(Kimura et al., 2006)
CSTR	4.0	30	Glycerol	2	-[c]	-[d]	(Kimura et al., 2006)
UASB	4.0	55	Sucrose	4	0.15	-[d]	(Lopes et al., 2007)
MBR	5.0	30	Formate	188	81	29	Chapter 3
Gaslift	5.5	30	Hydrogen	52	43 [f]	-[d]	(van Houten et al., 1995)
MBR	6.0	30	Formate	302	110	38	Chapter 2
Gaslift	6.0	30	Hydrogen	135	39 [f]	-[d]	(van Houten et al., 1995)
Gaslift	6.5	30	Hydrogen	135	28 [f]	-[d]	(van Houten et al., 1995)
Gaslift	7.0	30	Hydrogen	312	-[b]	-[d]	(van Houten et al., 1994)
Gaslift	7.0	30	Hydrogen	177	35 [f]	-[d]	(van Houten et al., 1995)
Gaslift	7.5	30	Hydrogen	208	26 [f]	-[d]	(van Houten et al., 1995)
Gaslift	8.0	30	Hydrogen	260	29 [f]	-[d]	(van Houten et al., 1995)

[a] Insufficient data to calculate biomass yield. [b] Calculated on day 43 at which the last VSS measurement was done. [c] Experiments were done in batch in which the conversion efficiency could not be calculated. [d] No biomass determined. [f] Estimated with the assumption that VSS is equal to biomass (Tchobanoglous et al., 2003).

We postulate that in addition to toxicity problems associated with low pH (such as protein denaturation that would have been reduced by the selection of acidotolerant SRB) the low biomass yield at acidic pH was caused by the energy requirement to maintain a near neutral cytoplasmic pH. Under acidic conditions, a a proton gradient exist over the cell membrane which results in protons leaking into the cell, acidifying the cytoplasm and requiring intracellular protons to be pumped out of the cytoplasm to maintain pH homeostasis. The intracellular pH of neutrophilic microorganisms is maintained at pH 7 whereas, acidophilic microorganisms have an internal pH between 4.6 and 7 (typical internal pH values are 4 to 5 pH units higher than their pH

optimum) (Baker-Austin and Dopson, 2007). A lower reactor liquor pH results in a higher proton gradient across the cell membrane and more energy is needed for maintenance and therefore less energy is available for growth. At pH 6.0 about 34% of the energy gained by sulfate reduction ($\Delta G \approx -144$ kJ mol^{-1}) was used for biomass production (-300 kJ mol^{-1}), indicating that 66% of the energy was used for maintenance activities such as maintaining the pH homeostasis. At pH 4.5 only 12% of the energy gained was used for biomass production, which decreased to 0.4 % at pH 4.0. This indicated that at pH 4.0, >99% of the energy gained by sulfate reduction was used for maintenance. Acidophilic iron oxidizers capable of growth at pH 0 have been identified (Edwards, 2000) that have several adaptations for growth at low pH including a membrane highly impermeable to protons, an internal positive membrane potential that inhibits proton flux into the cytoplasm and a large number of proton exporters (Baker-Austin and Dopson, 2007). However, it is unknown how the acidotolerant SRB identified in this study are adapted to the low pH.

In chapter 5 it is postulated that inhibition of undissociated sulfide and VFAs like acetate were related to the energy required to maintain pH homeostasis(Alexander et al., 1987). At low pH, VFAs and sulfide are in the neutral, un-dissociated form that easily diffuse into the cytoplasm where they dissociate, release a proton and acidify the cytoplasm in a similar manner as that occurs for acidophiles(Alexander et al., 1987). Undissociated sulfide is reported to be the inhibiting sulfide species(Moosa and Harrison, 2006; Reis et al., 1992) and at pH 5.0 >99 % of the sulfide is present in the undissociated form and thus, should not be more inhibiting at lower pH values. In this study, a low sulfide concentration was maintained by stripped the reactor liquid. The acetate concentration was around 0.1 mM at the beginning of run 3 (Fig. 5a) at pH 4.0 which increased to 3 mM (Fig. 5a) while the activity increased over the time, showing that acetate inhibition could not explain the low growth rate at pH 4.0.

The 16S rRNA gene sequences most closely related to SRB at pH 4.5 were *D. vulgaris* and *D. desulfuricans* whereas, gene sequences most closely related to *D. desulfuricans*, *Desulfosporosinus orientis*, and *D. alcoholovorans* were found at pH 4.0. The only other reported 16S rRNA gene sequences from SRB isolated at low pH are *Desulfosporosinus* sp. M1 isolated from a pH 3.8 to 4.2 geothermal area(Kimura et al., 2006) and *Desulfosporosinus sp*. and *Desulfitobacterium sp*. in pH 4 AMD (Church et al., 2007). Interestingly, all 3 reported studies of SRB at pH 4.0 have identified strains with 91.5 to 94.2% similarity to *Desulfosporosinus* spp. (Church et al., 2007; Kimura et al., 2006) suggesting species from this genus may be the most acidotolerant or acidophilic.

In this study it has been shown that high sulfate conversion rates can be reached under acidic conditions. This is interesting for the treatment of waste water from the mining and metallurgical industries which usually have a pH around 2-4(Johnson, 2000b). This would decrease costs associated with caustic addition to increase the influent pH as well as opening possibilities to selectively recover metals by varying the pH and sulfide concentration. The results from this study suggest that it will be feasible to run a single stage bioreactor at pH 4.0 to 4.5 in which metals such as copper, nickel or zinc could be selectively recovered from iron.

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5

Effect of sulfide removal on sulfate reduction at pH 5 in a hydrogen fed gas-lift bioreactor

Abstract

Biotechnological treatment of sulfate and metal-ions containing acidic wastewaters from mining and metallurgical activities utilizes sulfate reducing bacteria to produce sulfide that can subsequently precipitate metal-ions. Reducing sulfate at a low pH has several advantages above neutrophilic sulfate reduction. This study describes the effect of sulfide removal on the reactor performance and microbial community in a high rate sulfidogenic gas-lift bioreactor fed with hydrogen at a controlled internal pH of 5. Under sulfide removal conditions, 99 % of the sulfate was converted at a hydraulic retention time of 24 hours reaching a volumetric activity as high as 51 mmol sulfate L^{-1} d^{-1}. The absence of sulfide removal at a hydraulic retention time of 24 h resulted in an average H_2S concentration of 18.2 mM (584 mg-S L^{-1}). Sulfate removal was incomplete, which was probably due to sulfide inhibition. Molecular phylogenetic analysis identified a less diverse population in the presence of a high sulfide concentration.

Introduction

Biotechnological treatment of sulfate and metal-ions containing acidic wastewaters from mining and metallurgical activities utilize sulfate reducing bacteria (SRB) to produce sulfide. The generated sulfide can subsequently precipitate metal ions in both passive treatment systems including wetlands and permeable reactive barriers (Johnson and Hallberg, 2005) and active-systems such as sulfidogenic bioreactors (Weijma et al., 2002a). To date, the most widely used technology for treatment of these waste streams is chemical treatment with lime or caustic. However, chemical and passive biological treatments limit the potential for resource recovery from metal containing waste streams. Sulfidogenic bioreactors for treatment of acidic influent in near neutral sulfidogenic bioreactors (Kaksonen et al., 2004; Kaksonen et al., 2003; van Houten et al., 1994; Weijma et al., 2002b) and packed columns filled with sand (Christensen et al., 1996) or straw and compost mixes (Gibert et al., 2003; Morales et al., 2005; Waybrant et al., 2002) resulting in pH neutral effluents have been described. Some of these studies describe the sulfate reduction process as being acidophilic or acidotolerant, despite that the effluent pH, and thus the reactor liquor being pH neutral (Elliott et al., 1998; Jong and Parry, 2006).

By decreasing the lower limit of the operational pH, the potential for selective metal recovery is greatly increased as metal-sulfide formation is determined by the pH and sulfide concentration (Huisman et al., 2006; Tabak et al., 2003). In addition, low pH sulfidogenic bioreactors also reduce the need for caustic addition to increase the pH of the influent wastewater and as the produced sulfide is in the gaseous phase, it simplifies sulfide separation from the effluent for subsequent metal precipitation (Tabak et al., 2003; Veeken et al., 2003) or for elemental sulfur production by partial oxidation with oxygen (Buisman and Lettinga, 1990; Janssen et al., 1999).

Sulfide has an inhibitory effect on the bioreactor microbial community which is species dependent (Icgen and Harrison, 2006). Although the mechanism of sulfide inhibition is not fully understood, it is suggested that undissociated sulfide (H_2S) is the inhibiting compound (Colleran et al., 1995; Moosa and Harrison, 2006; O'Flaherty et al., 1998; Reis et al., 1992). As H_2S has a pKa of 7 at 30°C (Kawazuishi and Prausnitz, 1987), the products of sulfate reduction at neutral pH are equal amounts of H_2S and HS^- (Eqs. 1 and 2 using hydrogen as an electron donor). Whereas, at pH 5 about 99 % of the product is H_2S, indicating a potentially higher level of sulfide inhibition.

$$4H_{2(g)} + SO_{4(aq)}^{2-} + 2H_{(aq)}^{+} \rightarrow H_2S_{(aq)} + 4H_2O_{(l)} \quad (1)$$

$$4H_{2(g)} + SO_{4(aq)}^{2-} + H_{(aq)}^{+} \rightarrow HS_{(aq)}^{-} + H_2O_{(l)} \quad (2)$$

This study describes the effect of sulfide removal on the reactor performance and microbial community in a high rate sulfidogenic gas-lift bioreactor at a controlled pH of the reactor liquor of 5. The reactor was fed with hydrogen as electron donor and CO_2 as carbon source. The achieved sulfate reduction rate demonstrates sulfate reduction at pH 5 at industrially relevant rates.

Material and methods

Growth medium and inoculum.

A defined medium dissolved in demineralized water (all chemicals were of analytical grade; Merck, Darmstad, Germany) was used containing (g L^{-1}): Na_2SO_4, 6.65; KH_2PO_4, 0.41; NH_4Cl, 0.3; KCl, 0.37; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.11; $NaHCO_3$, 1; yeast extract, 0.01; and 1 mL L^{-1} of each of an acid and alkaline trace element solution (Stams et al., 1993). Sludge from a pH 5 formate fed membrane bioreactor was used as inoculum, which itself was inoculated with sludge from three full scale sulfidogenic wastewater treatment plants at neutral pH (chapter 3).

Reactor set-up.

Two 4 L liquid volume glass gas-lift bioreactors (GLBs) were controlled at 30˚C with a Julabo 12 (Julabo, Seelbach, Germany) and a Tamson T1000 waterbath (Tamson instruments, Zoetermeer, The Netherlands) for GLB 1 and 2, respectively. The GLBs had an internal settler of 0.5 L and an external settler of 4 L (Fig. 1). The external settler was cooled to 4^{o}C with a Julabo F10 cryostat (Julabo, Seelbach, Germany) to minimize bacterial activity in the settler. The influent was stored in a 100 L container that was continuously sparged with N_2 to maintain anaerobic conditions and was dosed into the GLBs with a Stepdos 08RC liquid membrane pump (FEM 08TT.18RC, KNF-Verder, Vleuten, The Netherlands).

Sulfide was removed from the gas phase by recycling through a 3 M ZnCl solution. H_2 and CO_2 were dosed via a Brooks thermal mass flow controller type 5850E with maximum flows of 30 and 7.5 L min^{-1} for H_2 and CO_2, respectively (Brooks instruments, Veenendaal, The Netherlands) connected to a Brooks control unit (Type 5878). The gas phase was recycled through the reactor liquid by a KNF-Verder N840.3FT.18 gas membrane pump (KNF Neuberger Inc., Freiburg, Germany). To ensure efficient mass transfer of the gases, a teflon sparger with 168 holes of 0.44 mm

was placed at the interface area between the liquid and recycled gas. The gas flow (4 L min^{-1}) was measured with a McMillan gas flow sensor (model 100, McMillan Company, Texas, USA). The excess gas was analyzed with an online gas analyzer (Advance Optima, ABB Automation Product GmbH, Frankfurt am Main, Germany). All tubing and connections were made of PTFE (Schott A.G., Mainz, Germany and Serto AG., Fuldabrück, Germany).

The pH was measured using a H63 electrode (Schotts Instruments, Mainz, Germany) and controlled at pH 5 with 4 M NaOH and 4 M HCl dosed by an EH Liquisys-P pH controller (Endress Hauser Inc., Reinach, Switzerland). The pH system was regularly checked and if necessary recalibrated.

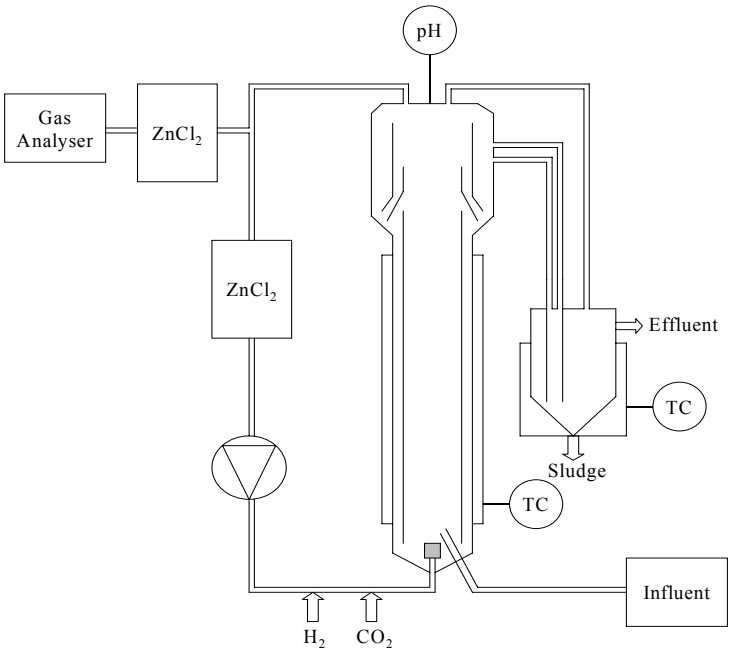


Figure 1. Schematic drawing of the gas-lift bioreactor with external settler, gas-recirculation, pH and temperature control (TC), $ZnCl_2$ stripping bottles, and gas analyzer

Experimental design. Duplicate GLBs under low sulfide conditions (GLLS) were operated at pH 5 in which sulfide was removed from the gas-phase by recycling the gas through a $ZnCl_2$ solution (regularly replaced to avoid saturation of the $ZnCl_2$). These two runs were carried out at various hydraulic retention times (HRTs), H_2, and CO_2 concentrations in the influent (Table 1). From day 19, both reactors had a H_2 flow of 95 mL min^{-1} and a CO_2 flow of 5 mL min^{-1}, with an HRT of 24 hours in run 1 until day 56, followed by a HRT of 18 hours until day 82. Whereas, in run 2 the

reactor had a HRT of 12 hours between day 19 and 31. A single pH 5 GLB under high sulfide conditions (GLHS; no sulfide removal from the gas-phase) was operated at a H_2 flow of 95 mL min^{-1} and a CO_2 flow of 5 mL min^{-1}.

Table 1: Hydraulic retention time, hydrogen flow rate, and CO_2 flow rate during the common period of both GLLS runs until day 19

Day	HRT [d]	H_2 [mL/min]	CO_2 [mL/min]
0 – 3	20	98	2
3 – 7	10	98	2
7 – 13	5	90	10
13 – 19	5	100	0

Physico-chemical analyses.
Sulfate was analyzed with ion-chromatography as described by Sipma et al. (Sipma et al., 2004). Volatile fatty acids (VFA) were analyzed on a Hewlett Packard series II gas chromatograph (Weijma et al., 2000). Sulfide was measured using the Dr. Lange sulfide kit LCK-653 and a Xion 500 spectrophotometer (Hach Lange GMBH, Düsseldorf, Germany). H_2, CO_2, and CH_4 were analyzed in the headspace by an online gas-analyzer (Advance Optima, ABB Automation Product GmbH, Frankfurt am Main, Germany). Total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed by standard methods (Clesceri et al., 1998). The particle size distribution of the sludge was analyzed with laser scattering image analysis (Coulter laser LS 230, Beckman Coulter, USA).

DNA Preparation, PCR amplification, DGGE, cloning, and sequencing. Duplicate samples from each of the bioreactors were taken and DNA isolated by either bead beating and the Wizard DNA Clean-Up System (Promega, Madison, WI) or sodium dodecyl sulfate and proteinase K treatment before phenol chloroform extraction (chapter 3). Both DNA preparations were PCR amplified using archaeal (ARC344F-GC and ARC915R) and bacterial (GM5F-GC and DS907R) specific primers (chapter 3). The amplified DNA fragments were separated by denaturing gradient gel electrophoresis (DGGE) using a denaturing gradient of 30 – 70 % denaturant, cloned into *Escherichia coli*, PCR amplified, and the 16S rRNA gene sequenced (Dopson and Lindström, 2004).

Phylogenetic analysis. The obtained 16S rRNA gene sequences were analyzed (Morales et al., 2005) using the chimera check program at the Ribosome Database Project II site (http://rdp.cme.msu.edu/html;) and the ARB program package (Ludwig

et al., 2004). The number of base pairs used for alignment and phylogenetic analysis for each of the 16S rRNA sequences is given in Table 2. The two chimera sequences were not included in the phylogenetic analysis.

Calculations.

Assumptions:

1. The amount of influent liquid into the reactor is the same as the amount of effluent ($\phi_{l,in} = \phi_{l,out}$), Therefore $\phi_{l,in}$ and $\phi_{l,out}$ can also be called ϕ_l.
2. The sulfate and acetate concentration in the effluent ($C_{s,out}$ and $C_{Ac,out}$) are assumed to be equal to the concentration in the reactor ($C_{s,r}$ and $C_{Ac,r}$), which is valid for a completely mixed system. However, biomass (C_x) is not assumed to be the same in the reactor or going out, because the internal settler affects solids, but not dissolved compounds and the liquid phase.

With assumption 1 and 2, the mass balance of sulfate will be:

$$\frac{dS}{dt} = \phi_l \cdot C_{s,in} - \phi_l \cdot C_{s,r} - R_s \cdot V_r \quad (1$$

Under steady state situation $\frac{dS}{dt} = 0$

$$r_v = \frac{\phi_l \cdot C_{s,in} - \phi_l \cdot C_{s,r}}{V_r} \quad (2$$

$$r_s = \frac{\phi_l \cdot C_{s,in} - \phi_l \cdot C_{s,r}}{C_{x,r}} \quad (3$$

$$HRT = \frac{V_r}{\Phi_l} \quad (4$$

$$SRT = \frac{C_{x,r}}{C_{x,out}} \cdot HRT = \frac{C_{x,r} \cdot V_r}{C_{x,out} \cdot \Phi_l} \quad (5$$

Nomenclature

Φ	Flow rate	[$L \cdot h^{-1}$]
C_s	Sulfate concentration	[$mmol \cdot L^{-1}$]
C_x	Biomass concentration	[$gVSS \cdot L^{-1}$]
HRT	Hydraulic retention time	[h]
r_v	Volumetric activity	[$mmol \cdot L^{-1} \cdot h^{-1}$]
V_r	Volume reactor	[L]
S	Sulfate	[-]
r_s	Specific activity	[$mmol \cdot gVSS^{-1} \cdot L^{-1}$]

SRT	Solid retention time	[h]

Subscript

in	fed to reactor
l	liquid phase
out	leaving the reactor
r	in the reactor

Results and discussion

Reactor performance at low-sulfide conditions (GLLS).

Sulfate reduction at pH 5 was shown in a continuous experiment for over 12 weeks in which the produced sulfide was removed from the gas-phase (Fig. 2g). Sulfide removal resulted in an average sulfide concentration in the GLLS of 3.3 mM (Fig. 2i). The relatively higher concentrations were most likely caused by saturation of the $ZnCl_2$ solution used for sulfide removal. During both GLLS runs, the major end products were sulfide, acetate, and biomass (methane never rose above 0.1 % of the gas phase).

During the first 19 days, both GLLS runs had identical operating conditions (Tables 1), which resulted in almost identical results (Fig. 2) demonstrating the reproducibility of the system. Both runs started at a HRT of 20 days to prevent biomass wash-out, after which the HRT was step-wise decreased (Table 1). Sulfate reduction started directly in both runs (Fig. 2g & 2h) and more sulfate was reduced than added in the first 7 days, decreasing the sulfate concentration in the reactor liquor to 0.2 mM on day 7 (Fig 2a & 2b). From day 7 to day 19, the sulfate remained almost depleted, indicating that all incoming sulfate was directly converted to sulfide (Fig. 2a & 2b).

At the start of both runs, CO_2 was dosed with a flow rate of 2 mL min^{-1} and was completely used at day 7, without VFA and methane being detected, suggesting that all the carbon ended up in the biomass. However, as autotrophic biomass growth is slow (Widdel, 1988) it was likely that acetate served as an intermediate for biomass production. Weijma *et al.* (2002a) showed that when CO_2 is used as the sole carbon source, enough acetate was produced for growth of heterotrophic SRBs. To prevent carbon limitation for growth the CO_2 feed was increased from 2 to 10 mL min^{-1} on day 7 (Table 1). This stimulated the activity of acetogenic bacteria, resulting in an increase in the acetate concentration to 41 mM (run 1; Fig. 2e) and 39 mM (run 2; Fig.

2f). The CO_2 feed was stopped between day 13 and 19 to prevent inhibition by acetate, which resulted in a rapid drop of the acetate concentration (Fig. 2e & 2f).

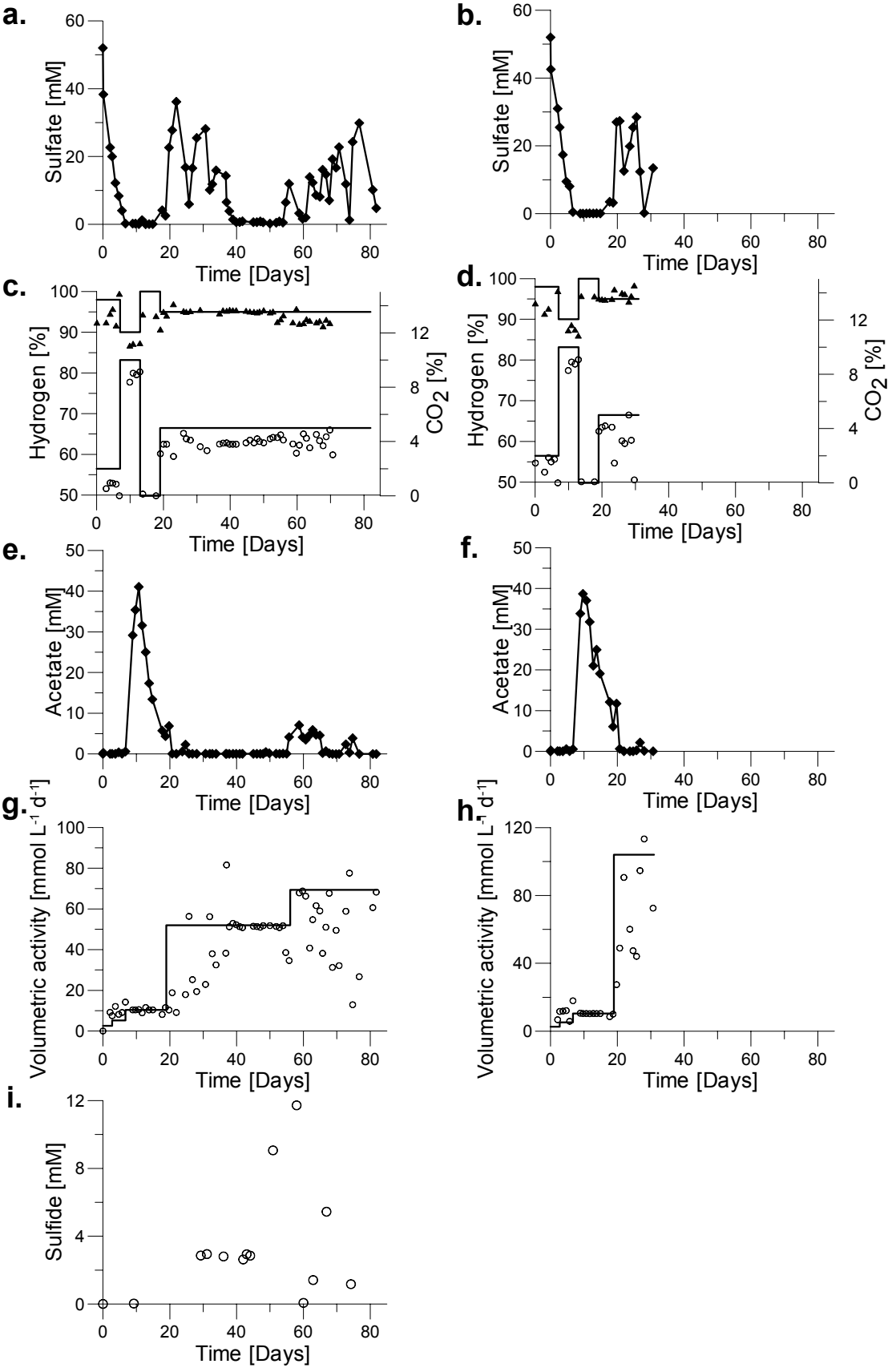


Figure 2. Data from the gas lift bioreactor at low sulfide conditions (GLLS) with: a) sulfate concentration (♦) in the reactor liquor of run 1; b) sulfate concentration (♦) in the reactor liquor of run 2; c) hydrogen (▲) and CO_2 (○) in the effluent and in the influent (—) of run 1; d) hydrogen (▲) and CO_2 (○) in the effluent and in the influent (—) of run 2; e) acetate concentration (♦) in the reactor liquor of run 1; f) acetate concentration (♦) in the reactor liquor of run 2; g) dosing rate of sulfate (—) and the volumetric activity (o) for run 1; h) dosing rate of sulfate (—) and the volumetric activity (o) for run 2; and i) sulfide concentration (○) in the reactor liquor of run 1.

Changing the HRT in run 1 at day 19 from 5 days to 24 hours resulted in a slow increase of the volumetric activity until day 40, at which a steady state was reached until day 54 (Fig. 2g). The sulfate concentration increased rapidly from day 19 onwards in run 1 followed by a rapid decrease in sulfate and depletion on day 40 (Fig. 2a). The average residual sulfate concentration during the steady state (day 40 to day 54) was 0.6 mM (standard deviations and numbers of replicates for steady state measurements are given in Table 3 and 4), resulting in a volumetric activity of 51.4 mmol L^{-1} d^{-1} and a 99 % sulfate conversion efficiency (Table 4). The VSS concentration of 0.13 g L^{-1} resulted in a specific activity of 395 mmol SO_4^{2-} g VSS^{-1} d^{-1} (Table 4) which seems to be a high rate compared to 110 and 81 mmol SO_4^{2-} g VSS^{-1} d^{-1} reached in a MBR at pH 6 and pH 5, respectively (chapter 2 and 3). The effect of biomass wall growth and attachment were not taken into account, which could explain the low biomass concentrations in the liquid phase and thus, the high specific activity. However, the short solid retention time (SRT) of 26 to 30 hours (Table 4) stimulated the high specific activity. The small sludge particle size during steady state (day 54) with a mean value of 7 μm and a mode (the particle size with the highest differential volume) of 22 μm (Fig. 3), created a large active surface area that resulted in a high specific activity. The sludge particle size of the effluent contained larger particles with a mean value of 13 μm and a mode of 24 μm than the particle size within the reactor liquor. In the replicate measurement analyzed directly after each other, the sludge particle size decreased to a mean value of 9 μm while retaining the mode of 24 μm (Fig. 3) possibly as the formed flocs were fragile and easily disrupted. This was supported by the visual observation that the sludge became larger and settled faster. The average acetate concentration in the reactor during steady state was 0.04 mM (Table 3). It has been shown that SRB outcompete methanogens in H_2 fed gas-lift bioreactors with excess sulfate (Weijma et al., 2002b) however, this was also the case in this study when sulfate was limiting. The low pH of the reactor liquid could have influenced this competition as well as the short SRT due to the slow settling velocity

of methanogen dominated sludge compared to sludge containing predominantly SRB (Weijma et al., 2002b).

Table 3: Steady state values of GLLS run 1 at a hydraulic retention time of 24 h (day 40-54)

Compound	Average	Standard deviation	n[a]
Sulfate reactor [mM]	0.62	0.19	11
Acetate reactor [mM]	0.042	0.042	11
H_2 out [%]	95.2	0.22	10
CO_2 out [%]	4.0	0.19	10
CH_4 out [%]	0.0	0.0	10
TSS reactor [g L^{-1}]	0.80	0.14	5
VSS reactor [g L^{-1}]	0.13	0.016	7
TSS out [g L^{-1}]	0.65	0.060	2
VSS out [g L^{-1}]	0.12	0.023	5

[a] number of measurements

Table 4: Calculations from steady state values of GLLS run 1 at a hydraulic retention time of 24 h (day 40-54)

Calculated parameter	value
Volumetric activity [mmol L^{-1} d^{-1}]	51.4
Specific activity [mmol $gVSS^{-1}$ d^{-1}]	395
Conversion efficiency [%]	98.8
SRT based on VSS [h]	26
SRT based on TSS [h]	30

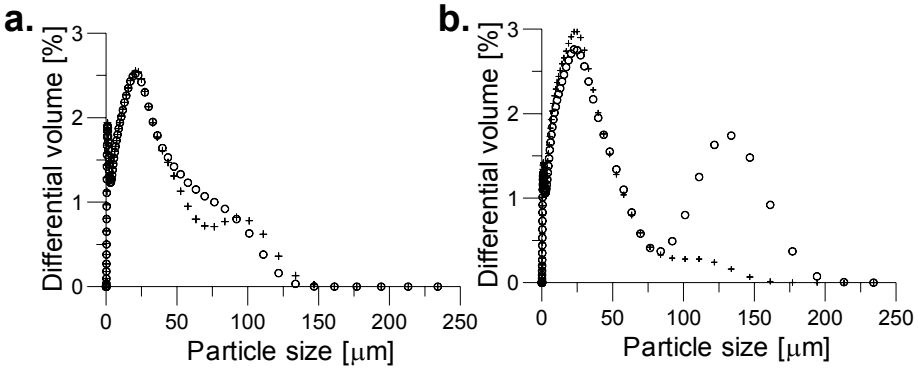


Figure 3. Particle size distribution of sludge taken from: a) the reactor and b) the effluent. The first measurement (o) was directly followed by the second measurement (+).

After a two week steady state with an HRT of 24 h in run 1, the HRT was set to 16 h from day 56 to 82, in which the volumetric activity slowly increased (Fig. 2g), while the sulfate concentrations fluctuated between 0 and 30 mM (Fig. 2a). The acetate concentration also fluctuated and reached a maximum concentration of 7 mM

(Fig. 2e). Methane remained absent during the whole run. As the volumetric activity fluctuated it suggests that with a SRT close to the HRT of 16 h the cells were on the limit of their growth rate.

The change in HRT in run 2 from 5 days to 12 h at day 19 resulted in fluctuations in the volumetric activity and hence, the sulfate concentration varied between 0 and 30 mM (Fig. 2b). Acetate remained below 2 mM (Fig. 2f), while the CO_2 concentration was between 3 to 5 % (Fig. 2d). GLBs have been operated at pH 7 with HRT's as low as 4 hours, with a combination of a three-phase-separator and external settler providing a SRT of 2.6 days (Esposito et al., 2003). In the present study, only a three-phase-separator was used for biomass retention allowing the system to be flexible and to rapidly adapt to the applied conditions.

Reactor performance at high-sulfide conditions (GLHS).

The absence of sulfide removal in GLHS had a major effect on the bioreactor performance (Fig. 4). Sulfate reduction started directly and sulfide concentrations increased to 30 mM (day 8), after which the sulfide concentration fluctuated (Fig. 4a). The sulfide concentrations most likely caused sulfide inhibition leading to incomplete sulfate removal (Fig. 4a). Acetate accumulated rapidly to 18 mM at day 2, after which it stabilized (Fig. 4b). The CO_2 concentration was low in the first 4 days due to CO_2 being used for acetate production and biomass growth, resulting in a hydrogen concentration in the headspace of 98 % in the first 4 days (Fig. 4c). The average sulfide concentration from day 1 onwards was 18.5 (± 8.5) mM (591 mg-S L^{-1}), which resulted at pH 5 in an H_2S concentration of 18.2 mM (584 mg-S L^{-1}).

Effect of sulfide on microbial population and reactor performance.

The GLLS and GLHS inocula originated from a membrane bioreactor at pH 5 (MBR5), from which 11 separate 16S RNA gene bands were identified by DGGE, cloned, and sequenced (chapter 3). Some of the MBR5 16S RNA gene sequences were similar to those present in GLLS and GLHS (Fig. 5 & Fig. 6). For instance, MBR5, GLLS and GLHS all contained 16S rRNA genes that aligned within *Bacteroidetes*, *Firmicutes*, and *δ-Proteobacteria* and a sequence aligning with the methanogenic archaea was found in GLHS and MBR5 (Fig. 6 & Table 2).

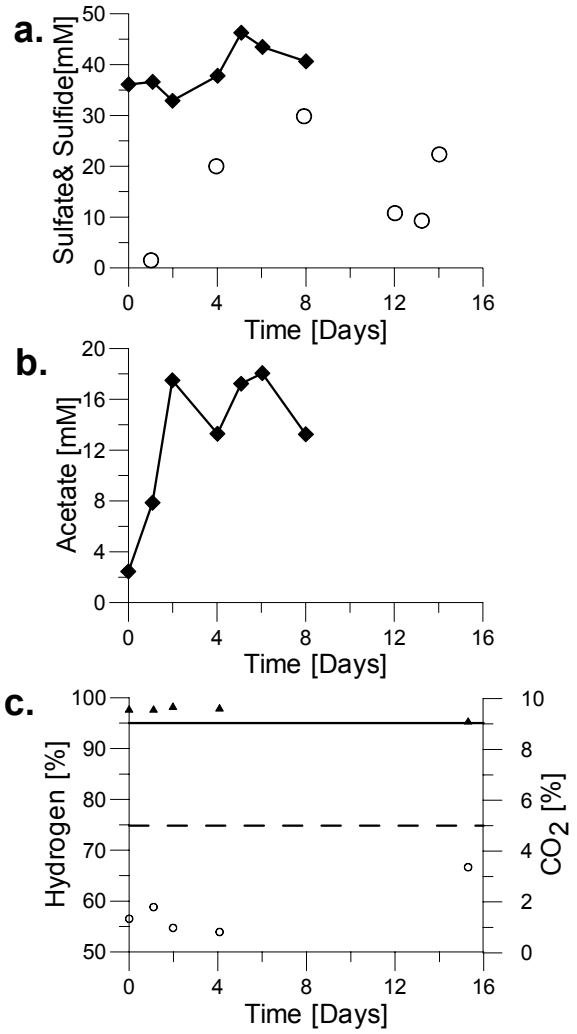


Fig. 4. Data from the gas lift bioreactor at high sulfide conditions (GLHS) with: a) sulfate (♦) and sulfide (○) concentration in the reactor liquor; b) acetate concentration (♦) in the reactor liquor; c) hydrogen (▲) and CO_2 (○) in the effluent and in the influent hydrogen (—) and CO_2 (- -).

Sulfide removal not only had a significant effect on the reactor performance but also on the microbial populations. Eleven clones were identified from GLLS after 81 days of operation (Fig. 5) of which 7 of the 16S rRNA gene sequences were closely related to sulfate reducing *Desulfovibrio* spp. Whereas, GLHS only contained 4 clones after 7 days of operation after inoculation with sludge from GLLS at day 53 (Table 2 and Fig. 6). Based on 16S rRNA gene similarities, the closest related named species to 6 of the 7 *Desulfovibrio* clones in GLLS was *D. carbinoliphilus* D41, a benzyl alcohol oxidizing SRB (NCBI database). Clone GLLS-8 was most closely related to *D. burkinensis* HDv isolated from an African rice field (Ouattara et al., 1999). Of the 4 clones identified in the GLHS, only GLHS-4 was distantly related to a *Desulfovibrio* sp. and was 83 % similar to *D. gigas* (Table 2 and Fig. 6). However, the ARB program aligned clone GLHS-4 in the *Spirochates* clade (Fig. 6) as it also had

81 % similarity to *Spirochaeta bajacaliforniensis* DSM16054T. Using the molecular technique fluorescent *in situ* hybridization (FISH) it was demonstrated that sulfide inhibition at pH 7.8 is SRB species dependent and that *Desulfovibrio* spp. (found in both GLLS and GLHS) were less sensitive to sulfide than some other families (Icgen and Harrison, 2006).

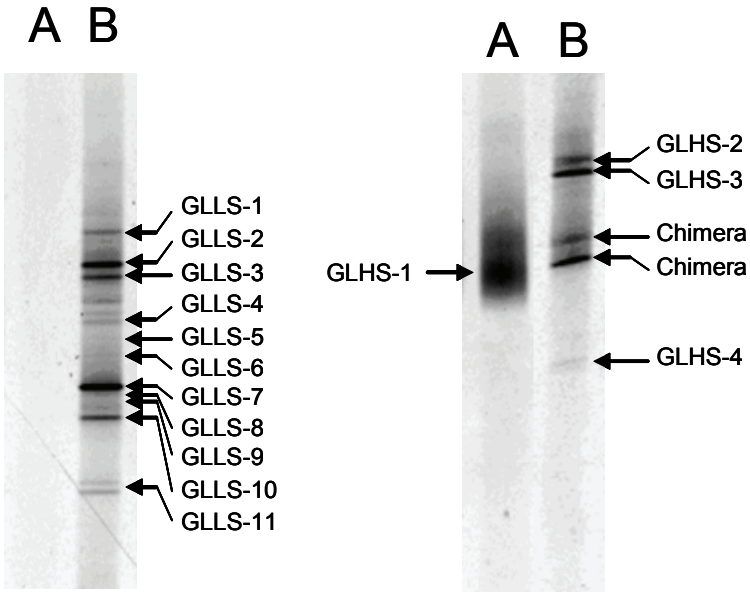
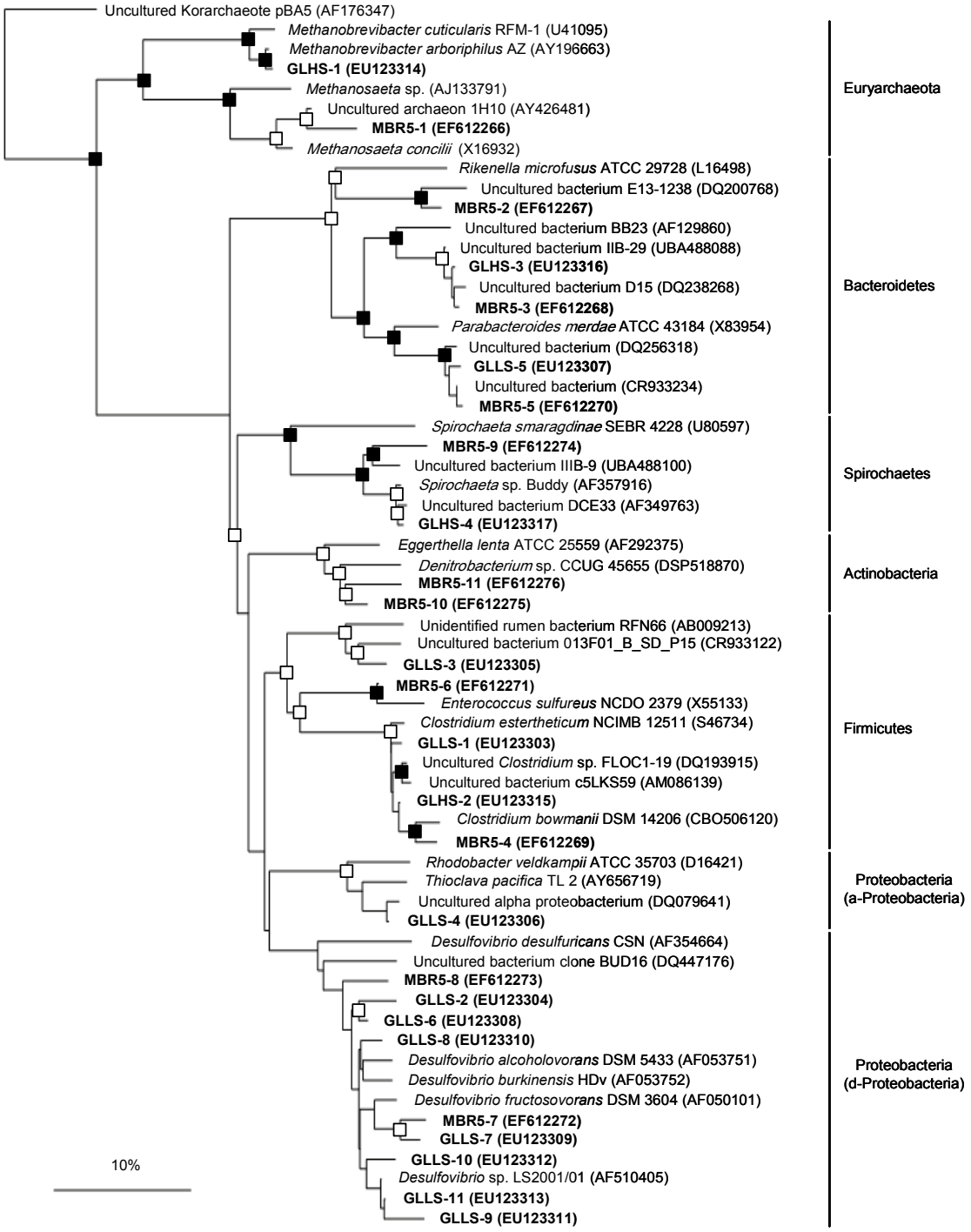


Fig. 5. DGGE gel of archaea (A) and bacteria (B) amplified from GLLS and GLHS mixed cultures prepared using the Wizard DNA Clean Up System. Two chimeric artefacts were identified that were not included in the phylogenetic analysis.



on partial 16S rRNA gene sequences of clones isolated from the GLLS, GLHS, and pH 5 membrane bioreactor used as inoculum (all in bold). Phylogenetic analysis was carried out by the maximum likelihood, distance neighbour joining, and DNA parsimony methods and the nodes supported by all 3 trees (■) and 2 trees (□) have been marked. Accession numbers are given in parenthesis. The scale bar corresponds to 10 % sequence similarity.

Table 2. Cloned 16S rRNA gene fragments from the bioreactors and their closest related named gene sequences in the NCBI database.

Clone	Closest named specie relative in database	Accession nr.[a]	% similarity[b]	Nr. of bases[c]
Gas lift bioreactor pH 5 run under low sulfide conditions				
GLLS-1	*Clostridium bowmanii* DSM 14206	AJ506120	98	558
GLLS-2	*Desulfovibrio carbinoliphilus* D41	DQ186200	94	580
GLLS-3	*Lactonifactor longoviformis*	DQ100449	94	539
GLLS-4	*Thioclava pacifica* TL 2	AY656719	98	545
GLLS-5	*Parabacteroides goldsteinii*	AY974070	94	578
GLLS-6	*Desulfovibrio carbinoliphilus* D41	DQ186200	96	532
GLLS-7	*Desulfovibrio carbinoliphilus* D41	DQ186200	96	583
GLLS-8	*Desulfovibrio burkinensis* HDv	AF053752	96	559
GLLS-9	*Desulfovibrio carbinoliphilus* D41	DQ186200	97	565
GLLS-10	*Desulfovibrio carbinoliphilus* D41	DQ186200	96	580
GLLS-11	*Desulfovibrio carbinoliphilus* D41	DQ186200	96	535
Gas lift bioreactor pH 5 run under high sulfide conditions				
GLHS-1	*Methanobrevibacter arboriphilus*	AB065294	98	550
GLHS-2	*Clostridium bowmanii* DSM 14206	AJ506120	98	548
GLHS-3	*Petrimonas sulfuriphila BN3*	AY570690	99	577
GLHS-4	*Desulfovibrio gigas*	DQ447183	83	586

[a]Accession number of the closest related named gene sequence obtained by BLAST comparison in the NCBI database (http://www.ncbi.nlm.nih.gov/).

[b]Percentage sequence similarity to the closest named relative in the NCBI database.

[c]Number of base pairs used for the BLAST comparison and alignment in the ARB program.

No consensus exists on the effect of sulfide on microbial populations in sulfidogenic bioreactors, possibly due to the many operational conditions involved. It is generally accepted that the H_2S molecule is the most inhibiting sulfide species at pH <7 (Moosa and Harrison, 2006; O'Flaherty et al., 1998) as H_2S can pass freely through the cell membrane (Speece, 1983). Once inside the cytoplasm it has been postulated that sulfide combines with iron in cytrochromes and other essential iron containing intracellular compounds (Madigan et al., 2000). In contrast to data from Okabe et al. (1995) and Reis et al. (1992) this suggests that sulfide inhibition would not be rapidly reversible upon sulfide removal. In acidophilic iron oxidizers it has been shown that small organic acids pass through the cell membrane and dissociate in the cytoplasm (as the cytoplasmic pH is above their pKa) thereby, releasing proton(s), destabilizing the proton motive force, and consequently inhibiting ATP generation (Alexander et al., 1987). Also, other inhibiting factors at low pH such as fatty acids (Reis et al., 1990) and protons (Alexander et al., 1987) would also be present. It is possible that sulfide could inhibit SRB in the same way as organic acids by entering the cytoplasm

as H_2S and dissociating to release a proton. This would result in much of the energy gained by sulfate reduction being used for pH homeostasis instead of growth and eventually leads to cell death. This alternative hypothesis for the mechanism of sulfide toxicity also explains the reported rapid alleviation of sulfide inhibition upon sulfide removal (Okabe et al., 1995; Reis et al., 1992). The relationship between maintenance energy and sulfide concentration has been shown in a *D. desulfuricans* continuous culture at pH 7 in which the cell yield (growth) was reduced by 50 and 75 % at approximately 250 mg L^{-1} sulfide (3.7 mM H_2S) and 437 mg L^{-1} sulfide (6.4 mM H_2S), respectively (Okabe et al., 1995). In the reactor liquor of GLHS, there was an average of 18.2 mM H_2S suggesting that compared to inhibition in the Okabe et al. study there would have been >75 % reduction in growth rate. Van Houten et al. (1994) showed that a hydrogen fed GLB at pH 7 could still be operated with 14 mM H_2S (28 mM of total sulfide). While an acetic acid fed batch reactor operated at pH 6.2 – 6.7 was completely inhibited at 16 mM (547 mg L^{-1}). Reduced growth rate or death of a particular species by sulfide inhibition would result in washout from the GLB and the decreased population diversity under high sulfide conditions (Table 2).

Other species identified from the GLBs include GLHS-1 that was most similar to a methanogenic archaeon with 98 % similarity to *Methanobrevibacter arboriphilus* isolated from paddy field soil (Asakawa et al., 1993) whereas, no archaea were found in the sludge under low sulfide conditions. This confirms the reactor performance results where methane was detected in the GLHS gas phase, but not in the GLLS gas phase. Clones related to acetogenic spp. were found in both reactors (GLLS-1 & GLHS-2) which were 98 % similar to the named species *Clostridium bowmanii* DSM14206. GLLS-3 aligned within the *Firmicutes* clade (Fig. 6) and was 94 % similar to the named species *Lactonifactor longoviformis* (Table 2). It is likely that the clones with high similarity to the *Firmicutes* produced the acetate measured in both the GLBs that is probably consumed by the SRB as a carbon source. Under both high and low sulfide conditions a clone related to the phylum *Bacteroides* was found, which were closely related to the named species *Parabacteroides goldsteinii* (GLLS-5) and *Petrimonas sulfuriphila* (GLHS-3). Microorganisms contained in the *Bacteroides* clade are fermentative suggesting they may degrade dead biomass. In GLLS, a clone related to the *α-Proteobacteria Thioclava pacifica* TL2 (Table 2) was found, which is a facultative autrotrophic marine sulfur-oxidizing bacteria isolated from a near shore sulfidic hydrothermal area in Papua New Guinea (Sorokin et al., 2005). It is unclear what metabolic role the *α-Proteobacteria* has in the GLB.

Conclusions

This study shows sulfate reduction can be achieved in a mesophilic GLB at pH 5 with and without sulfide removal from the gas phase. The removal of sulfide from the gas phase had a major impact on the reactor performance and microbial population. Under sulfide removal conditions, 99 % of the sulfate was converted at a HRT of 24 hours reaching a volumetric activity of 51 mmol sulfate L^{-1} d^{-1}. Incomplete removal of sulfate was observed when sulfide was not removed, which was probably due to sulfide inhibition. However, sulfate reduction was able to take place at a HRT of 24 hours with an average H_2S concentration of 18.2 mM (584 mg-S L^{-1}). At high sulfide concentrations a less diverse population was found than at low sulfide concentrations. Clones that aligned with *Desulfovibrio* spp. appeared to be the most tolerant sulfate reducers against the effect of the low pH. Finally, we postulate that sulfide inhibition at low pH is due to the dissociation of H_2S inside the cytoplasm resulting in destabilization of the cytoplasm leading to a higher maintenance and thus lower growth rates.

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6

Effect of the sulfide concentration on zinc bio-precipitation in a single stage sulfidogenic bioreactor at pH 5.5

Abstract

Dissolved zinc can be found in natural and process stream associated with the mining and metallurgical industry. These streams usually have a low pH. By using sulfate reducing bacteria, sulfide can be produced that can precipitate the zinc as zinc-sulfide, which could be separated and used for zinc production. In this study, the effect of the sulfide concentration on the precipitation of zinc was determined in a sulfate reducing gas-lift bioreactor operated at pH 5.5. The reactor was fed with hydrogen as electron donor for sulfate reduction. Zinc was precipitated as crystalline sphalarite. At a lower sulfide concentration in the reactor, larger zinc-precipitates were produced with better settling properties. The aqueous zinc concentration was between 0.3 - 0.8 μM during all the stable phases. While the solid zinc concentration increased to 104 mmol L^{-1}.

Introduction

The use of biotechnology for removal of metals from acid mine drainage (AMD) and recovery of metals (Cd, Co, Cu, Fe, Pb, Ni, Zn) from heap leachate is currently gaining interest from the mining and metallurgical industry (Huisman et al., 2006; Morin et al., 2006; Tabak et al., 2003; Weijma et al., 2002). A biotechnological technique to recover metals that uses sulfate reducing bacteria (SRB) follows a two-step procedure: 1) conversion of sulfate to sulfide in a bioreactor (SRB) in which an electron donor has to be added, and 2) precipitation of metals with sulfide in which insoluble metal-sulfides are formed that can be separated from the liquid. This two step process for zinc consist of:

Sulfate reduction: $SO_4^{2-} + 4H_2 \rightarrow S^{2-} + 4H_2O$ (1

Zinc precipitation: $S^{2-} + Zn^{2+} \rightarrow ZnS \downarrow$ (2

Net reaction: $ZnSO_4 + 4H_2 \rightarrow ZnS \downarrow +4H_2O$ (3

Compared to conventional chemical treatment of metal containing wastewater with lime and hydroxide, sulfide precipitation achieves much lower metal concentrations in the effluent and the effluent pH does not have to be increased to the extend of lime treatment to be effective (Feng et al., 2000). Lime treatment of zinc at a high pH is effective (<0,001 mg L^{-1} at pH 9.1), while ineffective at low pH (5.7 mg L^{-1} at pH 5.7) (Feng et al., 2000). In addition, metal-sulfide sludge is about 1/10 of the sludge volume formed by the lime-process (Huisman et al., 2006). The reuse potential of zinc/sulfate lime sludge is limited and usually has to be disposed of, while metal-sulfides can be used directly in the metallurgical industry (Weijma et al., 2002).

When sulfide is dosed through the gas-phase of liquid-phase into a contactor (Esposito et al., 2006; Veeken et al., 2003a; Veeken et al., 2003b), local high sulfide concentrations exist around the injection. The nucleation rate of metal-sulfides is high at high sulfide concentrations creating many nuclei, while the crystal growth rate is higher at low sulfide concentrations, creating larger metal-sulfide particles with better settling properties (Mersmann, 1999). Therefore, high concentrations should be avoided as they produce small metal-sulfide particles with poor settling properties.

In a sulfate reducing reactor, SRB act as dispersed sulfide injection points, creating a homogeneous sulfide concentration. When simultaneously sulfate reduction and metal precipitation occur in the same reactor, local over-saturation of sulfide can be avoided, creating large well settable ZnS particles (Veeken et al., 2003a). Additionally, reactor investment costs are reduced because only one reactor is needed.

Simulations sulfate reduction and zinc-sulfide precipitation has been demonstrated in a full scale gas-lift bioreactor under near pH neutral conditions (Boonstra et al., 1999; Weijma et al., 2002). However, mining and metallurgical waste and process streams are of low pH and by operating under acidic conditions costs for neutralization could be largely avoided. Additionally, under acidic conditions, the concentration of the active sulfide species in metal precipitation (S^{2-}) is low. This implies that that even at relatively high total sulfide ($H_2S + HS^- + S^{2-}$) concentrations, the crystal growth rate should be high compared to the nucleation rate (Mersmann, 1999), but biological induced sulfide precipitation under acidic conditions has not been demonstrated to date.

In the present study, simultaneous sulfate reduction at pH 5.5 and zinc precipitation are demonstrated in a gas-lift bioreactor. In addition, the effect of the sulfide concentration in the reactor liquor (by addition of excess sulfate) on the bio-precipitation of zinc is investigated.

Material and methods

Experimental design.

All experiments were done in a 4.0 L gas-lift bioreactor operated between pH 5.4 and 5.5 at 30˚C. Hydrogen (flow rate 16.2 L d^{-1}) was used as electron donor and CO_2 (flow rate 1.8 L d^{-1}) as carbon source. During the run, three stable phases were reached: stable phase 1 (S1; day 22-32 (excluding day 24 and 25 were the liquid pump had stopped dosing)), stable phase 2 (S2; day 59-66) and stable phase 3 (S3; day 72-77). A stable phase was assumed when the sulfide concentration in the reactor liquid and gas phase had stabilized and were maintained for 6-10 days. The hydraulic retention time (HRT) during the stable phases was 24.2 h with a zinc loading rate of 7.2 mmol L^{-1} d^{-1}. Zinc was dosed as $ZnSO_4$ resulting in a sulfate dosage of 7.2 mmol L^{-1} d^{-1} through the zinc feed. A sulfate excess was created by dosing Na_2SO_4 in a separate feed, at a dosing rate of 5.0 (S1), 0.5 (S2) and 0.05 (S3) mmol L^{-1} d^{-1}, resulting in a total sulfate load of 12.2 (S1), 7.7 (S2) and 7.25 (S3) mmol L^{-1} d^{-1}.

Experimental set-up.

A glass gas-lift bioreactor with a liquid volume of 4.0 L was operated at 30˚C by running water through a water jacket that was heated with a Tamson T1000 waterbath (Tamson instruments). The gas-lift bioreactor had an internal and external settler of 0.5 and 4 L, respectively. The external settler was cooled to 4^{o}C with a Julabo F10 cryostat to minimize bacterial activity in the settler. The pH was measured using a H63 electrode (Schotts Instruments) and controlled with 4 M NaOH and 4 M HCl dosed by a EH Liquisys-P pH controller (Endress Hauser Inc.). The pH system was regularly checked and if necessary recalibrated. The oxidation-reduction potential (ORP) was measured using a QIS electrode (Type O14/NS/12x250/DJ/KNO_3/1M/BNC) on a Radiometer readout unit (Type PHM210, Meterlab).

The metal containing influent was stored in a 100 L container that was continuously sparged with N_2 to maintain anoxic conditions while the Na_2SO_4 stream was stored in a 20 L container and flushed with N_2 at the beginning of the experiment. The metal and Na_2SO_4 medium were dosed into the GLB by a Stepdos 08RC and 03RC liquid membrane pump, respectively (FEM 08TT.18RC, KNF-Verder).

H_2 and CO_2 were dosed via a Brooks thermal mass flow controller type 5850E with maximum flows of 30 and 7.5 L min^{-1} for H_2 and CO_2, respectively (Brooks instruments) connected to a Brooks control unit (type 5878). The gas phase was recycled through the reactor liquid by a KNF-Verder N840.3FT.18 gas membrane pump. To ensure efficient mass transfer of the gases, a teflon sparger with 168 holes of 0.44 mm was placed at the interface between the solution and recycled gas. The gas recycle flow rate was measured using a SHO-rate (Type R2-15-C, Brooks instruments) and corrected to 4 L min^{-1}. All the tubing and connections were made of PTFE (Schott A.G., Mainz, Germany and Serto AG.).

Media.

The start-up medium contained the following components (g/L): $CaCl_2.2H_2O$ (0.11); KCl (0.37); KH_2PO_4 (0.41); $MgCl_2.6H_2O$ (0.1); Na_2SO_4 (1.48); NH_4Cl (0.3); yeast extract (0.01). All chemicals were Merck Analytical Grade. Trace elements were added from acid and basic stock solutions in final concentrations as reported in (Stams et al., 1993). After start-up and at stable phase, zinc was supplied in the same nutrient matrix as the start-up medium, but Na_2SO_4 was replaced by $ZnSO_4.H_2O$ (2.87 g/L).

The excess of sulfate was dosed through a $NaSO_4$ medium, with the same nutrient matrix as the start-up medium, but varying Na_2SO_4 concentrations.

Inocula. The inoculum contained 10 g wet Eerbeek sludge (Indrustrie Water Eerbeek, Eerbeek, The Netherlands), 10 g wet Nedalco sludge (Royal Nedalco, Bergen op Zoom, The Netherlands), 10 g wet Emmtec sludge (Emmtec, Emmen, The Netherlands), 100 ml supernatant of Zinifex sludge (Zinifex Budel Zinc, Budel, The Netherlands), 100 ml of sulfate reducing sludge taken from previous reactor runs at pH 5 (chapter 5). The sludge was crushed and mixed with a blender, except from the sludge taken from previous reactor runs, which was added without any treatment.

Analyses.

Zinc was analyzed with ICP-OES in which aqueous zinc (Zn_{aq}) was analyzed on samples filtered trough a 0.45 µm filter and solid zinc (Zn_{solid}) was analyzed by subtracting Zn_{aq} from the total zinc concentration (chapter 7). Sulfide was determined on a filtered sample (0.45 µm) using Dr. Lange sulfide kit LCK-653 and a Xion 500 spectrophotometer (Hach Lange GMBH). Sulfate and acetate were analyzed by ion chromatography according to (Sipma et al., 2004). Total suspended solids (TSS) were determined following (Clesceri et al., 1998). The biomass concentration by total-N (nitrogen) determination of washed solids using Dr. Lange total-N kit LCK 238 and the Xion 500 spectrophotometer and calculated with the general biomass composition $CH_{1.8}O_{0.5}N_{0.2}$ (Roels, 1983). The particle size distribution (PSD) of the solids was measured with a Beckman Coulter LS 230. Settling properties were determined with an Imhof cone and 100 ml reactor liquid. Reactor precipitates and solids were dried under N_2 atmosphere at room temperature, and examined for mineralogy by XRD (chapter 7). The solids were also analyzed on SEM-EDX (Type JEOL SEM-6480LV, Jeol, with an EDX analyser Noran system SIX, Thermo Electron Corporation) to examine crystal morphologies and semi-quantitative chemical analysis.

Chemical equilibrium model calculations.

The chemical equilibrium model PHREEQC 2.0 for Windows was used to calculate the sulfide speciation and the Zn-speciation from the measured sulfide and Zn_{aq} concentration, respectively. The following sulfide and zinc species were considered to be included in the model: Zn^{2+}, $ZnCl^{+}$, $ZnCl_2$, $ZnCl_3^{-}$, $ZnCl_4^{2-}$, $ZnCO_3$, $Zn(CO_3)_2^{2-}$, $ZnHCO_3^{+}$, $ZnHS^{+}$, $Zn(HS)_2$, $ZnOH^{+}$, $Zn(OH)_2$, $Zn(OH)_3^{-}$, $Zn(OH)_4^{2-}$, $ZnSO_4$, H_2S, HS^{-}, and S^{2-}. All equilibrium constants were taken from the PHREEQC data base, except from the formation of $ZnHS^{+}$ and $Zn(HS)_2$ out of Zn and HS^{-}, with a log K of -

6.5 and 14.0, respectively (Smith and Martell, 1976). In addition, the saturation index (SI) of sphalerite (ZnS) was calculated, which is defined as:

$$SI = \log\left[\frac{(Zn^{2+})(S^{2-})}{K_{SP}}\right] \quad (4)$$

With K_{SP} = -22.0 for sphalerite, and with (Zn^{2+}) and (S^{2-}) the activities for Zn^{2+} and S^{2-}. The SI is negative in case of under-saturation, and positive with super-saturation.

Results

Reactor performance during the start-up period.

The bioreactor was, after inoculation, operated in batch for the first 7 days, to allow the sulfate reducing population to adapt to the applied conditions. Sulfate reduction started at day 3, as indicated by the decrease in sulfate concentration (Fig. 1c) and increase in sulfide concentration (Fig. 1e). Sulfate depleted at day 7 and as a response sulfide increased to 9 mM, showing that the sulfate reduction rate had increased. Zinc and sulfate were therefore dosed to the reactor from day 7, at a rate of 4.4 mmol SO_4 L^{-1} day^{-1} and 1.4 mmol zinc L^{-1} day^{-1}. A sulfide excess was maintained throughout the run to assure a low concentration of toxic free zinc in the reactor liquor. The zinc load was gradually increased during the start-up period, while monitoring the sulfide concentration in the reactor liquid. From day 8, the sulfide concentration decreased rapidly below 2 mM (Fig. 1e) and the amount of Zn_{solid} in the reactor increased (Fig. 1d) indicating ZnS precipitation.

The sulfate concentration decreased rapidly and sulfate was depleted by day 8 (Fig. 1c), which resulted in low sulfide concentrations from day 8 (Fig. 1e). As a response the Zn_{aq} concentration increased from 0 to 47 µM from day 7 to 10 (Fig. 1f). The Na_2SO_4 loading rate was therefore increased to 6.0 mmol L^{-1} day^{-1} and as a result, more sulfide was generated (Fig. 1e) and the Zn_{aq} concentration decreased to 8 µM on day 18 (Fig. 1d). From day 7, the HRT was gradually decreased from 133 to 38 h at day 18, by gradually increasing the $ZnSO_4$ feed to reach 7.2 mmol L^{-1} day^{-1} on day 18 (Fig. 1b).

Reactor performance during period I with 5 mM excess sulfate.

From day 19, the HRT was set on 24 h by increasing the Na_2SO_4 feed and changing its sulfate concentration to reach a total sulfate load of 12.2 mmol L^{-1} day^{-1}. A stable

phase (S1) was reached at day 22 and maintained until day 32, with an interruption on day 24-25 because the metal dosing had stopped unintentionally (Fig 2a). During S1, the sulfide concentration was between 1.3 – 3.0 mM and the sulfate concentration between 0.04 – 3.5 mM (average values and standard deviation in Table 1). The sulfate conversion was sub-optimal after day 25 (87.1 ± 12.2%) as compared to the period before day 23 (100%), but this did not affect the zinc recovery efficiency. The Zn_{aq} concentration ranged during S1 between 0.5 – 2.0 µM except for one high value (12 µM) measured at the end of S1 (Fig. 1d). In the effluent, 0.5 – 1.5 µM Zn_{aq} was measured and the gas phase contained 2428 ± 875 ppmv H_2S (Table 1), leading to a loss of 4.7-7.0 % of the produced sulfide via the gas effluent. After S1 on day 32, the reactor was stopped for two hours to clean the gas distributor.

Table 1. Results of stable phases S1, S2, and S3.

Parameters	Unit	S1	S2	S3
pH	.	5.48	5.46	5.42
ORP	mV	-391	-331	-537
sulfide	mM	2.2 ± 0.7	0.10 ± 0.02	0.008 ± 0.005
sulfate	mM	1.6 ± 1.5	0.16 ± 0.3	0.006 ± 0.003
Zn_{aq} reactor	µM	0.78 ± 0.46	0.74 ± 0.46	0.32 ± 0.15
Zn_{solid} reactor	mmol L^{-1}	16.5 ± 5.4	103 ± 27	104 ± 28
Zn_{aq} effluent	µM	0.65 ± 0.6	2.4 ± 0.8	8.6 ± 4.6
Zn_{solid} efffluent	mmol L^{-1}	2.7 ± 2.6	0.6 ± 0.3	0.6 ± 0.3
log[S^{2-}]		-11.0 ± 0.1	-12.4 ± 0.1	-13.6 ± 0.3
Biomass	g L^{-1}	0.43 ± 0.12	1.27 ± 0.44	1.42 ± 0.21
PSD mode	µm	12.9 ± 0.7	17.9 ± 1.5	22.0 ± 1.8
PSD d10[a]	µm	4.0 ± 0.1	6.2 ± 0.5	7.9 ± 1.0
PSD d50[b]	µm	13.2 ± 0.6	16.8 ± 0.7	19.7 ± 2.1
PSD d90[c]	µm	43.6 ± 2.1	41.7 ± 2.5	41.2 ± 3.6
H_2S headspace	ppmv	2428 ± 875	698 ± 201	20.4 ± 12.2
H_2(g) used	L g^{-1} Zn	26.6±1.1	22.4±0.7	20.7±0.8
SI Sphalerite		1.2 ± 0.7	2.0 ± 0.4	2.8 ± 0.2
Volumetric activity	mmol L^{-1} d^{-1}	10.6 ± 1.4	7.5 ± 0.3	7.2 ± 0.0
Specific activity	mmol $gBiomass^{-1}$ d^{-1}	27.6 ± 12	6.7 ± 2.7	5.2 ± 0.7
Retention time ZnS	d	6.5 ± 6.2	15.3 ± 6.5	13.2 ± 5.2
Zinc recovery efficiency	%	>99.9 ± 0.01	>99.9 ± 0.01	>99.9 ± 0.00

[a] 10 % of the particles were smaller than. [b] 50 % of the particles were smaller than. [c] 90 % of the particles were smaller than.

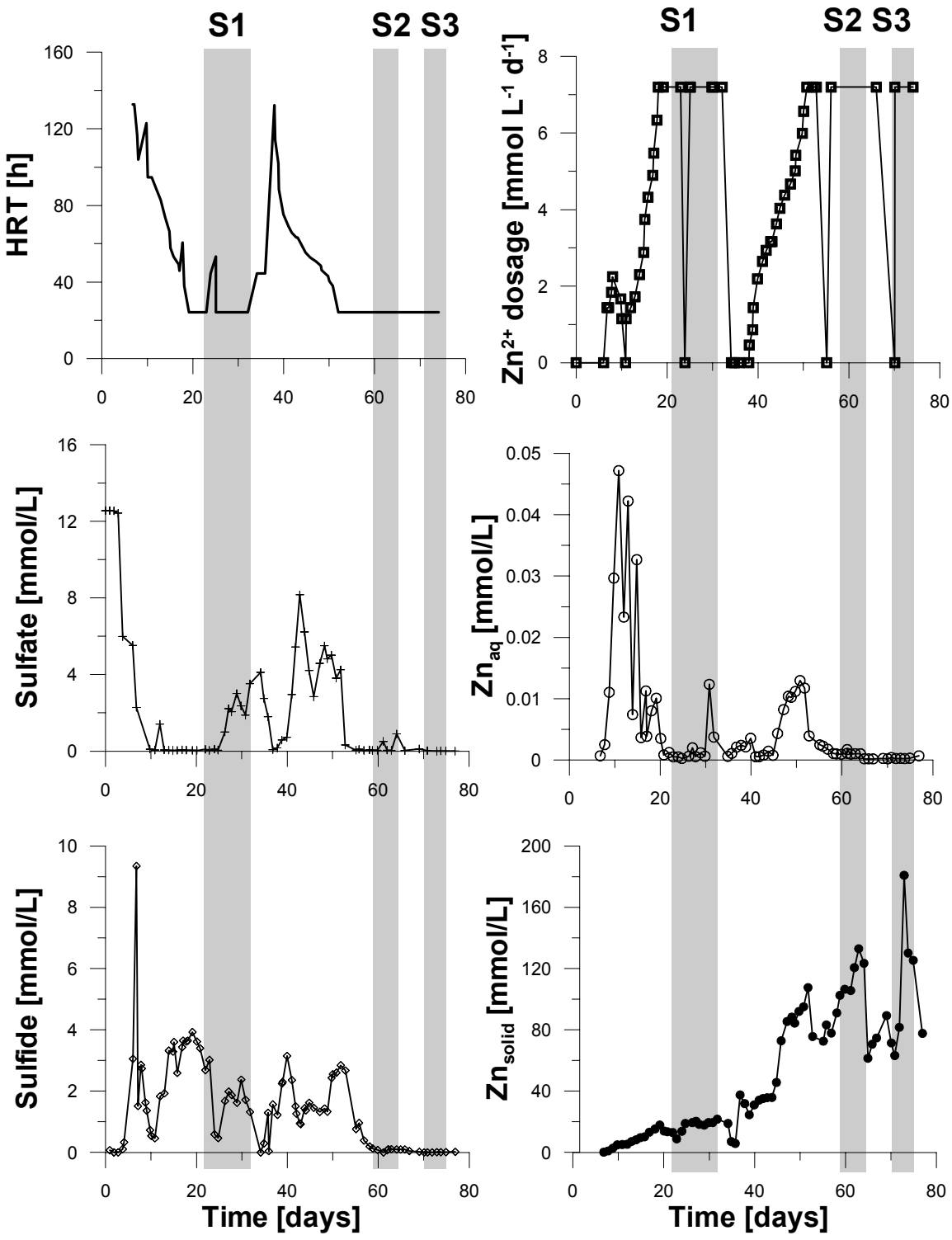

Figure 1. Course of (a) HRT, (b) sulfate, (c) sulfide, (d) Zn^{2+} dosage, (e) soluble zinc (Zn_{aq}), and (f) suspended zinc (Zn_{solid}) during the reactor run. Stable phases are labeled S1, S2 and S3 (as in text) and the marked in grey planes.

Reactor performance during period II with 0.5 mM excess sulfate.
After the restart, the sulfate load via the Na_2SO_4 stream was set on 2.2 mmol L^{-1} day^{-1} with a HRT of 44 hours (Fig. 1a). On day 37, the sulfide concentration had increased to 1.6 mM indicating sulfate reduction at a sufficient rate to dose zinc at a rate of 0.5 mmol L^{-1} day^{-1}. The sulfate load was increased to 2.2 mmol L^{-1} day^{-1} on day 43, causing a sulfate peak between day 43 and day 52 (Fig. 1c). On day 52, the zinc loading rate was set to 7.2 mg L^{-1} day^{-1} (Fig. 1b) and the Zn_{aq} concentration remained below 15 µM from day 43-52 (Fig. 1d). This indicated that the system produced sufficient sulfide to precipitate the zinc. The sulfate load was set at 7.7 mmol L^{-1} day^{-1} at a HRT of 24 h on day 52 (Fig. 1a). During S2 (day 59-66), the sulfide concentration remained between 0.8-0.9 mM (Fig. 1e), resulting in lower sulfide in the gas phase (698 ± 201 ppmv) compared to S1 (Table 1). The sulfate concentration fluctuated around 0.04 mM, with two extreme values of 0.5 and 0.9 mM on day 61 and 64 (Fig. 1c). During S2, a very low Zn_{aq} concentration of 0.8 µM was maintained (Fig. 1d) and the effluent Zn_{aq} concentration of 0.7 µM (Table 1).

Reactor performance during period III with 0.05 mM excess sulfate.
After finishing S2 at day 66, the sulfate load was put on 7.25 mmol L^{-1} day^{-1} at a HRT of 24 h (Fig. 1a) and from day 72 to 77 a stable phase (S3) was maintained (Table 1). The low sulfate excess resulted in low sulfide concentrations in the reactor liquor (<0.02 mM) and headspace (20.4 ± 12.2 ppmv; Table 1). The Zn_{aq} concentration of 0.03 µM was low compared to the previous stable phases (Table 1). However, the Zn_{aq} concentration in the effluent was higher than in the previous stable phases (8.6 µM; Table 1).

Effect of the sulfide concentration on zinc bio-precipitation during the stable phases. The more sulfate was dosed, the more sulfate remained in the reactor liquor (ranging from 1.6 to 0.006 mM; Table 1), and the higher the sulfide concentration in the reactor liquor (Table 1). The higher sulfide concentration was due to an increased volumetric sulfate reducing activity when more sulfate was dosed, resulting in more sulfide being produced. The volumetric sulfate reducing activity ranged from 11 to 7 mmol L^{-1} d^{-1} during S1 to S3 (Table 1). When more sulfide was present in the reactor liquor, the H_2S concentration in the headspace increased.

The specific sulfate reducing activity of the biomass decreased from 28 to 5 mmol $gBiomass^{-1}$ d^{-1} from S1 to S3, probably because the stable phases were sequentially run and the solids were retained in the system. The biomass was part of the precipitates and partly encapsulated in the precipitates and therefore less active. Due

to the high retention time of the ZnS between 6.5 to 15.3 d (Table 1), a high biomass concentration could be maintained in the reactor liquor. The Zn_{solid} concentration in the reactor increased from S1 to S3, which is probably related to the length of the experiment. However, the particles became larger when the sulfide concentration was lower and could thus be better retained (Table 1). Accumulation of Zn_{solid} predominantly occurred between stable phases (Fig. 1f).

The Zn_{aq} concentration in the reactor decreased slightly from 0.8 – 0.3 µM which showed that the recovery efficiency during all the stable phases was >99.9 %. The Zn_{solid} concentration in the effluent of the reactor (after the internal settler, but before the external settler) first decreased from 2.7 to 0.6 mmol L^{-1} for S1 to S2 and then stabilized, while the Zn_{aq} in the effluent increased from S1 to S3, unlike to the Zn_{aq} in the reactor liquor.

Characterisation of reactor solids.

XRD analyses of the reactor solids show that crystalline sphalerite was formed (confidence 95%) throughout the experiment and the degree of purity increased from S1 to S3. SEM-EDX analyses revealed that the Zn:S ratio was 1 (Table 2), confirming that the Zn precipitate was sphalerite. The solids retrieved from S1 also contained relatively large amounts of salts (KCl, NaCl) and biomass, as confirmed by XRD, SEM-EDX, and biomass determination. In S2 and S3, at least 80% of the reactor solids consisted from ZnS. The d_{10}-d_{90} (the mid 80% range) particle size (Table 1) of reactor solids was between 3.9-46.3 µm, but overall particle size increased gradually from S1 to S3 as shown by the higher mode, median and mean of the particle size distribution (PSD; Table 1, Fig. 2).

Table 2. Sludge characteristics and SEM-EDX analysis

Parameter	Unit	S1	S2	S3
Zn	atom %	20.7	31.0	32.0
S	atom %	21.2	29.0	29.0
C	atom %	36.8	31.2	31.0
Biomass/ ZincSOLID	%	21.4 ± 1.8	10.2 ± 3.8	11.9 ± 4.3
Settling rate	$m.h^{-1}$	1.02	1.95	2.94
50% settling	sec	700	n/a	300
Sludge water content[a]	vol%	94	93	92

[a] determined after 24 hours of settling in still water in a Imholf column

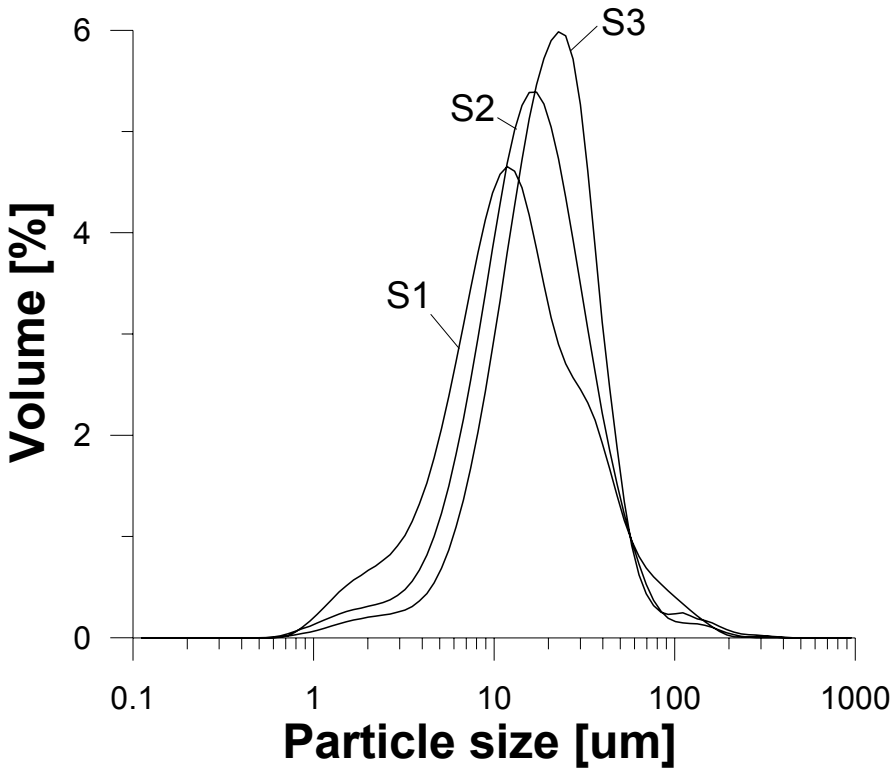

Figure 2. Particle size distribution of the precipitates in the reactor during stable phase 1,2 and 3 (S1, S2 and S3).

Discussion

Bio-precipitation of zinc in a single stage bioreactor at pH 5.5.

This study shows that a bioreactor with simulations sulfate reduction and zinc precipitation can operate for several weeks at pH 5.5, with a >99.9% recovery efficiency at a zinc load of 7.2 mmol L^{-1} d^{-1} during the stable phases. Similar zinc recovery efficiencies (>99.9 %) are reported by using Na_2S (Veeken et al., 2003a) and biogenic sulfide (effluent of a sulfate reducing reactor; (Esposito et al., 2006)) in a continuous stirred tank reactor (CSTR) at pH 6.3 - 6.5 and room temperature. The sulfate conversion rates reached during the stable phases were between 7 - 11 mmol L^{-1} d^{-1}, but were limited by the sulfate loading rate. Previous experiments have shown that the gas-lift bioreactor used in this study was able to handle sulfate loading rates of 51 mmol L^{-1} d^{-1} at pH 5.0 (chapter 5) and sulfate reduction rates up to 188 mmol L^{-1} d^{-1} have been demonstrated in a membrane bioreactor at pH 5.0 (chapter 3).

The Zn_{aq} concentrations in the reactor were well below these values during all stable phases (0.3- 0.8 μM), and no toxic effects of aqueous zinc have been experienced, which is in accordance with Poulson et al. (Poulson et al., 1997) who reported an EC_{100} (concentration at which 100% of the culture is inhibited) of 199 μM for zinc.

Utgikar et al. (Utgikar et al., 2001) reported an EC_{100} value of 306 µM for zinc, which was only slightly higher than the EC_{50} values (concentration at which 50 % of the culture is inhibited) of 252 µM, showing that aqueous zinc concentrations close to the EC_{100} were already inhibiting. The Zn_{aq} concentrations during the stable phases in the reactor (Table 1) were among the lowest found in literature. Esposito et al. (Esposito et al., 2006) reported effluent concentrations as low as 11 µM at pH 6.3 for a CSTR system using biogenic sulfide for zinc removal, and Veeken et al. (Veeken et al., 2003a) reported effluent zinc concentrations of <0.5 µM at pH 5.4-7.8 in a similar CSTR experiment.

Effect of sulfide concentration on zinc speciation.

PHREEQC calculations show that $Zn(HS)_2$ and free Zn^{2+} were the only two relevant zinc species in the reactor liquor next to ZnS (Table 3). The formation of zinc–sulfide complexes has been reported (Esposito et al., 2006; Veeken et al., 2003a) and shows that even a small sulfide excess increases the total soluble zinc concentration by the formation of complexes at pH 6.2-7.8. In our reactor, free Zn^{2+} emerged as an important secondary species at very low sulfide excess in S3 (Fig 3a), because there is insufficient HS^- to bind all zinc as $Zn(HS)_2$. This is because at pH 5.5, only 5% of the sulfide is present as HS^- and 95% as H_2S, and the latter does not complex with zinc. Therefore, the low sulfide concentration in S3 leads paradoxically to increased super-saturation of sphalerite (Fig. 3b), due to increased free Zn^{2+} speciation at the cost of $Zn(HS)_2$.

Table 3. Calculated zinc and sulfide speciation during stable phases (calculated with PHREEQC).

Species	Unit	S1	S2	S3
Zn^{2+}	mol L^{-1}	$1.24*10^{-11}$	$7.27*10^{-10}$	$4.69*10^{-08}$
$Zn(HS)_2$	mol L^{-1}	$3.68*10^{-06}$	$7.41*10^{-07}$	$3.02*10^{-07}$
HS^-	mol L^{-1}	$1.11*10^{-04}$	$4.66*10^{-06}$	$3.20*10^{-07}$
H_2S	mol L^{-1}	$2.10*10^{-03}$	$9.49*10^{-05}$	$7.13*10^{-06}$
Zn^{2+}	% of Zn_{aq}	<<0.01	0.1	17.2
$Zn(HS)_2$	% of Zn_{aq}	>99.99	99.9	82.2
HS^-	% of S_{tot}	5.0	4.7	4.3
H_2S	% of $^aS_{tot}$	95.0	95.3	95.7

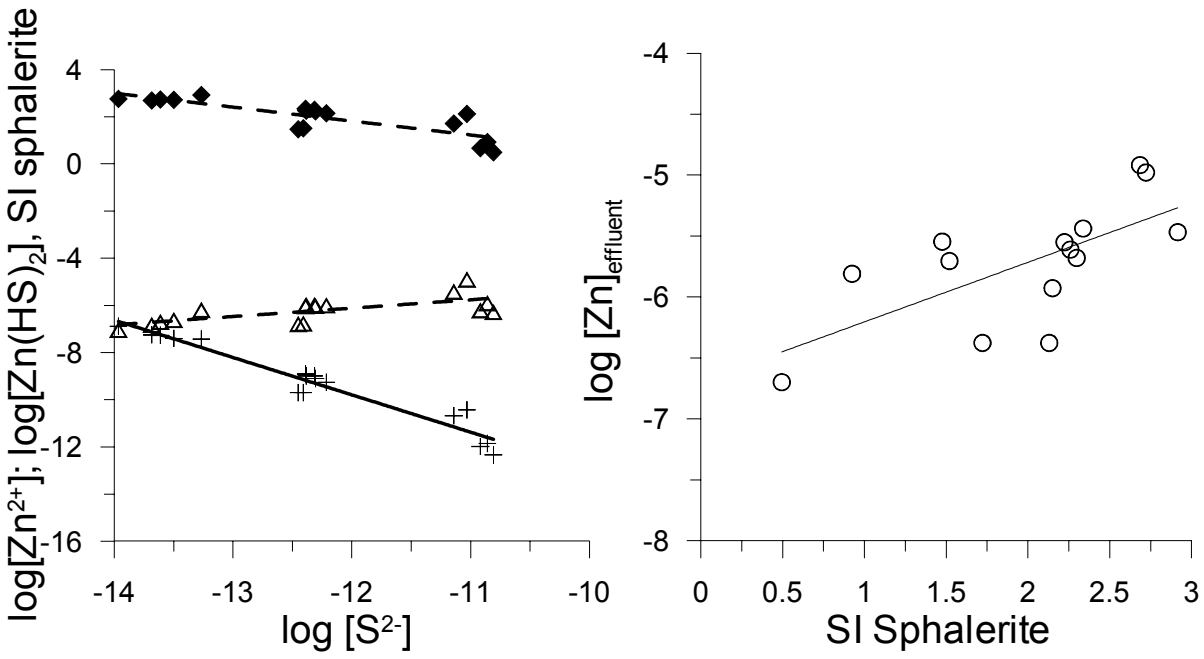

Figure 3. Scatter plots of modeled values for (a) Zinc speciation (log value of concentration in mmol L^{-1}) for Zn^{2+} (+), $Zn(HS)_2$ (Δ), and saturation index (SI) of sphalerite (♦) in reactor liquid versus log[S^{2-}], and (b) soluble zinc in the effluent (O) vs. the SI in the reactor.

Zinc-sulfide precipitates characteristics.

The re-use and recovery potential of the zinc-sulfide precipitates depends on its purity and settling tendency. The produced zinc precipitate was crystalline to a high degree, which is favorable for dewatering and increases commercial values. The settling properties of the sludge depend on the PSD of the zinc-particles in the reactor and the tendency to form aggregates in the settler under low shear conditions. At lower sulfide concentration, the zinc-particles increase in size, and the size distribution becomes more homogeneous as demonstrated by a higher, sharper peaks (Fig. 2; Table 1). Veeken et al. (Veeken et al., 2003a) demonstrated that the particle size logarithmically decreased with the S^{2-} concentration. The zinc-precipitates formed in S2 and S3 have therefore a higher quality than the zinc-particles of S1. The zinc-precipitates at higher sulfide concentrations settled much slower then at low sulfide concentration (Table 2). Assuming a particle density of 4100 g/cm^3 for sphalerite (Veeken et al., 2003a) and a laminar vertical up-flow of 18.15 cm/h in the internal settler at stable phase, the internal settler would following Stokes Law retain only particles larger than 5.5 µm in the reactor. The PSD show that in S1, 18 % of the particles were smaller than 5.5 µm, and this fraction decreased in S3 to only 5.5 % (Fig. 2). The settling properties were also influenced by aggregation of Zn particles, which only occurs under low shear

conditions like in the settlers. The vigorous mixing in the reactor most likely prevented formation of particles larger than 40 μm in the reactor (Fig. 2).

Impact of this study on bioprecipitation/biocystallisation of zinc.

In this study, simultaneous sulfate reduction and zinc precipitation were performed in a single gas-lift bioreactor at pH 5.5. A single bioreactor system saves on investment costs, because no additional contactor is required. At lower sulfide concentration, larger ZnS particles are formed with better settling properties. By operating the process as low as at pH 5.5, caustics will be saved for influent pH adjustment.

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7

Selective recovery of nickel from a nickel-iron solution using microbial sulfate reduction in a gas-lift bioreactor

Abstract

Process streams with high concentrations of metals and sulfate are characteristic for the mining and metallurgical industry. The present study aims to selectively recover nickel from a nickel-iron containing solution at pH 5.0, using a single stage bioreactor that combines simultaneous low pH sulfate reduction and metal-sulfide formation in a single reactor system. The results show that nickel was selectively precipitated in the bioreactor at pH 5.0 and the precipitates contained maximally for 83 % of the metal content of nickel. The nickel-iron precipitates were partly crystalline and had a metal/ sulfur ratio of 1, suggesting these precipitates were NiS and FeS. Experiments that focus on nickel recovery at pH 5.0 and 5.5 reached a recovery of >99.9 % resulting in a nickel effluent concentration < 0.05 μM. This study shows that selective metal precipitation in a single stage low pH sulfate reducing bioreactor has potential to produce metal-sulfides that can be used by the metallurgical industry as resource for metal production.

Introduction

The mining and metallurgical industry produces streams with high concentrations of metals and sulfate as process streams (e.g. bioleaching (Rawlings et al., 2003)), waste streams (e.g. acid mine drainage (Johnson, 2000)) or metallurgical wastewater (Weijma et al., 2002). Bioleaching of nickel laterite ore, produces a solution with high concentrations (several grams per litre) of nickel and iron (Alibhai et al., 1993; Tzeferis and Agatzini-Leonardou, 1994). Nickel is a valuable metal used for the production of austenitic stainless steel, super alloys or non ferrous alloys, steel alloys, rechargeable batteries and catalysts (Kurama, 2007). Nickel is toxic to the environment so should be removed from it and with hydroxide precipitation nickel can only be removed until 0.9 to 40 μM at pH 12.5 and 6.7, respectively (Feng et al., 2000).

Nickel can be precipitated and recovered from these waste and process streams as insoluble nickel-sulfide (NiS)(Huisman et al., 2006; Veeken et al., 2003a). At low sulfide concentrations, the crystal growth rate of NiS particles will be larger then the nucleation rate (formation of new particles), resulting in large NiS particles with good settling qualities (Mersmann, 1999). When sulfide is dosed to a reactor, a local high concentration of sulfide will exist around the injection point, resulting in small NiS particles, which a worse settling quality. When sulfide is produced in the reactor by sulfate reducing bacteria (SRB), an evenly distributed sulfide concentration will be reached and at the same time sulfate will be removed from the waste stream. Sulfate reduction and metal-sulfide precipitation can occur simultaneous in one reactor system, reducing investment costs, simplifying process design and avoiding the transport of toxic sulfide to the reactor. A sulfate reducing bioreactor treating zinc containing wastewater at neutral pH has been demonstrated at full scale (Weijma et al., 2002).

By controlling the pH and sulfide concentration, metals can be recovered from a multi-metal waste stream as pure metal-sulfide based on the difference in solubility of each metal-sulfide (Huisman et al., 2006; Tabak et al., 2003). At pH <5 nickel can be recovered at NiS while iron would not precipitate as FeS. High rate sulfate reduction has recently been shown at pH 5 (chapter 3 and 5), which suggest that it would be feasible to recover nickel as nickel-sulfide from a nickel-iron solution in a single stage bioreactor.

This study focuses on the selective recovery of nickel as NiS from a mixed nickel-iron solution by using microbial sulfate reduction at pH 5.0. A single stage gas-lift bioreactor was used with hydrogen as electron donor for microbial sulfate reduction. First, the recovery of nickel by NiS precipitation was studied at pH 5.5. Secondly, the selective recovery of nickel from a nickel/iron solution was studied at pH 5.0. Both the reactor performance and the product formation are presented in this study.

Material and methods

Experimental design.

All experiments were done in a gas-lift bioreactor that was operated under mesophilic conditions (30^0C). In the first run, the formation of nickel-sulfide from nickel-sulfate was studied at pH 5.5, while in the second run, the selective recovery of nickel-sulfide from a nickel-sulfate and iron-sulfate containing solution was studied at pH 5.0. The lowering of the reactor pH was done to prevent iron-sulfide formation and therefore to be able to selectively recover nickel. In both runs, hydrogen was dosed as electron donor and CO_2 as carbon source at a 90/10 % ratio, with a total flow rate of 12.5 ml min^{-1}. Both runs started with a batch phase without addition of the metals in order to initiate biological sulfate reduction and sulfide build up. The dosing rate of metals and sulfate as well as the applied hydraulic retention time (HRT) are given in Table 1.

Reactor set-up.

A glass gas-lift bioreactor with a liquid volume of 4.0 L was operated at 30˚C by running water through a water jacket that was heated with a Tamson T1000 waterbath (Tamson instruments). The gas-lift bioreactor had an internal and external settler of 0.5 and 4 L, respectively. The external settler was cooled to 4^oC with a Julabo F10 cryostat to minimize bacterial activity in the settler. The pH was measured using a H63 electrode (Schotts Instruments) and controlled with 4 M NaOH and 4 M HCl dosed by a EH Liquisys-P pH controller (Endress Hauser Inc.). The pH system was regularly checked and if necessary recalibrated. The oxidation-reduction potential (ORP) was measured using a QIS electrode (Type O14/NS/12x250/DJ/KNO_3/1M/BNC) on a Radiometer readout unit (Type PHM210, Meterlab).

The metal containing influent was stored in a 100 L container that was continuously sparged with N_2 to maintain anoxic conditions while the Na_2SO_4 stream was stored in a 20 L container and flushed with N_2 at the beginning of the experiment. The metal and Na_2SO_4 media were dosed into the gas-lift bioreactor by a Stepdos 08RC and 03RC liquid membrane pump, respectively (FEM 08TT.18RC, KNF-Verder).

Table 1: Operational parameters of the gas-lift bioreactor during run 1 fed with nickel-sulfate at pH 5.5 and 2 fed with nickel/iron-sulfate at pH 5.0.

Time [d]	Nickel dosing rate [mM d^{-1}]	Iron dosing rate [mM d^{-1}]	Sulfate dosing rate [mM d^{-1}]	HRT [h]
		Run 1		
0	0	0	0	∞
9.0	0.6	0	3.6	189
9.8	1.4	0	4.4	138
10.1	3.4	0	9.4	63
10.9	4.6	0	10.6	53
12.0	1.5	0	7.5	88
13.9	2.8	0	8.7	70
14.8	3.4	0	9.4	63
15.1	4.3	0	10.3	55
15.8	5.2	0	11.2	49
16.8	5.8	0	11.8	46
17.1	6.1	0	12.1	44
17.8	6.7	0	12.7	42
18.1	7.7	0	13.6	38
		Run 2		
0	0	0	0	∞
5.9	0.5	0.5	1.0	98
6.9	1.0	1.0	2.0	49
7.8	2.0	2.0	3.9	24

H_2 and CO_2 were dosed via a Brooks thermal mass flow controller type 5850E with maximum flows of 30 and 7.5 L min^{-1} for H_2 and CO_2, respectively (Brooks instruments) connected to a Brooks control unit (type 5878). The gas phase was recycled through the reactor liquid by a KNF-Verder N840.3FT.18 gas membrane pump (KNF-Verder). To ensure efficient mass transfer of the gases, a teflon sparger with 168 holes of 0.44 mm was placed at the interface between the solution and recycled gas. The gas recycle flow rate was measured using a SHO-rate (Type R2-15-C, Brooks instruments) and corrected to 4 L min^{-1}. All the tubing and connections were made of PTFE (Schott A.G., Mainz, Germany and Serto AG.).

Media.

Defined mineral media in demineralized water were used (all chemicals were of analytical grade and supplied by Merck). All media contained (all g L^{-1}): KH_2PO_4, 0.41; NH_4Cl, 0.3; KCl, 0.37; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.11; $NaHCO_3$, 1; yeast extract, 0.01; and 1 mL L^{-1} of each of an acid and alkaline trace element solution (Stams et al., 1993). The start-up medium contained 10.4 mM Na_2SO_4, the Na_2SO_4

solution contained 33.0 mM of Na_2SO_4, the nickel solution contained 17.0 mM of $NiSO_4$ and the nickel-iron solution contained 2.0 mM of $NiSO_4$ and 2.0 mM of $FeSO_4$.

Inoculum.
The inoculum in both runs consisted of 10 g wet Eerbeek sludge (Indrustie Water Eerbeek, Eerbeek, The Netherlands), 10 g wet Nedalco sludge (Royal Nedalco, Bergen op Zoom, The Netherlands), 10 g wet Emmtec sludge (Emmtec, Emmen, The Netherlands), 100 ml supernatant of Zinifex sludge (Zinifex Budel Zinc, Budel, The Netherlands) and 100 ml of sulfate reducing sludge taken from previous reactor runs at pH 5 (chapter 5). The sludges were crushed with a blender, except for the sludge taken from previous reactor runs.

Physico-chemical analyses.
Total metal concentrations were measured, after dissolving them in 7.5 M HCl/ 2.5 M HNO_3 and subsequently diluted 50x, with inductive coupled plasma optical emission spectrometry (ICP-OES) with a Vista MPX CCD simultaneous ICP-OES (Varian). Aqueous metal concentrations were analysed after filtration (0.45 µm) in the same way for total metals except for the dilution factor of 5x.

Reactor precipitates were dried under N_2 atmosphere at room temperature, and examined for mineralogy and crystallinity by X-ray diffraction (XRD) using a Panalytical X'Pert Pro diffractometer (Panalytical, Almelo, The Netherlands) with nickel-filtered CuKα radiation (tube operating at 40 kV and 40 mA). Crystallographica Search-Match Version 3.0.0.1 peak matching software (Oxford Cryosystems) was used to interpret the diffractograms. The same precipitates were also analyzed using scanning electron microscopy (SEM Type JEOL SEM-6480LV, Jeol) and energy dispersive X-ray analyser (EDX Type Noran system SIX, Thermo Electron Corporation) to examine crystal morphologies and semi-quantitative chemical analysis. The particle size distribution of the precipitates was analyzed with laser scattering image analysis (Coulter laser LS 230, Beckman Coulter).

Sulfate was analyzed with ion-chromatography as described by Sipma et al. (2004). Total sulfide was analyzed in filtrated samples using Dr. Lange sulfide kit LCK-653 and biomass concentration by total–N (nitrogen) determination of washed solids using Dr. Lange total-N kit LCK 238 which were analysed on a Xion 500 spectrophotometer (Hach Lange GMBH). The biomass was calculated using the general formula for biomass composition $CH_{1.8}O_{0.5}N_{0.2}$ (Roels, 1983). Volatile fatty

acids (VFA) were analyzed on a Hewlett Packard series II gas chromatograph (Weijma et al., 2000).

Calculations.

Nickel and iron recovery (or removal efficiency), was calculated from the ICP data using equation 1 and 2:

$$\text{Nickel recovery} = \frac{Ni_{solid}}{Ni_{tot}} \cdot 100\% = \frac{Ni_{solid}}{(Ni_{solid} + Ni_{aq})} \cdot 100\% \quad (1$$

$$\text{Iron recovery} = \frac{Fe_{solid}}{Fe_{tot}} \cdot 100\% = \frac{Fe_{solid}}{(Fe_{solid} + Fe_{aq})} \cdot 100\% \quad (2$$

Metals that passed through a 0.45 µm filter were considered as aqueous metals (Ni_{aq} and Fe_{aq}) and the solid metals (Ni_{solid} and Fe_{solid}) were calculated by subtracting the aqueous metals from the total amount of metal. The nickel and iron recovery was also modelled using a chemical modelling program (OLI stream analyser 2.0, OLI systems Inc.) using the measured pH and total concentration of nickel, iron and sulfide as input.

Results

Nickel-sulfide formation in a nickel-sulfate fed gas-lift bioreactor. The first 9 days of run 1 were operated in batch to allow SRB to adapt to the applied conditions. From day 7 to day 10, the sulfate concentration in the reactor decreased due to sulfate reduction, resulting in accumulation of sulfide in the reactor to 10 mM at day 10 (Fig. 1). The biomass concentration increased between day 7 and 9, from 0.07 to 0.12 g L^{-1} biomass, confirming that the microorganims proliferated in the reactor (Table 2).

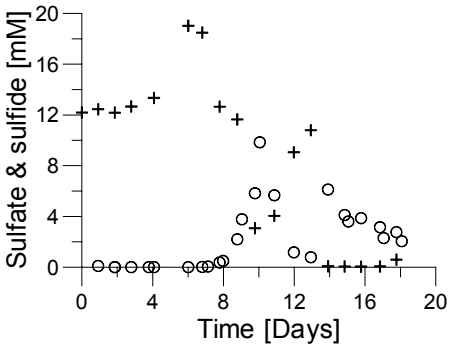

Figure 1: Sulfate (o) and sulfide (+) concentration in the reactor during run 1 fed with nickel-sulfate at pH 5.5 and 30°C.

Table 2: Total-N and biomass concentration in the reactor liquor of run 1 fed with nickel-sulfate at pH 5.5 and 2 fed with nickel/iron-sulfate at pH 5.0.

Time [d]	Total-N [mg L^{-1}]	Biomass [g L^{-1}]
	Run 1	
7	8.22	0.07
9	13.22	0.12
	Run 2	
1	24.6	0.22
5	29.9	0.26
9	9.6	0.08
10	10.0	0.09
11	11.2	0.10

Nickel-sulfate dosing started on day 9 (Table 1) when 4 mM sulfide was present in the reactor. The dosage rate of the nickel-sulfate solution was carefully increased in a stepwise manner (Table 1) to prevent toxic nickel concentrations by an overload of metal compared to sulfide production. The sulfide concentration dropped to 0.8 mM at day 13 due to incomplete sulfate reduction (Fig. 1). The Ni_{aq} concentration increased to 110 µM (Fig. 2a), as a response to the low sulfide concentration. At day 14, sulfate was again completely reduced increasing the sulfide concentration to 6 mM and the Ni_{aq} concentration dropped to 5 µM at day 14 (Fig. 2a). The sulfide concentration slowly decreased to 2 mM at day 18, while sulfate was nearly depleted from day 14 onwards (Fig. 1). The acetate concentration increased to 8 mM at day 10 and was depleted by day13 (data not shown).

The nickel-sulfate loading rate was increased from 0.6 mM d^{-1} at day 9 to 7.7 mM d^{-1} at day 18 (Table 1), resulting in a gradual decrease of the HRT from 189 h to 38 h (Table 1). The Ni_{solid} concentration in the reactor increased slowly to 13 mM at day 18 (Fig. 2a), showing that the internal settler retained the nickel-sulfide precipitate. The Ni_{aq} concentration fluctuated over time with a maximum Ni_{aq} concentration of 110 µM on day 13 (Fig. 2a). The nickel recovery varied between 92.8 % and 98.5 % until day 14, than became stable between 99.6 and > 99.9% (Fig. 2b).

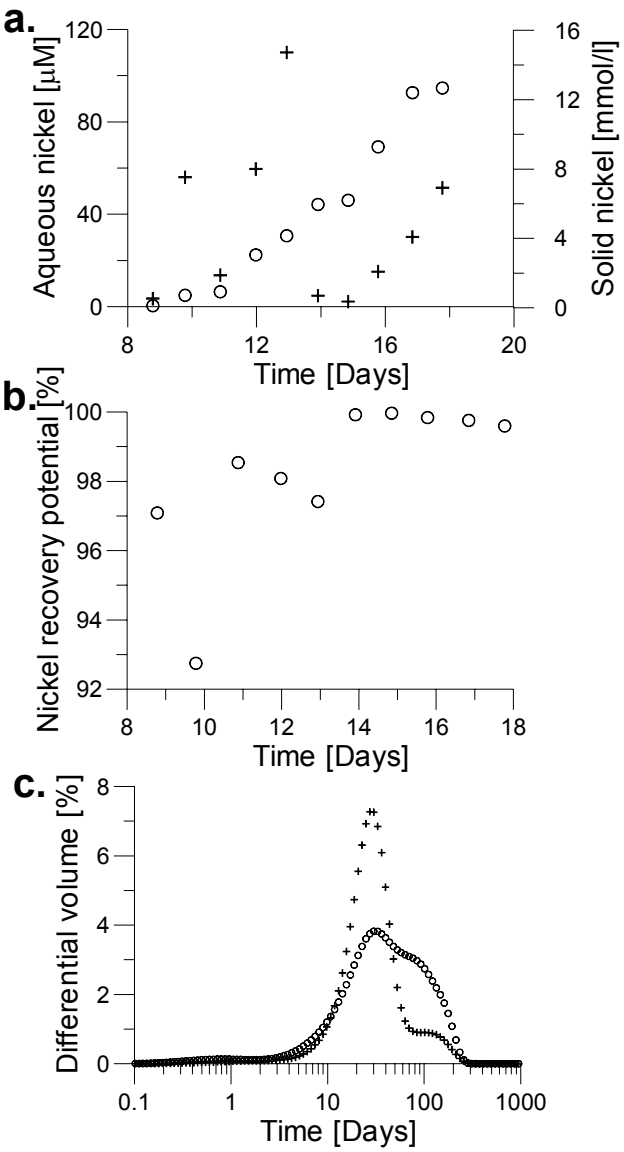

Figure 2: Nickel concentrations in the gas-lift bioreactor during run 1 fed with nickel-sulfate at pH 5.5 and 30°C with a) the concentration of aqueous (+) and solid nickel (o), b) the nickel recovery (o) and c) the particles size distribution of the sludge at day 9 (o) and 12 (+).

Selective recovery of nickel-sulfide from a nickel/iron-sulfate fed gas-lift bioreactor. The second run was operated in batch mode for the first 6 days. The sulfate concentration slowly decreased from day 1 onwards, with a rapid decrease in sulfate concentration from day 4, indicating increased sulfate reduction rate (Fig. 3a). Sulfate reduction resulted in a sulfide concentration of 0.4 - 0.6 mM between day 1 and 4, and to 2.6 mM on day 5, due to the increase in sulfate reduction rate at day 4 (Fig. 3a). The biomass concentration increased slightly from 0.22 to 0.26 g L^{-1} between day 1 and 5, showing there was growth (Table 2).

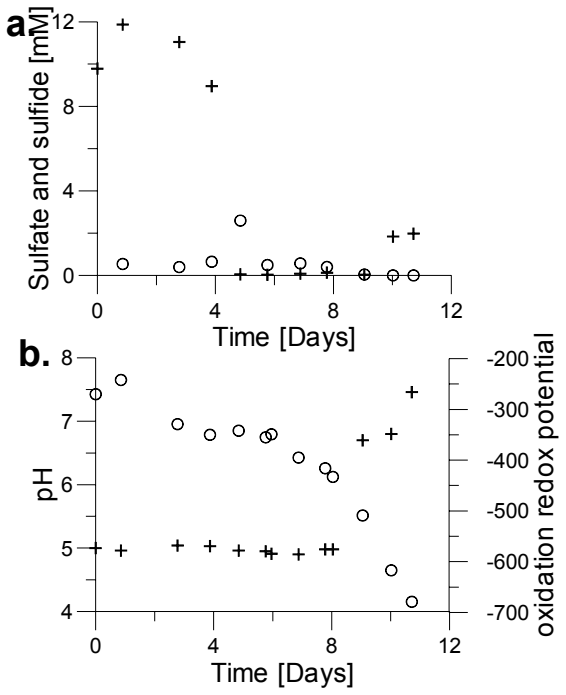

Figure 3: Reactor performance of the gas-lift bioreactor from run 2 fed with nickel/iron-sulfate at pH 5.0 and 30°C with a) sulfate (+) and sulfide (o) concentration in the reactor and b) pH (+) and oxidation-reduction potential (o).

From day 6 onwards, nickel-sulfate and iron-sulfate medium was dosed in a Ni:Fe ratio of 1 (Table 1). All incoming sulfate was converted (Fig. 3a) and nickel reacted with sulfide to form solid nickel-sulfide, while only part of the iron was precipitated as solid iron-sulfide (Fig. 4b). Ni_{solid} and Fe_{solid} accumulated in the reactor and reached 3 mM for both metals at day 11 (Fig. 4b; 5a). The Ni_{aq} and Fe_{aq} concentration were relatively high after the start of the metal dosage at day 7 and 8 (Fig. 4a), with a maximum Ni_{aq} and Fe_{aq} concentration in the reactor of 17 and 699 μM, respectively (day 8; Fig. 4a). The Ni_{aq} and Fe_{aq} concentration have the same trend over time, however, the Fe_{aq} concentration is about 40 times higher than the Ni_{aq} concentration (Fig. 4a). The biomass concentration decreased from 0.26 to 0.08 g L^{-1} between day 5 and 9 (Table 2) due to biomass washout. From day 9, the biomass concentration increased slightly to 0.09 g L^{-1} on day 10 and 0.10 g L^{-1} on day 11 (Table 2). The acetate concentration was low the first 4 days, after which the concentration started to increase reaching a peak concentration of 12 mM at day 7 (data not shown). From day 7 onwards, the acetate concentration decreased again and was depleted from day 11 (data not shown).

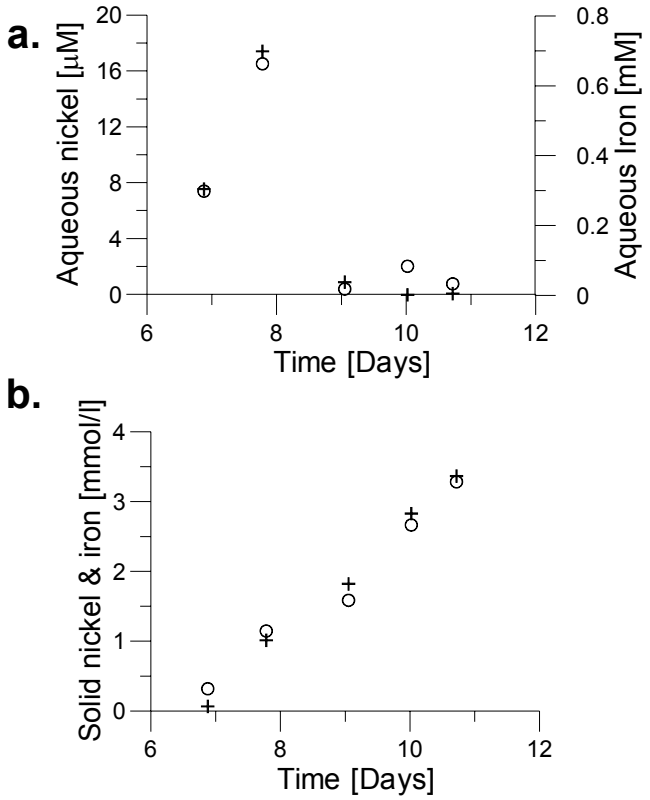

Figure 4: Results of the nickel and iron concentration in run 2 fed with nickel/iron-sulfate at pH 5.0 and 30°C with a) the concentration of aqueous nickel (0) and iron (+) and b) the concentration of solid nickel (o) and iron (+).

The oxidation-reduction potential (ORP) went from –270 mV at startup to -329 mV at day 3, when sulfate reduction had started (Fig. 3b). The ORP decreased when metals were dosed from day 6 gradually to –679 mV at day 11 (Fig 3b). The pH was controlled during the second run between pH 5.0 and 4.9 until day 8. From day 8, metal-sufides formed a coating on the membrane of the pH electrode caused the electrode to malfunction. The pH was measured externally and showed a pH gradient from 4.9 to 7.5 from day 8 to 11 (Fig. 3b), allowing to study the effect of pH on the selective recovery of nickel from a nickel-iron solution.

Metal-precipitate characterisation. The particle size distribution (PSD) of the reactor solids at the start of the metal dosing from run 1 (day 9) shows a mid 80 % PSD (the particles between the d_{10} and the d_{90}) between 10 and 264 µm and had a mode (highest % differential volume) of 32 µm (Fig. 2c). After metals had been dosed for 3 days (day 12), the PSD was narrower with the mid 80 % PSD between 13 and 73 µm and a mode of 30 µm (Fig. 2c).

XRD analyses of the nickel-iron precipitates from the reactor and external settler in run 2 showed that they were partly crystalline. Mineral structure analyses with XRD did suggest the presence of Millerite (NiS), Ni_4S_3, Polydymite (Ni_3S_4) and Mackinawite (FeS). SEM-EDX analyses of run 2 showed that the metal:sulfur ratio was 1.0 (Table 3), indicating the presence NiS and FeS. The oxygen in the precipitates was most likely from biomass, suggesting that the biomass:metal ratio slowly increased over time (Table 3). The metal content of the precipitates in the settler at day 9 in run 2 consisted for 63 % of nickel (Table 3). When the biomass and salts would not be considered, 41 volume % of the precipitates consist of nickel, 24 % of iron and 35% sulfur. Pure nickel sulfide would contain 65 volume % nickel. The precipitates in the settler of day 11 contained 33 volume % of nickel, because all the nickel and iron were recovered at day 10 and 11 (Table 3; Fig. 4). The precipitates in the reactor at day 11 contained 42 volume % nickel, because the pH was lowered to 5.0 at the end of the run, liberating iron from the precipitates.

Table 3: SEM-EDX full spectrum scan results, average the percentage atoms of elements of three measurements with standard deviations

	Settler day 9	Settler day 11	Reactor day 11
Nickel [%]	6.5 ±0.53	8.0 ±0.45	11.9 ±0.20
Iron [%]	3.9 ± 0.31	7.8 ±0.44	6.4 ± 0.21
Sulfur [%]	10.1 ±0.92	15.7 ±1.97	19.1 ± 0.89
Oxygen [%]	6.8 ±1.26	11.0 ±0.50	15.3 ± 0.63
Ratio Oxygen/Metals	0.65	0.70	0.88
Ratio Sulfur/Metals	1.0	1.0	1.0
Ratio nickel/metals	0.63	0.51	0.65

Discussion

This study show that nickel can selectively recovered over iron by using microbial sulfate reduction at low pH. At day 7 of run 2 about 98 % of the nickel and 18 % of the iron were recovered, resulting in a precipitate with a metal content consisted for 83 % of nickel. The results from run 2 shows that iron recovery increases with pH, but nickel recovery is hardly affected by pH (Fig. 5a). A small variation around pH 5.0 gives a big difference in iron recovery. This is in accordance with modelled results of a stream of 1 mM of $NiCl_2$ and $FeCl_2$ and 1.5 mM of Na_2S over a pH gradient (modelled with OLI; Fig. 5b). The modelled results show that above a pH of 5.5 almost all the iron is recovered as FeS, while below a pH of 4.8 almost no iron is recovered as FeS (Fig. 5b). The modelled results show that under the applied conditions, nickel could be selectively recovered over iron at a pH <4.8.

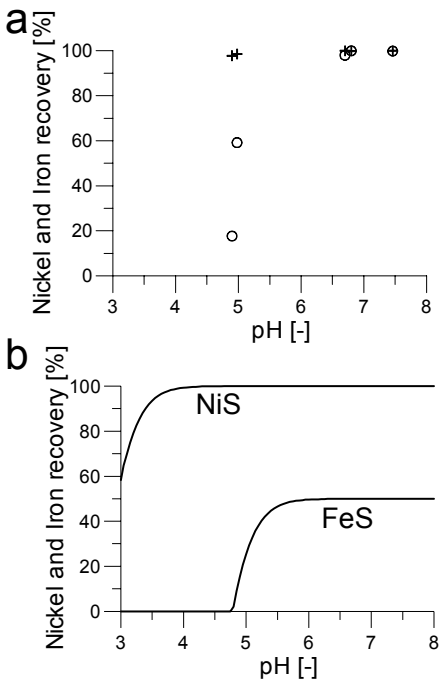

Figure 5: Recovery of nickel (+) and iron (o) with a) measured data and b) modeled data with OLI stream analyser 2.0 (OLI systems Inc.).

Nickel can be precipitated as nickel-sulfide in a single bioreactor at pH 5.5 and 5.0, with nickel recovery >99.9 %. This shows that it is possible to simultaneously reduce sulfate and precipitate nickel, which simplifies the recovery process saving investments cost and making separate sulfide generation redundant. By operating a bioreactor at low pH, the need to add caustic to raise the pH of the reactor liquor will decrease, reducing operational costs. Furthermore, the density of sulfide precipitate is 6 to 10 times higher compared the alternative process hydroxide precipitation (Huisman et al., 2006), decreasing storage and transport expenses. The Ni_{aq} concentration in the reactor went below 0.05 μM (detection limit of the ICP-AES), which is 9 – 400 times lower than hydroxide precipitation at pH 12.5 and 6.7, respectively (Feng et al., 2000).

Ni_{aq} concentrations up to 110 μM did not seem to be toxic to the SRB, which is in accordance with reported EC_{100} (metal concentration at which 100 % of the culture is inhibited) values for Ni_{aq} of 170 μM (Hao et al., 1994; Poulson et al., 1997). Fortin et al. (Fortin et al., 1994) showed that a *Desulfotomaculum* sp. was resistant to 6.1, 7.7 and 9.4 mM nickel in the presence of respectively 0, 1.8 and 3.6 mM iron, suggesting that the nickel toxicity was lower in the presence of iron.

The modelled metal recoveries (calculated with measured data) gave similar results as the measured metal recovery, but the model predicted that the recovery of both metals

should be higher then the measured values (Table 4). This could be due to small metal-sulfide particles which passed through the 0.45 µm membrane and would therefore be considered as Ni_{aq} and unrecoverable. However, the PSD of run 1 at day 12 only 0.2 % of the particles were found to be smaller than 0.45 µm. Nickel could also have formed soluble complexes with sulfide, which are not in the database of the modelling program.

Table 4: The measured and modelled (with OLI) nickel and iron recovery percentages.

Time	pH	Sulfide in reactor liquor	Metal recovery			
			Measured		Modelled	
[days]		[mM]	Ni [%]	Fe [%]	Ni [%]	Fe [%]
7	4.9	0.57	97.7	17.7	>99.9	28.9
8	5.0	0.40	98.6	59.2	>99.9	84.2
9	6.7	0.04	>99.9	98.0	>99.9	99.9
10	6.8	0.00	>99.9	99.9	>99.9	99.7
11	7.5	0.00	>99.9	99.8	>99.9	94.8

Veeken et al. (Veeken et al., 2003b) recovered various metals in a continuous contactor dosing chemically produced Na_2S by controlling the sulfide concentration and pH. A low effluent concentration was reached for most metals, except for nickel. The high Ni_{aq} concentration at a 30 min HRT was ascribed to the low reaction rate of nickel-sulfide formation due to lack of nucleation seeds. In the present study the biomass most probably functioned as nucleation seeds, increasing the reaction rates. Tabak et al. (Tabak et al., 2003) reported a six-stage continuous metal precipitator process fed with AMD from the Berkeley Pit (Butte, Montana, USA) in which metals were precipitated with sulfide produced in a separate bioreactor. Various metals were recovered from the AMD stream, however, nickel was not recovered due to the low concentration.

This study showed that nickel can be selective recovered from a nickel-iron solution at pH 5.0. However, to be able to completely recover nickel selectively from iron the pH should be lower than 4.8. This study can be used to develop new processes for recovery of metals from wastewater in the mining and metallurgical industry.

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8

Concluding remarks and outlook

The thesis aimed at developing processes for selective metal recovery from mixed metal waste and process streams using microbial sulfate reduction under acidic conditions. The development of this process at industrial interesting rates requires knowledge on microbiology, chemistry and process technology (Fig.1).

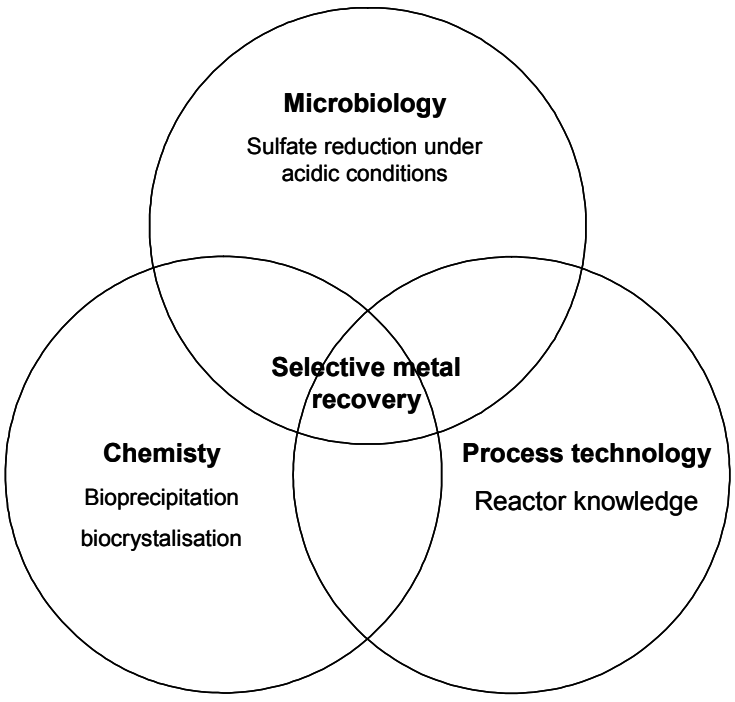

Figure 1: Scientific fields needed to be able to selectively recover metals from mixed metal containing waste and process streams.

1 Sulfate reduction under acidic conditions

1.1 Introduction

Sulfate reduction as low as 3.8-4.0 has been reported (Kimura et al., 2006), however, the achieved rates limit industrial application and growth has not been shown at these low pH values. Studies performed in batch bottles usually rely on phosphate buffering which are weak at low pH resulting in a rapid increase in pH when activity is present. The initial pH could also be increased by addition of biomass, biomass lyses, and excretion of compounds from the biomass. Batch assays become more reliable when the pH is regularly measured and corrected, but that is rarely done. Studies in column experiments usually have an acidic influent but a pH neutral effluent suggesting sulfate reduction is done in the pH neutral part of the column. These systems are usually densely packed and can be considered as plug-flow systems with gradients, suggesting sulfate reduction could take place in pH neutral pockets in the acidic part of the column.

Studies on acidophilic or acidotolerant populations from acid mine drainage exist but studies on enrichment of microorganisms under sulfidogenic acidic conditions are rare (Church et al., 2007; Kimura et al., 2006). Especially from long term bioreactor runs at a controlled pH like in this thesis.

1.2 Knowledge gained on sulfate reduction under acidic conditions

In this study, high rate sulfate reduction has been demonstrated at pH 6.0, 5.0, 4.5 and 4.0 in a membrane bioreactor by using formate and hydrogen as electron donor (Table 1). The activity of the sulfate reducing bacteria (specific activity) was relatively constant from pH 6.0 to 4.0, while the volumetric activity increased with the pH. This was due to the biomass yield on sulfate that increased with the pH. In chapter 4 it was postulated that the low biomass yield on sulfate at low pH was caused by the amount of energy needed to maintain the pH homeostasis instead of being used for growth.

Table 1: Results from the membrane bioreactor runs from this thesis

pH	Temp [^{o}C]	Electron donor	Volumetric activity [mmol L^{-1} SO_4 d^{-1}]	Specific activity [mmol SO_4 $gVSS^{-1}$ d^{-1}]	Biomass yield [gVSS kg^{-1} SO_4]	Chapter
6.0	30	Formate	302	110	38	2
5.0	30	Formate	188	81	29	3
4.5	30	Hydrogen	111	79	14	4
4.0	30	Formate	151	64	-	4
4.0	30	Hydrogen	6	85	0.4	4

The removal of sulfide from the reactor liquor at pH 5.0 had a major impact of the reactor performance and microbial population. In chapter 5 it was demonstrated that when sulfide was not removed from the reactor liquor, sulfate removal was incomplete and the population decreased from 11 to 4 identified species compared to a reactor in which the sulfide was removed from the reactor liquor. In chapter 5, it was postulated that undissociated sulfide that passes through the cell membrane, acidifying the cytoplasm. The excretion of sulfide from the cytoplasm requires energy, thus decreasing the amount of energy available for growth. This inhibition mechanism is similar to the effect of protons as described in chapter 5. This shows that sulfide and protons do not reduce the volumetric activity but increase the maintenance energy, leaving less energy for growth.

Because undissociated acids like acetate are inhibiting at low pH (Reis et al., 1990), they are not suitable as electron donor for sulfate reduction under acidic conditions. Electron donors like ethanol or glycerol are also less suitable as electron donor for sulfate reduction at low pH because incomplete sulfate reducers than produce next to sulfide also acetate. Formate is also inhibiting at low pH, but as shown in chapter 2, 3 and 4 will be converted to hydrogen, which is not inhibiting. The use of hydrogen as electron donor is preferred. CO_2 has previously (Esposito et al., 2003; van Houten et al., 1994; Weijma et al., 2002) and in this study shown to be a good carbon source for hydrogen fed sulfate reducing bioreactors. Part of the CO_2 is most likely converted to acetate that will then be used as carbon source. When the CO_2 concentration was high, excess acetate was produced, but the acetate concentration could be reduced by decreasing the CO_2 concentration (chapter 4).

Sulfidic bioreactors under acidic conditions, showed diverse population of sulfate reducers, acetogens and methanogens, which would be expected from these reactors. But also other populations were present which were most likely fermentative bacteria living on dead biomass or intermediates produced by the other microorganism in the population. The data in chapter 4 suggest that these organisms are all acidotolerant species and not acidophilic.

1.3 Future research in sulfate reduction under acidic conditions.

For future research, it will be interesting to investigate the possibility of sulfate reduction at pH <4.0. This could be investigated with the current population, however, it does not seem likely that acidophilic or extreme acidotolerant SRB are present in the sludge. It would therefore be more interesting to test samples from ecosystems like acid mine drainage sites or acid mine lakes that contain high concentrations of

sulfate and have a low pH in which SRB, which are adapted to these conditions, could be present.

Inocula from other high rate bioreactors could also be tested because these inocula could contain acidophilic or extreme acidotolerant SRB that could have a higher biomass yield at low pH. If these SRB are present, it will be in low concentrations and they need a considerable amount of time to adapt and proliferate. This was also seen in the present study were the lag phase increased when the pH dropped.

During the experiments in this thesis enrichments of acidotolerant SRB, which are active at pH 4.0 were gained. It would be interesting to isolate these SRB to be able to study the effect of process conditions which could help in understanding populations dynamics in bioreactors under acidic conditions. Specifically, it would be interesting to study the effect of sulfide, acetate and protons under defined and sterile conditions. To measure the internal pH of these acidotolerant species could prove valuable.

The membrane bioreactor used in this study was ideal for retaining biomass and therefore high activities could be reached even at pH 4 (chapter 4). The system became however instable due to these high rates. It would be interesting to run a gas-lift bioreactor at low pH conditions with a carrier material to support long sludge retention times. This could enable to run a gas-lift bioreactor at a low hydraulic retention time, but still at a high sulfate reducing rate.

2. Bio-cystallisation of metals

2.1 Introduction.

Bio-crystallisation can be defined as the interplay between the microbiology and the chemical process of crystallisation. In natural ecosystems the metal cycles are controlled by biogeochemical processes. These biogeochemical processes could also be used in controlled bioreactors to recover metals from waste streams. The advantage of using microbiology to produce metal crystals can be to increase the rate of the process, the purity of the crystal or to be able to operate under milder process conditions. The knowledge gained under controlled conditions can be used to explain phenomenon in nature or to develop new processes for metal recovery. Cost could be reduced by using bio-crystallisation to produce metals by reducing the amount of chemicals and energy required. Not only metals but also metalloids can form crystals (Table 2). In indirect bio-crystallisation metals are outside the cell produced as metal-

sulfides. While in direct bio-crystallisation metalloids are precipitated inside the cell and excreted.

Table 2. Examples of metal-crystals divided based on chemical appearance form

Metal-sulfides		Elements	
Antimony	Sb_2S_3	Gold	Au
Arsenic[a]	As_2S_3	Mercury	Hg
Cadmium,	CdS	Palladium	Pd
Chromium[a]	Cr_2S_3	Selenium	Se
Cobalt	CoS	Silver	Ag
Copper	CuS		
Iron	FeS		
Lead	PbS		
Manganese[a]	MnS		
Mercury	HgS		
Molybdenum[a]	MoS_2, Mo_2S_3		
Nickel	NiS		
Scandium	Sc_2S_3		
Tin[a]	SnS, SnS_2		
Titanium[a]	TiS, TiS_2, Ti_2S_3		
Thallium	Tl_2S		
Vanadium[a]	VS_2, V_2S_3		
Zinc	ZnS		

[a] If present in oxidized form, the metal itself is first biologically reduced before precipitation with sulfide

2.2 Knowledge gained on bio-crystallisation of metals in this thesis.

In this thesis simultaneous sulfate reduction and metal precipitation was investigated in a single reactor operated at low pH (chapter 6 and 7). This will save investment costs because a separate contactor for crystallisation would be avoided and the sulfide concentration is evenly disturbed. In a contactor sulfide is injected into the system creating gradients in the sulfide and metal concentrations, while in a single stage bioreactor the sulfide is produced by the SRB present in the reactor functioning as sulfide injection points. This could provide conditions in which the metal-crystals could slowly grow and become crystalline as has been shown by Veeken et al. (Veeken et al., 2003a) for Na_2S and in chapter 6 for biological produced sulfide. The lower the sulfide concentrations the higher the crystal growth rate will be, while a

high sulfide concentration increases the nucleation rate. In chapter 6 it was demonstrated that at low sulfide concentrations larger metal particles are produced which settle better and decrease the cost of dewatering. The bioprecipitation of nickel was studied in chapter 7 with and without the presence of iron in the reactor liquor. The results show that nickel could be precipitated as nickel sulfide at pH 5.0 - 5.5, but was not as crystalline as the zinc-sulfide.

2.3 Future research on metal bio-crystallisation.

There are still a lot of unknowns in the field of bio-crystallisation e.g. the effect biology has on crystallisation, how to influence crystallisation and how to use it. One of the aims in bio-crystallisation is to increase the particle size of crystalline particles to make them easier to dewater which would save cost. Furthermore, how to increase the crystallinity of the metal precipitate.

It is therefore interesting to know what the effect of different process conditions is like pH, temperature and the influence of medium composition is on metal-particles. The products can be characterised based on composition, particle size, homogeneity, morphology and stability. The effect of the biomass or bio-active particles excreted by the cells could also be studied, as well as the effect of turbulence and mixing, which will determine which bioreactor types are suitable to study bio-crystallisation and could be used for recovery of metals. It would also be interesting to control the type of crystal produced, making it more stable or easier to re-use.

3. Selective recovery of metals

3.1 Introduction

Metals can be selectively recovered from mixed metal streams by varying the pH and sulfide concentration which has been demonstrated with sulfide from chemical Na_2S (Veeken et al., 2003b), sulfate reduction (Tabak et al., 2003) and sulfur reduction (Huisman et al., 2006). The precipitation of metals is influenced by the pH and the sulfide concentration.

Figure 2 and 3a show the effect of pH on the recovery percentage of different metals. Copper already precipitates at extreme low pH, while zinc doesn't precipitate until pH 1.3 under the applied conditions. Nickel will start precipitating at higher pH values then zinc, while iron precipites only at values above 4.6.

Figure 3b shows the effect of the sulfide concentration on the nickel recovery percentage. The higher the pH values the lower the pH will be at which nickel is

already precipitated. The recovery curve is also steeper at higher sulfide concentrations.

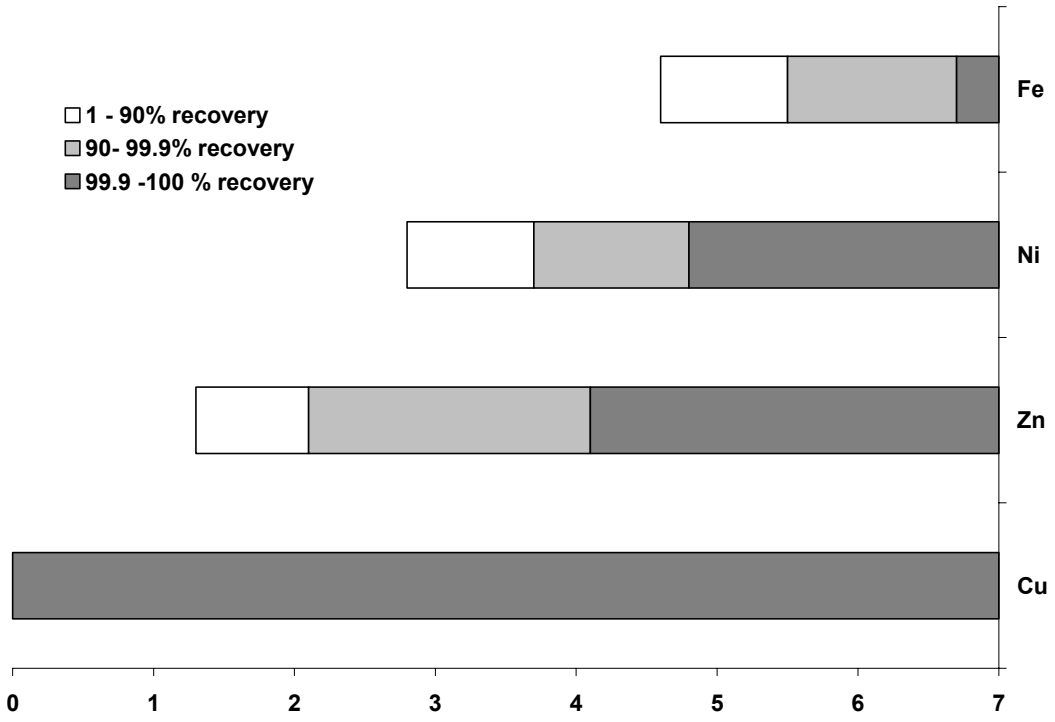

Figure 2: Effect pH on recovery/removal percentage (modeled in OLI stream analyzer 2.0 with 1mM metal; 1mM Na_2S)

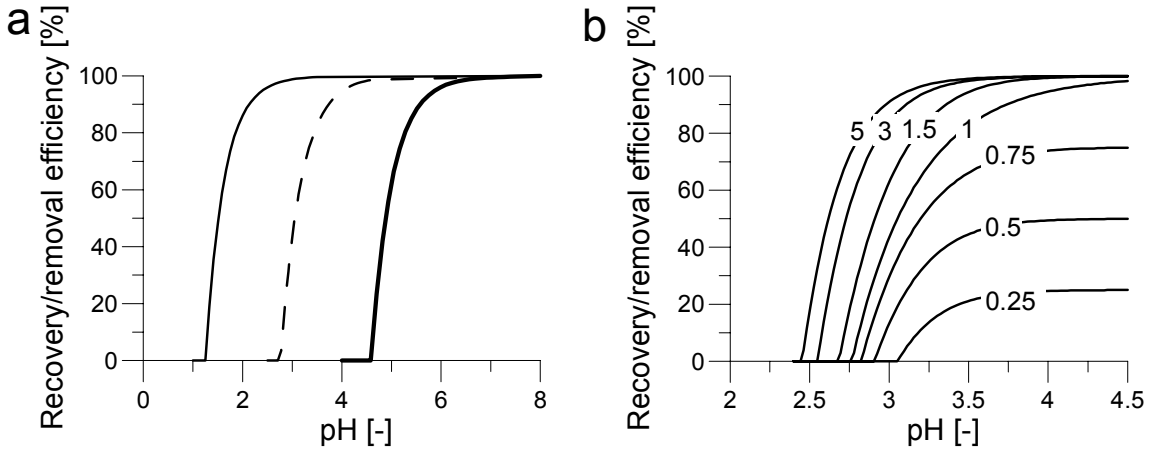

Figure 3. Recovery or removal efficiency versus the pH with a) the effect of pH on zinc (—) nickel (--) and iron (▬)(1 mM of metal; 1mM of sulfide) and b) the effect of various sulfide concentrations (depicted in mM in the graph) on nickel precipitation as nickel-sulfide (1mM nickel; all calculations were modeled in OLI stream analyzer 2.0)

3.2 Selective metal recovery in this thesis.

In chapter 7, a single stage gas-lift bioreactor was fed with a nickel-iron influent to study the selective recovery potential of nickel at pH 5.0. The results show that nickel could be selective recovered from iron, but that a lower pH or sulfide concentration would have resulted in a better separation between nickel and iron.

3.3 Future research on selective recovery of metals.

For selective metal precipitation to be successful in a single stage bioreactor system in which sulfate reduction and metal are simultaneously taken place, the microbial population should be able to stand high concentrations of the metal that will not be precipitated in the first step. It will therefore be important to study the effect of metal-ions on the microbial population. The selection for metal-resistant microorganisms could be part of this.

Next to studying nickel and iron, it will be interesting to study a whole range of metals and combinations. The pH series that is needed for more metals is depended on which metals and what the kinetic properties of those metals is. To be able to separate metals as zinc and copper from nickel the pH of the reactor liquid should be 2.5. This means that sulfate reduction should be achieved at this pH to be able to run a single stage bioreactor system.

4. Final remarks

This thesis showed that acidic sulfate reduction is an interesting tool for the selective recovery of metals from mining and metallurgical waste and process streams. Future research in needed to expend the possibilities shown by this thesis to develop recovery processes for all metals.

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Summary

This thesis aimed at developing processes for selective metal recovery from mixed metal waste and process streams using microbial sulfate reduction under acidic conditions. High rate sulfate reduction has been demonstrated at pH 6.0 (chapter 2), 5.0 (chapter 3), 4.5 and 4.0 (chapter 4) by using formate and hydrogen as electron donor. The specific activity of the sulfate reducing bacteria was relative constant over the whole pH gradient, while the volumetric activity increased with the pH. The microbial population under low pH sulfate reducing conditions was determined at pH 5 (chapter 3), 4.5 and 4.0 (chapter 4), showing a population of acidotolerant sulfate reducing bacteria, methanogens, acetogens and fermentative bacteria were present in the reactors. The removal of sulfide from the reactor liquor at pH 5.0 had a major impact on the reactor performance and microbial population. In chapter 5 it was demonstrated that when sulfide was not removed from the reactor liquor, sulfate removal was incomplete and the population decreased from 11 to 4 identified species compared to a reactor in which the sulfide was removed from the reactor liquor. Simultaneous sulfate reduction and metal precipitation in a single reactor was investigated at low pH. In chapter 6 it was demonstrated that at low pH larger metal-sulfide particles are produced which settle better and decrease the cost of dewatering. In chapter 7, a single stage gas-lift bioreactor was fed with a nickel-iron influent to study the selective recovery potential of nickel at pH 5.0. The results show that nickel could be selective recovered from iron, but that a lower pH or sulfide concentration would have resulted in a better separation between nickel and iron. This thesis showed that acidic sulfate reduction is an interesting tool for the selective recovery of metals from mining and metallurgical waste and process streams.

Samenvatting

Dit proefschrift heeft als doel om processen te ontwikkelen voor selectieve metaal herwinning uit afvalwater and processtromen die meerdere metalen bevatten, door gebruik te maken van sulfaat reductie onder zure omstandigheden. Sulfaat reductie met hoge omzettingssnelheden is gedemonstreerd bij pH 6.0 (hoofdstuk 2), 5.0 (hoofdstuk 3), 4.5 en 4.0 (hoofdstuk 4) door gebruik te maken van formiaat en waterstof als elektron donor. De activiteit van de sulfaat reducerende bacteriën (specifieke activiteit) was relatief constant over de gehele pH gradiënt, terwijl de volumetrische activiteit toeneemt met de pH. De microbiële populatie is bepaald onder lage pH sulfaat reducerend condities bij pH 5.0 (hoofdstuk 3), 4.5 en 4.0 (hoofdstuk 4), waarbij de aanwezigheid van zuur tolerante sulfaat reducerende bacteriën, methaan producerende, acetaat producerende en fermentatieve bacteriën werden gedemonstreerd in de reactor. De verwijdering van sulfide uit de reactor vloeistof bij pH 5.0 had een grote impact op de werking van de reactor en de microbiële populatie. In hoofdstuk 5 werd getoond dat wanneer sulfide niet werd verwijderd uit de reactor vloeistof, sulfaat niet compleet wordt verwijderd en de populatie verkleinde van 11 naar 4 geïdentificeerde species in vergelijking tot een reactor waarbij sulfide wel werd verwijderd uit de reactor vloeistof. Simultane sulfaat reductie en metaal neerslag was onderzocht in een enkele reactor bij lage pH. In hoofdstuk 6 werd getoond dat bij een lage pH grotere metaal-sulfide deeltjes werden gevormd welke beter neerslaan, wat de kosten voor het ontwateren verkleind. In hoofdstuk 7 werd een nikkel en ijzer bevattende oplossing toegevoegd aan een gaslift bioreactor om de selectieve scheiding potentiaal van nikkel bij pH 5.0 te bestuderen. De resultaten laten zien dat nikkel selectief gescheiden kan worden ijzer, maar dat een lagere pH of sulfide concentratie zou leiden tot een nog betere scheiding van nikkel en ijzer. Dit proefschrift laat zien dat sulfaat reductie onder een lage pH interessant is voor de selectieve herwinning van metalen uit afval- en proces water van de mijnbouw en metaalindustrie.

List of scientific publications

M.F.M. Bijmans, R.J.W. Meulepas, P.N.L. Lens, C.J.N Buisman. Sulfate reduction for inorganic water treatment: a review. Submitted for publication

M.F.M. Bijmans, T.W.T. Peeters, P.N.L Lens, C.J.N. Buisman. High rate sulfate reduction at pH 6 in a pH-auxostat submerged membrane bioreactor fed with formate. Water research, In Press, 2008

M.F.M. Bijmans, F. Ennin, M. Dopson, P.N.L. Lens, C.J.N. Buisman. Effect of sulfide removal on sulfate reduction at pH 5 in a hydrogen fed gas-lift bioreactor. Journal of Microbiology and Biotechnology, In Press, 2008

M.F.M. Bijmans, M. Dopson, T.W.T. Peeters, P.N.L. Lens, C.J.N. Buisman. Sulfate reduction at pH 5 in a high-rate membrane bioreactor: Reactor performance and microbial community analyses. Submitted for publication

M.F.M. Bijmans, H.M.T. de Vries, P.N.L. Lens, M. Dopson C.J.N. Buisman. Sulfate reduction at pH 4.0 for treatment of mining and metallurgical streams. In preparation

M.F.M. Bijmans, P.J. van Helvoort, P.N.L. Lens, C.J.N. Buisman. Effect of sulfide concentration on biological and chemical biocrystallisation of zinc. In preparation

M.F.M. Bijmans, P.J. van Helvoort, P.N.L. Lens, C.J.N. Buisman. Bioprecipitation of nickel-sulfide in a sulfidogenic gas-lift bioreactor. Submitted for publication

S.A. Dar, M.F.M. Bijmans, I. Dinkla, B. Geurkink, P.N.L. Lens M. Dopson. Population dynamics of single stage sulfidogenic bioreactors treating synthetic Zinc and Nickel-containing waste streams. In preparation

S. Hoekema, M.F.M Bijmans, M. Janssen, J. Tramper and R. H. Wijffels. A pneumatically agitated flat-panel photobioreactor with gas re-circulation: anaerobic photoheterotrophic cultivation of a purple non-sulfur bacterium. International Journal of Hydrogen Energy, Volume 27, Issues 11-12, 2002, Pages 1331-1338

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Nu ben ik zeker heel veel mensen vergeten. Voor die mensen:

……..	……...
(Vul hier je naam in)	(schrijf hier een dankwoord)

Martijn

Curriculum vitae

Martijn Bijmans was born on the 22nd of May 1978 in Roermond (the Netherlands). He finished the HAVO in Roermond in 1995. From 1995 to 1997 he did the middle laboratory education (MLO) in Eindhoven (The Netherlands) in the field of biology and medical laboratory research, followed by the higher laboratory education (HLO) in Eindhoven until 2002. He then started his university carrier by studying Biotechonology in Wageningen (the Netherlands) from 2002 to 2004 with process technology as specialisation. He started his Ph.D. project on 15 April 2008 at the sub-department of Environmental Technology Wageningen University. The BioMinE project had 38 partners from 15 countries and was financed by the European Union.

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

CERTIFICATE

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE), declares that

Martijn Franciscus Maria Bijmans

Born on: *22 May 1978* at: *Roermond, The Netherlands*

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Place: *Wageningen* Date: *16 May 2008*

the Chairman of the SENSE board	the SENSE Director of Education
Prof. dr. R. Leemans	Dr. C. Kroeze

The SENSE Research School declares that Mr. Martijn Franciscus Maria Bijmans has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 42 ECTS, including the following activities:

SENSE PhD courses:

- **Environmental Research in Context**
- **Research Context Activity: "Review article for a broad audience on Resource Recovery using Biotechnology and organization of Sulfur Days on 2 -3 November 2005, Wageningen, the Netherlands"**
- **Euro Summer school: Closing water and resources cycles: options for gas treatment**
- **Advanced Course on environmental Biotechnology**
- **Biological processes in Environmental Technology**

Other Phd and MSc courses:

- **Biochemical engineering principles**
- **3rd International advanced course on process design and operation**
- **Ore dressing and environmental geochemistry of mine waste management**
- **Course: Oral Presentation**
- **Techniques for writing and presenting scientific papers**
- **Writing a grant proposal**

Management Skills:

- **Head of the environmental safety lab**
- **Organisation of Sulfur symposium**

Oral Presentations:

- **Third European Bioremediation Conference, 4-7 July 2005, Chania, Greece**
- **9th International Mine Water Congress, 5-7 September 2005, Oviedo, Spain**
- **Sulfur symposium, 2-3`November 2005, Wageningen, The Netherlands**
- **IBS 2007, 17th International Biohydrometallurgical symposium, 2-5 September 2007, Frankfurt, Germany**
- **NBC 2008, Biotechnology: crossing borders, 13-14 March 2008, Ede, The Netherlands**
- **SENSE symposium Sensible water technology, 12 -13 April 2007, Leeuwarden, The Netherlands**

Deputy director SENSE
Dr. A. van Dommelen

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