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**Bioreactor Applications Utilizing Mesophilic Sulfate-Reducing
Bacteria for Treatment of Mine Wastewaters at 9–35 °C**



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Reducing Bacteria for Treatment of Mine Wastewaters at
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ABSTRACT

The exploitation of low-grade ores, treatment of wastewaters of mining activities, hydrometallurgical recovery processes and bioremediation of metal contaminated environment require novel and economical bioprocesses. Biotechnology has recently been introduced to mining technology, including for example bioleaching and biological metal recovery processes. The biological processes are a low cost option to traditional mining and metallurgical processes. The exploitation of metals is being focused to low-grade ores and to deposits located at high altitudes and northern regions having demanding environmental conditions. The mining operations are expensive, and introduction of bioprocess technology to mining processes may increase the profits of the operation. On the other hand, the mine wastewaters and metallurgical effluents produced in active mines also at low ambient temperatures have to be treated, and there is limited information on the bioprocess operation at sub-optimal temperatures. Acid mine drainage (AMD) is continuously being produced in old mines, also in those located at cold regions. The quantity of AMD production may be large, although the temperature may affect on the rate of the AMD formation. Heating of a bioreactor and wastewater stream or AMD to the optimal temperature of the biological treatment process may not be feasible, thus the low temperature biological mine wastewater treatment is a compromise between the microbial activity, temperature and reactor size.

Biological sulfate reduction provides simultaneous treatment of the major pollutants of acid mine drainage and mine wastewater: sulfate and metal concentrations are decreased, metals are precipitated as low soluble sulfides and the acidity of the solution is neutralized by biologically generated alkalinity. In a chemical process all these steps would require several unit processes and careful control of pH. The biological mine wastewater treatment with sulfate reduction has several benefits when compared to chemical precipitation with lime: the metal and sulfate concentrations in the biologically treated effluent are lower, and the produced sludge is more stable, dense and has high re-use potential.

The objective of the present study was to develop sulfate reducing bioprocess technology for mine wastewater and AMD treatment. The limiting factors in the use of sulfate reducing bioreactors for mine wastewater treatment can be divided to two categories: 1) the costs of the bioreactor operation due to electron donor and heating and 2) the limitations of the sulfate reducing bacteria (SRB), which do not tolerate high metal concentrations and acidity. Because the tolerance of SRB for mine wastewater treatment can be resolved with reactor technological solutions, e.g. dilution and solution recycling in the process, the aim was to focus on studying the electron donors and activity of SRB at sub-optimal temperatures.

There is limited number of publications describing low temperature sulfate reducing bioprocesses. In present study, a low temperature formate-fed sulfate reducing fluidized-bed bioreactor (FBR) treated synthetic and real mine wastewater at 9°C with stable sulfate reduction rate of 8-14 mmol SO₄²⁻ L⁻¹ d⁻¹, and high metal precipitation, 5.4 mmol Fe L⁻¹ d⁻¹ (99% precipitation), was achieved. The microbial community and the active species of the low temperature sulfidogenic FBR were analyzed with denaturing gel gradient electrophoresis (DGGE). The results showed that this reactor was dominated by a mesophilic SRB *Desulfomicrobium* sp., which was also the active species in the reactor. Therefore, the long-time operation at low temperature resulted in enrichment of psychrotolerant mesophilic SRB. Since formate is not a commercially feasible electron donor, further experiments were made with hydrogen-fed membrane bioreactors (MBR) and gas-lift bioreactors (GLB) at 9°C,

resulting in sulfate reduction rates of 6.9 and 6.2 mmol $\text{SO}_4^{2-} \text{L}^{-1} \text{d}^{-1}$ in these reactors, respectively. The specific sulfidogenic activities in these bioreactors were 1.6-33 mmol $\text{SO}_4^{2-} \text{g VSS}^{-1} \text{d}^{-1}$, demonstrating that high biomass activity can be achieved in low temperature. Sulfate reduction consumed majority of the electrons in these reactors, while acetate production from homoacetogenesis consumed a minor part of the electrons when the temperature was low and the reactor retention time was long. The temperature dependency of the sulfate reduction of the enrichment culture used in the low temperature bioreactor was analyzed, and the optimal temperature was 31°C, demonstrating that this was a psychrotolerant mesophilic enrichment culture. Therefore, the following membrane bioreactor experiments included also operation at 15 and 30-35°C with a reference mesophilic enrichment culture. The operation at these temperatures showed that mesophilic SRB processes can be operated at sub-optimal temperatures, but the activity is decreased by 10-40 % at 15°C when compared to optimal temperature. The activity of SRB at sub-optimal temperature is limited by transport and oxidation rate of the electron donor, because the electron flow to sulfate reduction and specific sulfidogenic activity decrease with the temperature.

Mine wastewater treatment at 35°C was studied using fluidized-bed bioreactor fed with ethanol-lactate mixture. The sulfate reduction rate was high and stable, being 62-100 mmol $\text{SO}_4^{2-} \text{L}^{-1} \text{d}^{-1}$, and the metal precipitation rates were 11 mmol $\text{Fe L}^{-1} \text{d}^{-1}$ (99% precipitation) and 1 mmol $\text{Zn L}^{-1} \text{d}^{-1}$ (99% precipitation). This experiment included also biological hydrogen sulfide production experiment, where sulfide production rate of 73 mmol $\text{H}_2\text{S L}^{-1} \text{d}^{-1}$ was obtained. The sulfate reduction rate in this FBR was limited by the acetate oxidation rate, which was at maximum 50 mmol acetate $\text{L}^{-1} \text{d}^{-1}$. Therefore, the acetate oxidation kinetics of this reactor process was studied, and kinetic constants for acetate oxidation were defined. The K_m , affinity for acetate was 63 μmol , indicating high affinity for acetate. The maximum acetate oxidation rate, V_{max} , was 0.76 $\mu\text{mol g VSS}^{-1} \text{min}^{-1}$. These results demonstrate that although the enrichment of acetate oxidizing SRB is slow. The acetate oxidation rate controls the treatment capacity of the bioreactor fed with an organic electron donor.

Pure electron donors, such as ethanol and hydrogen are expensive. Therefore, low-cost options as electron donors are needed. Therefore, the amenability of reed Canary grass (*Phalaris arundinacea*) plant material hydrolyzate as electron donor for mine wastewater treatment was studied. The experiments were performed with a fluidized-bed bioreactor, and sulfate reduction rate of 21-34 mmol $\text{SO}_4^{2-} \text{L}^{-1} \text{d}^{-1}$ and metal precipitation of was 15 mmol $\text{Fe L}^{-1} \text{d}^{-1}$ (99% precipitation) were achieved, although the acetate oxidation rate limited the process. Also the suitability of the dry reed Canary grass plant material as substrate for sulfate reduction was demonstrated in batch assays with H_2S yield of 0.8 mmol $\text{H}_2\text{S g}^{-1}$ plant material. For comparison, the H_2S yield with the hydrolyzate was 6.2 mmol $\text{H}_2\text{S g}^{-1}$ plant material.

In summary, the experiments conducted for this thesis increased the knowledge on the achievable sulfate reduction rates and treatment capacity of mesophilic SRB at sub-optimal temperatures with several bioreactor types. It was demonstrated that mesophilic SRB could be enriched and long-time maintained in active state at low temperature sulfidogenic bioreactors. The microbiology and metabolic capacities of mesophilic SRB at decreased temperatures were studied. The suitability of sulfidogenic fluidized-bed bioreactors for mine wastewater treatment and biological hydrogen sulfide production was demonstrated with a number of electron donors, including also a potential low-cost electron donor, the plant material hydrolyzate.

TIIVISTELMÄ

Köyhien malmien hyödyntäminen, kaivostoiminnan jätevesien käsittely, hydrometallurginen metallien talteenotto sekä metalleilla saastuneen ympäristön kunnostus vaativat uusia ja taloudellisesti edullisia bioprosesseja. Biotekniikkaa on hyödynnetty kaivosalalla esimerkiksi bioliuotuksessa ja metallien talteenottoprosesseissa. Biologiset yksikköprosessit ovatkin edullisuutensa vuoksi kilpailukykyinen vaihtoehto perinteisille metallurgisille prosesseille. Malmien etsintää laajennetaan jatkuvasti köyhempiin malmeihin, jotka sijaitsevat vaativissa ympäristöolosuhteissa esimerkiksi korkealla vuoristossa tai kylmillä seuduilla. Kaivostoiminta on kallista, joten bioprosessitekniikkaa hyödyntämällä voidaan kasvattaa köyhän malmin prosessoinnista saatavaa tuottoa. Käsittelyä vaativia kaivosjätevesiä muodostuu aktiivisesti toimivissa sekä suljetuissa kaivoksissa jatkuvasti myös kylmissä olosuhteissa. Kylmiä kaivosjätevesiä käsittelevistä bioprosesseista on tällä hetkellä vain vähän tietoa. Aktiivisesti toimivissa kaivoksissa jäteveettä syntyy jatkuvasti suuria määriä, mutta alhainen lämpötila voi vaikuttaa jossain määrin kaivosjätevesien muodostumisnopeuteen jo suljetuissa kaivoksissa. Kaivosjätevesiä käsittelevän bioreaktorin ja kaivosjäteveden lämmittäminen biologiselle käsittelyprosessille optimaaliseen lämpötilaan ei ole taloudellista, joten kylmässä lämpötilassa tapahtuva biologinen kaivosjätevesien käsittelyprosessi on aina kompromissi mikrobien aktiivisuuden, lämpötilan ja bioreaktorin koon välillä.

Biologinen sulfaatinpelkistys mahdollistaa kaivosjätevesien tärkeimpien ympäristöä kuormittavien komponenttien samanaikaisen käsittelyn: sulfaatti- ja metallipitoisuudet laskevat, metallit saostuvat niukkaliukoisiksi sulfideiksi ja hapan liuos neutraloituu biologisesti tuotetulla alkaliniteetilla. Kemiallisessa prosessissa kaikkien näiden vaiheiden toteuttaminen vaatii useita osaprosesseja sekä tarkkaa pH:n säätöä. Biologisella kaivosjätevesien käsittelyllä sulfaatinpelkistäjäbakteerien avulla on useita etuja kemialliseen kalkkisaostukseen verrattuna: biologisella käsittelyllä saavutetaan alhaisemmat metalli- ja sulfaattipitoisuudet, sekä syntyvä metallisulfidisakka on stabiilimpaa, paremmin laskeutuvaa ja sitä voidaan hyödyntää ottamalla talteen metalleja sakasta.

Tämän väitöstyön tavoitteena oli kehittää biologiseen sulfaatin pelkistykseen perustuvia bioprosessitekniisiä ratkaisuja kaivosjätevesien käsittelyyn. Tällä hetkellä sulfaattia pelkistävien bioreaktorien hyödyntämistä rajoittavat tekijät voidaan jakaa kahteen kategoriaan: 1) reaktorin käytöstä aiheutuvat kustannukset, mukaan lukien elektronidonori ja lämmitys sekä 2) sulfaattia pelkistävien bakteerien rajallinen sietokyky käsiteltävän jäteveden sisältämille suurille metallipitoisuuksille ja happamuudelle. Koska bakteerien sietokykyyn liittyvät rajoitteet voidaan ratkaista käyttämällä oikeanlaista reaktoriteknologiaa, kuten prosessien sisäisiä liuoskiertoja ja laimentamista, keskityttiin tässä väitöstyössä tutkimaan elektronidonoreita ja sulfaattia pelkistävien bakteerien aktiivisuutta alhaisissa lämpötiloissa.

Kylmässä toimivista sulfaattia pelkistävästä bioreaktoreista on vain vähän tutkimustietoa. Kokeissa osoitettiin, että formaattia käyttävä leijupetireaktori (FBR) käsitteli synteettistä ja oikeaa kaivosjäteveettä 9°C lämpötilassa vakaalla sulfaatinpelkistysnopeudella (8-14 mmol SO₄²⁻ L⁻¹ d⁻¹) ja suurella metallien saostusnopeudella (5.4 mmol Fe L⁻¹ d⁻¹, 99 % saostui). Tämän reaktorin mikrobiyhteisö ja aktiiviset mikrobilajit analysoitiin käyttäen denaturoivaa geeligradienttielektroforeesia (DGGE). Analyysitulokset osoittivat, että kylmässä toimivaa bioreaktoria hallitsi mesofiilinen *Desulfomicrobium*-laji, joka oli myös reaktorissa aktiivisesti toimiva laji. Nämä tulokset osoittivat, että pitkäaikainen toiminta kylmässä lämpötilassa rikasti bioreaktoriin kylmää sietäviä mesofiilisiä sulfaatinpelkistäjä-bakteereita.

Kylmän lämpötilan bioreaktori jatko-kokeet suoritettiin 9°C lämpötilassa toimivilla vetyä käyttävillä membraanibioreaktoreilla (MBR) ja kaasunosteisella bioreaktorilla (GLB), koska edellisessä kokeessa käytetty formaatti ei ole teolliseen mittakaavaan soveltuva elektronidonori korkean hintansa vuoksi. Membraani- ja kaasunosteisilla reaktoreilla saavutettiin sulfaatinpelkistysnopeudet 6.9 (MBR) ja 6.2 mmol SO₄²⁻ L⁻¹ d⁻¹ (GLB). Kylmässä lämpötilassa toimivien bioreaktoreiden spesifinen sulfaatinpelkistys aktiivisuus oli 1.6-33 mmol SO₄²⁻ g VSS⁻¹ d⁻¹, mikä osoitti, että suuri aktiivisuus voidaan saavuttaa myös alhaisessa lämpötilassa. Sulfaatinpelkistys kulutti pääosan (50-85 %) elektroneista näissä reaktoreissa, mutta asetaatin tuotanto homoasetogeneesissa prosesseissa kulutti osan elektroneista, kun lämpötila oli alhainen ja reaktorin viipymä pitkä. Tutkittaessa 9°C lämpötilassa toimineen bioreaktorin mikrobiviljelmän sulfaatin pelkistykseen lämpötilariippuvuutta, havaittiin, että mikrobiviljelmän optimilämpötila oli 31°C. Tämän vuoksi membraanibioreaktoreilla toteutettiin kokeita myös 15-35°C lämpötiloissa käyttäen referenssinä toista mesofiilistä mikrobiviljelmää. Näissä kokeissa osoitettiin, että mesofiilisiä sulfaatinpelkistäjäbakteereita voidaan käyttää myös optimilämpötilaa alhaisemmissa lämpötiloissa, mutta biomassan aktiivisuus on noin 10-40 % alhaisempi 15°C lämpötilassa verrattuna 30-35°C:n optimilämpötilaan. Kokeissa elektronivirta sulfaatinpelkistykseen sekä spesifinen sulfaatinpelkistysnopeus laskivat, kun reaktorin lämpötilaa laskettiin. Sulfaattia pelkistävien bakteerien aktiivisuutta optimilämpötilan alapuolella rajoitti täten niiden kyky kuljettaa ja hapettaa elektronidonoria.

Kaivosjätevesien käsittelyä tutkittiin myös leijupetireaktorilla 35°C:n lämpötilassa käyttäen etanoli-laktaattiseosta. Sulfaatinpelkistysnopeus oli tässä kokeessa 62-100 mmol SO₄²⁻ L⁻¹ d⁻¹ ja metallien saostusnopeudet 11 mmol Fe L⁻¹ d⁻¹ ja 1 mmol Fe L⁻¹ d⁻¹ (99% saostusteholla). Tämä koejakso sisälsi myös biologisen rikkivedyn tuotannon osuuden, jossa saavutettiin 73 mmol H₂S L⁻¹ d⁻¹ sulfidin tuottonopeus. Sulfaatinpelkistysnopeutta näissä kokeissa rajoitti asetaatin hapetusnopeus, vaikka hapetusnopeus oli suurimmillaan 50 mmol asetaattia L⁻¹ d⁻¹. Tämän vuoksi leijupetireaktorin biomassan asetaatin hapetuskinetiikka tutkittiin määrittämällä kineettiset vakiot asetaatin hapetukselle. Sulfaattia pelkistävän biomassan affiniteetti asetaatille (K_m) leijupetireaktorissa oli 63 μmol, mikä kuvaa biomassan kykyä käyttää asetaattia alhaisissa pitoisuuksissa. Asetaatin maksimihapetusnopeudeksi (V_{max}) määritettiin 0.76 μmol g VSS⁻¹ min⁻¹. Nämä tulokset osoittavat asetaattia hapettavien sulfaatinpelkistäjäbakteerien rikastamisen reaktoriolosuhteissa olevan hidas prosessi. Asetaatin hapetuskapasiteetti kontrolloi orgaanista elektronidonoria käyttävien sulfaattia pelkistävien bioreaktorien käsittelyprosessin kokonaiskapasiteettia.

Edullisia ja vaihtoehtoisia elektronidonoreita sulfaatinpelkistäjäbakteereille tarvitaan, koska etanoli ja vety ovat kemikaaleina kalliita. Tämän vuoksi vaihtoehtoisena elektronidonorina ruokohelven (*Phalaris arundinacea*) kasvimateriaalihydrolysaatin käyttöä kaivosjätevesien käsittelyyn. Leijupetireaktorilla tehdyssä kokeessa saavutettiin korkea sulfaatinpelkistysnopeus (21-34 mmol SO₄²⁻ L⁻¹ d⁻¹) sekä metallien saostus (15 mmol Fe L⁻¹ d⁻¹, 99% saostusteho). Asetaatin hapetusnopeus rajoitti tätä reaktoriprosessia. Lisäksi testattiin kuivan ruokohelvi kasvimateriaalin soveltuvuus substraatiksi, ja rikkivetysaanto oli 0.8 mol H₂S g⁻¹ kasvimateriaalia, kun hydrolysaatille vastaava arvo oli 6.2 mol H₂S g⁻¹ kasvimateriaalia.

Yhteenvedon voidaan todeta, että tämän väitöstyön tutkimustulokset tuottivat uutta tietoa mesofiilisten sulfaatinpelkistäjä-bakteerien sulfaatinpelkistysnopeuksista ja biologisesta jätevesien käsittelykapasiteeteista optimilämpötilan alapuolella. Koetulokset osoittivat, että mesofiilisiä sulfaatinpelkistäjäbakteereita voidaan rikastaa ja säilyttää aktiivisesti toimivina pitkiä ajanjaksoja alhaisissa lämpötiloissa toimivissa bioreaktoreissa. Lisäksi tutkittiin

mesofiilisten sulfaatinpelkistäjä-bakteerien mikrobiologiaa ja metabolista kapasiteettia alhaisessa lämpötilassa. Leijupetireaktoreiden soveltuvuus biologiseen kaivosvesien käsittelyyn sekä biologiseen rikkivedyn tuotantoon osoitettiin käyttäen useita elektronidonoreita, joista yksi oli myös edullinen sekä potentiaalinen kasvimateriaalihydrolysaatti.

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LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers, and referred to in this thesis by the roman numerals

- I Auvinen H, Nevatalo LM, Kaksonen AH, Puhakka JA. 2009. Low temperature (9°C) AMD treatment in a sulfidogenic bioreactor dominated by mesophilic *Desulfomicrobium* species. *Biotechnology and Bioengineering* 104(4): 740-751. Copyright: Wiley-Liss Inc. a subsidiary of John Wiley & Sons, Inc.
- II Nevatalo LM, Bijmans MFM, Lens PNL, Kaksonen AH, Puhakka JA. 2010. The effect of sub-optimal temperature on specific sulfidogenic activity of mesophilic SRB in a H₂-fed membrane bioreactor. *Process Biochemistry* 45: 363-368 Copyright: Elsevier B.V.
- III Nevatalo LM, Bijmans MFM, Lens PNL, Kaksonen AH, Puhakka JA. 2010. Hydrogenotrophic sulfate reduction in a gas-lift bioreactor operated at 9°C. *Journal of Microbiology and Biotechnology* 20(3): 615-621. Copyright: The Korean Society for Microbiology and Biotechnology.
- IV Nevatalo LM, Mäkinen AE, Kaksonen AH, Puhakka JA. 2010. Biological hydrogen sulfide production in an ethanol-lactate fed fluidized-bed bioreactor. *Bioresource Technology* 101 (1): 276-284. Copyright: Elsevier B.V.
- V Lakaniemi A-M, Nevatalo LM, Kaksonen AH, Puhakka JA. 2010. Mine wastewater treatment using *Phalaris Arundinacea* plant material hydrolyzate as substrate for sulfate-reducing bioreactor. *Bioresource Technology* 101 (11): 3931-3939. Copyright: Elsevier B.V.

THE AUTHORS CONTRIBUTION

Paper I: Hannele Auvinen performed the experimental work. Laura Nevatalo planned the experiment, wrote the paper and is the corresponding author. She participated in the experimental work and interpreted the results.

Paper II: Laura Nevatalo performed the experimental work, wrote the paper and is the corresponding author. The experiments were planned by Laura Nevatalo, Martijn Bijmans and Piet Lens.

Paper III: Laura Nevatalo performed the experimental work, wrote the paper and is the corresponding author. The experiment was planned by Laura Nevatalo, Martijn Bijmans and Piet Lens.

Paper IV: Laura Nevatalo and Annukka Mäkinen performed the experimental work. Laura Nevatalo planned the experiment, wrote the paper and is the corresponding author.

Paper V: Aino-Maija Lakaniemi performed the experimental work. Laura Nevatalo planned the experiment, wrote the paper and is the corresponding author. She participated in the experimental work and interpreted the results.

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ABBREVIATIONS

Ac	Acetate
Acetyl-CoA	Acetyl-CoenzymeA
AMD	Acid mine drainage
APS	Adenosine-5'-phosphosulfate
ARD	Acid rock drainage
ATP	Adenosine triphosphate
CAP	Cold acclimation proteins
CIP	Cold inducible protein
COD	Chemical oxygen demand
COD _s	Chemical oxygen demand of soluble compounds
CSP	Cold shock protein
DGGE	Denaturing gel gradient electrophoresis
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
DOC	Dissolved organic carbon
DS	Dissolved sulfide
<i>dsr</i>	Sulfite reductase gene
<i>dsrB</i>	Sulfite reductase gene (β -subunit)
EGSB	Expanded granular sludge blanket reactor
FBR	Fluidized-bed bioreactor
GLB	Gas-lift bioreactor
HRT	Hydraulic retention time
K _m	Michaelis constant (affinity towards substrate)
K _s	Monod saturation constant (affinity constant for growth)
MBR	Membrane bioreactor
ORP	Oxidation or reduction potential
PCR	Polymerase chain reaction
Q	Flow rate
RCGH	Reed Canary grass hydrolyzate
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SBR	Sequencing batch reactor
SRB	Sulfate reducing bacteria
SRP	Sulfate reducing prokaryotes
SRR	Sulfate reduction rate
SSA	Specific sulfidogenic activity
STR	Stirred tank reactor
T _d	Biomass doubling time
T _{opt}	Optimal growth temperature
TSS	Total suspended solids
TS	Total solids
UASB	Up-flow anaerobic sludge blanket reactor
VFA	Volatile fatty acids
V _{max}	Maximum specific (oxidation) rate
VS	Volatile solids
VSS	Volatile suspended solids
Y	Growth yield

1 Microbiology of sulfate reducing prokaryotes

Sulfate and metal containing wastewaters originating from the mining and metallurgical industries are a major source of pollution (Gazea et al. 1996, Johnson and Hallberg 2005). The formation of acid mine drainage (AMD) as result of exposure of pyrite (FeS_2) to water and oxygen, followed by biological iron oxidation by iron oxidizing bacteria and leaching of other sulfidic minerals have been reviewed by Johnson and Hallberg (2005). As the prevention of AMD formation is difficult (Johnson and Hallberg 2005), the focus should be on AMD control and treatment. Generation of AMD will continue several decades after the mine has been closed, and, therefore, the treatment process has to sustain long time operation (Gazea et al. 1996). Sulfate, one of the main components of AMD, is non-toxic, stable in the environment and chemically inert (Widdel 1988). Besides sulfate, AMD is acidic and contains several heavy metals (Tabak et al. 2003). Sulfate discharge unbalances the natural sulfur cycle, and sulfide produced from sulfate has major toxic effects in the environment (van Houten 2006). Chemical precipitation of AMD with lime removes sulfate as gypsum and metals are precipitated as hydroxides (Gazea et al. 1996), but this process will result in a residual concentration of sulfate due to the high solubility of gypsum, and the metal hydroxide sludge has no re-use potential and requires safe disposal (Huisman et al. 2006, Lopez et al. 2009). However, AMD and mine wastewaters can be treated using sulfate-reducing prokaryotes (SRP), which reduce sulfate to sulfide requiring a suitable electron donor. The sulfide produced by SRP precipitates soluble metals as their sulfides (Johnson 2000). Simultaneously the solution pH increases due to alkalinity production from sulfidogenic electron donor oxidation, while the metal sulfide formation produces also some acidity (Johnson 2000, Kaksonen et al. 2003). The applications utilizing SRP for the treatment of AMD and mine wastewaters include active systems (e.g. bioreactors) and passive systems, such as passive barriers and wetlands (Johnson and Hallberg 2005).

SRP are anaerobic bacteria and archaea that use sulfate as electron acceptor in energy yielding dissimilatory sulfate reduction (Widdel and Pfennig 1984, Rabus et al. 2006). SRP are also able to reduce other oxidized sulfur compounds (Widdel 1988). The SRP consist of 220 species in 60 genera (Barton and Fauque 2009). The SRP are classified according to optimum growth temperature, capability to oxidize acetate, the DNA GC content, cell shape, motility and presence of desulfoviridin and cytochromes (Widdel 1988). Natural environments for SRP are marine and freshwater sediments and the intestine of animals and man (Widdel and Pfennig 1984), but SRP also exist in industrial systems, such as anaerobic digesters, oil fields and paper factories (Rabus et al. 2006) and even in the harsh conditions of deep-sea hydrothermal vents (Jeanthon et al. 2002).

1.1 Phylogeny

Sulfate reducing archaea and bacteria are divided in six different classes: *Archaeoglobi*, *Thermoprotei*, *Thermodesulfobacteria*, *Nitrospira*, *Deltaproteobacteria* and *Clostridia* (Garrity et al. 2004). The majority of the described species are bacteria, and the few known archaea that reduce sulfate and belong to the hyperthermophilic genera *Archaeoglobus* and *Crenarchaeota* (Itoh et al. 1998, Itoh et al. 1999, Rabus et al. 2006). The sulfate reducing archaeal and bacterial genera are listed in Table 1.

Table 1. The sulfate reducing archaea and bacteria genera.

Phylum	Class	Family	Genus	Reference
AI <i>Crenarchaeota</i>	<i>Thermoprotei</i>	<i>Thermoproteaceae</i>	<i>Caldivirga</i>	Itoh et al. (1999)
			<i>Thermocladium</i>	Itoh et al. (1998)
AI <i>Euryarchaeota</i>	<i>Archaeoglobi</i>	<i>Archaeoglobaceae</i>	<i>Archaeoglobus</i>	Stetter (1988)
BIII <i>Thermodesulfobacteria</i>	<i>Thermodesulfobacteria</i>	<i>Thermodesulfobacteriaceae</i>	<i>Thermodesulfobacterium</i>	Jeanthon et al. (2002)
			<i>Thermodesulfator</i>	Moussard et al. (2004)
BVIII <i>Nitrospira</i>	<i>Nitrospira</i>	<i>Nitrospiraceae</i>	<i>Thermodesulfovibrio</i>	Henry et al. (1994)
BXII <i>Proteobacteria</i>	<i>Deltaproteobacteria</i>	<i>Desulfobacteraceae</i>	<i>Desulfobacter</i>	Widdel (1981)
			<i>Desulfatibacillum</i>	Cravo-Laureau et al. (2004)
			<i>Desulfatiferula</i>	Cravo-Laureau et al. (2007)
			<i>Desulfatirhabdium</i>	Balk et al. (2008)
			<i>Desulfobacterium</i>	Bak and Widdel (1986)
			<i>Desulfobacula</i>	Rabus et al. (1993)
			<i>Desulfobotulus</i>	Kuever et al. (2005)
			<i>Desulfocella</i>	Brant et al. (1999)
			<i>Desulfococcus</i>	Widdel (1981)
			<i>Desulfofaba</i>	Knoblauch et al. (1999)
			<i>Desulfofrigus</i>	Knoblauch et al. (1999)
			<i>Desulfoluna</i>	Suzuki et al. (2008)
			<i>Desulfonema</i>	Widdel (1981)
			<i>Desulfosarcina</i>	Widdel (1981)
			<i>Desulfospira</i>	Finster et al. (1997)
			<i>Desulfotignum</i>	Kuever et al. (2001)
		<i>Desulfobulbaceae</i>	<i>Desulfobulbus</i>	Widdel (1981)
			<i>Desulfocapsa</i>	Janssen et al. (1996)
			<i>Desulfofustis</i>	Friedrich et al. (1996)
			<i>Desulfopila</i>	Suzuki et al. (2007)
			<i>Desulforhopalus</i>	Isaksen and Teske (1996)
			<i>Desulfotalea</i>	Knoblauch et al. (1999)
			<i>Desulfurivibrio</i>	Sorokin et al. (2008b)
		<i>Desulfofarculaceae</i>	<i>Desulfofarculus</i>	Kuever et al. (2005)
		<i>Desulfohalobiaceae</i>	<i>Desulfohalobium</i>	Ollivier et al. (1991)
			<i>Desulfonatronospira</i>	Sorokin et al. (2008a)
			<i>Desulfonatronovibrio</i>	Zhilina et al. (1997)
			<i>Desulfonauticus</i>	Audriffin et al. (2003)
			<i>Desulfothermus</i>	Kuever et al. (2005)
			<i>Desulfovermiculus</i>	Belyakova et al. (2006)
		<i>Desulfomicrobiaceae</i>	<i>Desulfomicrobium</i>	Rozanova et al. (1988)
		<i>Desulfonatronumaceae</i>	<i>Desulfonatrorum</i>	Pikuta et al. (1998)
		<i>Desulfovibrionaceae</i>	<i>Desulfovibrio</i>	Kluyver and van Niel (1936)
		<i>Synthrophobacteraceae</i>	<i>Desulfacinum</i>	Sievert and Kuever (2000)
			<i>Desulfoglaeba</i>	Davidova et al. (2006)
			<i>Desulforhabdus</i>	Oude Elferink et al. (1995)
			<i>Desulfovirga</i>	Tanaka et al. (2000)
			<i>Synthrobacter</i>	Chen et al. (2005)
			<i>Thermodesulforhabdus</i>	Beeder et al. (1996)
		<i>Syntrophaceae</i>	<i>Desulfobacca</i>	Oude Elferink et al. (1999)
			<i>Desulfomonile</i>	De Weerd et al. (1990)
BXIII <i>Firmicutes</i>	<i>Clostridia</i>	<i>Peptococcaceae</i>	<i>Desulfosporosinus</i>	Stackenbrand et al. (1997)
			<i>Desulfirospora</i>	Kaksonen et al. (2007a)
			<i>Desulfotomaculum</i>	Campbell and Postgate (1965)
		<i>Thermoanaerobacteraceae</i>	<i>Desulfovirgula</i>	Kaksonen et al. (2007b)
		<i>Thermodesulfobiaceae</i>	<i>Thermodesulfobium</i>	Mori et al. (2003)

Most of the SRP are mesophilic, but thermophilic, hyperthermophilic and psychrophilic species have also been described (Stetter 1988, Henry et al. 1994, Knoblauch et al. 1999, Jeanthon et al. 2002, Moussard et al. 2004). Several mesophilic sulfate-reducing bacteria (SRB) have been characterized as psychrotolerant. The only hyperthermophilic sulfate reducing species are archaea belonging to the genus *Archaeoglobus* (Stetter 1988) and *Crenarchaeota* (Itoh et al. 1998, Itoh et al. 1999). The characteristics of the psychrotolerant and psychrophilic SRP and the effects of temperature decrease are described more in detail in Chapter 2. A one important classification criterion for SRP is the ability to oxidize acetate: the complete oxidizers are able to oxidize acetate to CO₂, but the incomplete oxidizers lack the enzymes for acetate oxidation (Widdel and Pfenning 1984, Schauder et al. 1986). Optimal

growth temperatures of selected SRP species and their ability to oxidize acetate are listed in Table 2.

Table 2. The optimal growth temperature (T_{opt}) of selected SRP species and their ability to oxidize acetate: complete oxidizers are labelled with (+) and incomplete oxidizers with (-).

Species	T_{opt} (°C)	Acetate oxidation	Reference
Gram-negative mesophilic SRP			
<i>Desulfobulbus</i>	28-39	-	Widdel and Pfenning (1984)
<i>Desulfomicrobium</i>	25 - 40	-	Castro et al. (2000)
<i>Desulfovibrio</i>	25 - 40	-	Widdel and Pfenning (1984)
<i>Desulfobacter</i>	28-32	+	Widdel and Pfenning (1984)
<i>Desulfobacterium</i>	20 - 35	+	Castro et al. (2000)
<i>Desulfococcus</i>	15 - 36	+	Widdel and Pfenning (1984)
<i>Desulfomonile</i>	37	+	Castro et al. (2000)
<i>Desulfonema</i>	28 - 32	+	Castro et al. (2000)
<i>Desulfosarcina</i>	28-33	+	Widdel and Pfenning (1984)
Gram-positive sporulating SRP			
<i>Desulfotomaculum</i>	25 - 65	+/-	Castro et al. 2000
Psychrotolerant mesophilic SRP			
<i>Desulforhopalus vacuolatus</i>	18 - 19	-	Isaksen and Teske (1996)
<i>Desulfofrigus fragile</i>	18	-	Knoblauch et al. (1999)
<i>Desulfotalea arctica</i>	18	-	Knoblauch et al. (1999)
<i>Desulfovibrio ferrireducens</i>	23	-	Vandieken et al. (2006b)
<i>Desulfovibrio frigidus</i>	20 - 23	-	Vandieken et al. (2006b)
<i>Desulfobacter hydrogenophilus</i>	29 - 32	+	Widdel (1987)
<i>Desulfobacter psychrotolerans</i>	20	-	Tarpgaard et al. (2005)
<i>Desulfobacterium autotrophicum</i>	25-28	+	Brysch et al. (1987), Rabus et al. (2002)
Psychrophilic SRP			
<i>Desulfofrigus oceanense</i>	10	+	Knoblauch et al. (1999)
<i>Desulfofaba gelida</i>	7	-	Knoblauch et al. (1999)
<i>Desulfotalea psychrophila</i>	10	-	Knoblauch et al. (1999)
Thermophilic SRP			
<i>Thermodesulfobacterium</i>	65 - 70	-	Castro et al. (2000)
Hyperthermophilic sulphate-reducing archaea			
<i>Archaeoglobus</i>	64-92	-	Castro et al. (2000)

1.2 Physiology

1.2.1 Sulfate transport and sulfate reduction

The characteristics of SRP have been reviewed in detail by Widdel (1988), and more recently by Myuzer and Stams (2008), Rabus et al. (2006) and Barton and Fauque (2009) and. Reduction of sulfate to hydrogen sulfide requires eight electrons (Thauer et al. 1977). The only product is hydrogen sulfide; the intermediate sulfur compounds are not excreted (Rabus et al. 2006). The sulfate reduction with an electron donor proceeds according to equation (Widdel 1988):



The sulfate reduction reactions take place in the cytoplasm, thus the cells must have an efficient sulfate transport system (Cypionka 1987, Cypionka 1989). The driving force of sulfate transport is an ion gradient, which is mediated by protons in the freshwater SRP species and sodium ions in the marine species (Cypionka 1987, Warthmann and Cypionka 1990). The sulfate molecule has to be activated prior to reduction (Peck 1962). This is done with ATP by ATP-sulfurylase, and the product is adenosine-5'-fosfosulfate (APS) (Peck 1962). APS is reduced by APS reductase to bisulfite and AMP (Peck 1962). The reduction of bisulfite to sulfide requires six electrons, and two pathways have been proposed: 1) direct reduction with six electrons (Lee et al. 1973, Huynh et al. 1984a) or 2) trithionate pathway, in which the reduction proceeds with two electron steps, where trithionate and thiosulfate are intermediates (Fitz and Cypionka 1990). The enzymes catalyzing both pathways have been

identified, but the intermediate products of the trithionate pathway have seldom been observed (Lee et al. 1973). The genes coding for the dissimilatory sulfite reductase enzyme (*dsr*) catalyzing the direct reduction of sulfite with six electrons have been identified from all known SRB species (Barton and Fauque 2009), therefore it can be utilized also as a molecular marker to identify SRB.

1.2.2 Fermentation and alternative electron acceptors

Some SRP species can ferment lactate, pyruvate, malate, fumarate fructose and glycerol in the absence of sulfate or another electron acceptor (Brysch et al. 1987, Ollivier et al. 1988, Knoblauch et al. 1999). Some SRP are able to dismutate (disproportionate) sulfite and thiosulfate to sulfate and sulfide (Badziong et al. 1978), but this inorganic fermentation yields only little energy, and becomes favorable only at a pH above 8 (Belkin et al. 1985).

Sulfite and thiosulfate also serve as electron acceptors for most SRP (Badziong and Thauer 1978, Widdel 1988), and elemental sulfur is reduced by some species (Widdel 1988, Barton and Fauque 2009). The reduction of sulfur oxyanions dithionite, trithionate and tetrathionate by SRP have seldom been studied, but Fitz and Cypionka (1990) reported that trithionate is incompletely reduced by *Desulfovibrio* sp. Also nitrate (Widdel 1988) and Fe (III) (Knoblauch et al. 1999) may serve as electron acceptors for some SRP, if no sulfate is available. *Desulfovibrio desulfuricans* spp. has been reported to oxidize thiosulfate and sulfite while nitrate is reduced to ammonia, but this reaction occurs only under electron donor limitation (Krekeler and Cypionka 1995). Nitrite and nitrate may serve as electron acceptors coupled to sulfide oxidation by *D. desulfuricans* spp. (Krekeler and Cypionka 1995). The presence of nitrite and nitrate prevented reduction of sulfur compounds for *D. desulfuricans* (Krekeler and Cypionka 1995), but some SRP are resistant to nitrite inhibition due to activity of the nitrite reductase enzyme (Haveman et al. 2004). Some SRP also perform reductive dehalogenation and reduction of the nitroaromatic compounds, arsenate, chromate and uranium (Rabus et al. 2006, Barton and Fauque 2009). The reduction of metals does not necessarily support the growth of SRP (Muyzer and Stams 2008).

1.2.3 Electron donors

SRP utilize a large number of electron donors, most of which are small molecular weight organic compounds that in natural habitats are excreted by fermentative bacteria (Widdel 1988, Rabus et al. 2006). The organic electron donors also serve as carbon source for SRP (Widdel 1988, Rabus et al. 2006). The most common electron donors are hydrogen, monocarboxylic acids (acetate, propionate, and higher fatty acids), dicarboxylic acids (succinate, malate, fumarate) ethanol, lactate and also phenyl-substituted organic acids (Rabus et al. 2006, Barton and Fauque 2009). Some SRP also utilize methanol, glycerol, fructose, glucose, amino acids, choline, furfural, phenols, nicotinate, indole, acetone, anisates, triethanoamine, toluene, benzoate, cresols, xylenes, chlorinated ethanes, trinitrotoluene, dinitrophenols and benzaldehyde (Rabus et al. 2006, Barton and Fauque 2009).

The growth of SRP is usually faster with electron donors that have a simple formula and high free energy change, such as ethanol, lactate, formate and hydrogen (Thauer et al. 1977, Rabus et al. 2006). With more complicated electron donors, such as aromatic compounds, the growth rates are lower (Rabus et al. 2006). Acetate has a simple molecular formula, but the free energy from sulfidogenic acetate oxidation is low (Thauer et al. 1977) and many SRP are unable to oxidize acetate (Widdel 1988). The electron donors and carbon sources used in the present study (hydrogen, formate, ethanol, lactate, acetate, sugars and plant material) are discussed in detail below.

Hydrogen

Most of the SRP are able to use hydrogen as electron donor (Widdel 1988, Rabus et al. 2006). Hydrogen is the energetically most favorable electron donor for SRP due to the high free energy change ($\Delta G^{0'}$) of sulfidogenic oxidation (Thauer et al. 1977). Growth on hydrogen requires an external carbon source, which is either CO₂ (autotrophic growth) or CO₂ and acetate (heterotrophic growth) (Brysch et al. 1987). The energy yield from sulfidogenic hydrogen oxidation is dependent on the partial pressure of H₂, i.e. the energy gained from this reaction is very dependent on the concentration (Rabus et al. 2006). The oxidation of hydrogen proceeds according to equation (Thauer et al. 1977):



The use of hydrogen requires activity of hydrogenases, which are responsible for the uptake of H₂ and the cleavage of the molecule (Teixeira et al. 1986, Casalot et al. 2002):



SRP have three major types of hydrogenases. In *Desulfovibrio* sp. the hydrogenases are [Fe] hydrogenase (Huynh et al. 1984b), [NiFe] hydrogenase (Teixeira et al. 1986) and [NiFeSe] hydrogenase (Rieder et al. 1984). The role and location of the hydrogenases in the SRP cells varies even within same genera (Casalot et al. 2002). The periplasmic hydrogenases are part of the machinery used to build the proton motive force across the cell membrane (Odom and Peck 1981).

Formate (Formic acid)

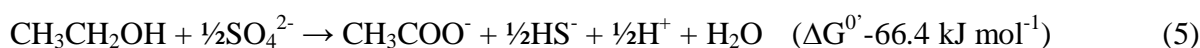
The redox potential of redox couples 2 H⁺/H₂ and HCO₃⁻/HCOO⁻ are both around -0.41 V, thus SRP are usually able to utilize formate if they oxidize H₂ (Brysch et al. 1987, Rabus et al. 2006). There are only a few exceptions to this rule. *Desulfobulbus propionicus*, *Desulfobacter hydrogenophilus* and *Desulfovibrio baarsii* use either formate or H₂ (Widdel 1988). The oxidation of formate proceeds as follows (Thauer et al. 1977):



Desulfovibrio sp. and some other SRP have special formate dehydrogenases (Odom and Peck 1981), except *Desulfobacter* sp. (Schauder et al. 1986). These SRP are able to oxidize acetate, but also to grow on formate, because formate hydrogenases are part of C₁/CO pathway involved in acetate oxidation, and the active site of formate dehydrogenase enzyme is presumably in the cytoplasmic side of the cell membrane (Schauder et al. 1986). Formic acid has a pK_a of 3.75, and it is less lipophilic than acetate, thus the formate transport to the cell is not mediated by membrane diffusion (Rabus et al. 2006). In bioreactors formate may be used in the form of hydrogen, since formate dissociates also spontaneously to H₂ and CO₂, and formate dissociation made sulfate reduction thermodynamically feasible (Bijmans et al. 2008a).

Ethanol

Ethanol is oxidized by most SRP either completely to CO₂ or incompletely to acetate (Rabus et al. 2006). The sulfidogenic oxidation of ethanol proceeds via acetaldehyde to acetate (Rabus et al. 2006). The ethanol oxidation to acetate proceeds as follows (Thauer et al. 1977):



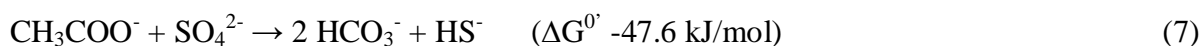
Lactate

Lactate is a commonly used substrate in the culturing of SRP because it is oxidized by most SRP either completely or incompletely to acetate (Widdel 1988). The net ATP yield from incomplete lactate oxidation is zero (Peck 1966), therefore the energy from incomplete oxidation is presumably linked to chemiosmotic energy conservation (Rabus et al. 2006). There is evidence on lactate cycling via H₂ for *Desulfovibrio* sp. (Odom and Peck 1981). The sulfidogenic lactate oxidation proceeds according to equation (Thauer et al. 1977):



Acetate

Acetate that results from the oxidation of an organic electron donor is either excreted from the cell or oxidized completely to CO₂ (Postgate 1984). Some SRP are unable to oxidize acetate because they do not have the necessary enzymes to oxidize acetyl-CoA (Schauder et al. 1986). The acetate oxidation proceeds as follows (Thauer et al. 1977):



Some SRP prefer to use acetate and utilize it efficiently, but other SRP species prefer to use alternative electron donors and their capability to use acetate is limited, although they are able to oxidize it (Imhoff-Stuckle and Pfenning 1983). Acetate oxidizing SRP may excrete some of the formed acetate, because acetyl-CoA is formed at faster rate than it is being oxidized to CO₂ (Imhoff-Stuckle and Pfenning 1983). The acetate oxidizing SRP may also be unable to use free acetate, the reason for this is unknown, but the rate of sulfidogenic acetate oxidation is also dependent on the pathway used by the SRP (Rabus et al. 2006). Methanol, formate, hydrogen (Nanninga and Gottschal 1987) and glycolate (Friedrich and Schink 1995) do not directly yield acetate as intermediate product in sulfidogenic electron donor oxidation.

SRP have two pathways for acetate oxidation (Schauder et al. 1986). *Desulfobacter* sp. couples the activation of acetate to acetyl-CoA in the tricarboxylic acid cycle (part of the citric acid cycle) (Brandis-Heep et al. 1983). This does not require input of ATP, and is therefore energetically favorable (Brandis-Heep et al. 1983). Species other than *Desulfobacter* sp. use the carbon monoxide dehydrogenase pathway, which does not utilize the citric acid cycle and is therefore slower and requires the input of ATP (Schauder et al. 1986). This pathway is the reverse version of homoacetogenic reaction, in which acetate is formed from CO₂ and H₂, the acetyl-CoA is cleaved into CO and a bound methyl group, both of which are then oxidized to CO₂ (Schauder et al. 1986).

The energy yield from sulfidogenic oxidation of acetate is less dependent on the concentration than the oxidation of H₂ (Rabus et al. 2006). When SRP are oxidizing higher fatty acids, some acetate may be excreted, because β-oxidation and acetyl-CoA oxidation do not proceed at the same rate (Schauder et al. 1986). Because of the imbalance of the acetate production and oxidation rates in the cell, the acetate oxidation in a sulfidogenic bioreactor may be difficult to balance.

Sugars

The ability to use sugars is rarely observed among SRP. *Desulfotomaculum nigrificans* has been reported to be able to grow with fructose and glucose as electron donors, and also to ferment these in absence of sulfate (Klempers et al. 1985). *Desulfotomaculum nigrificans* was able to grow only with autoclaved glucose, which was presumably due to conversion of glucose to a more usable form, e.g. fructose (Klempers et al. 1985). Also *Desulfovibrio*

fructosovorans and *Desulfovibrio salexigens* were reported to be able to utilize fructose in the presence and absence of sulfate (Ollivier et al. 1988, Zellner et al. 1989). *Desulfurispora thermophila* was able to use mannose, glucose and fructose as electron donors for sulfate reduction (Kaksonen et al. 2007a). The equation for sulfidogenic glucose oxidation is (Thauer et al. 1977):



Unidentified SRP species were reported to have saccharolytic activity by Joubert and Britz (1987). These SRP fermented carbohydrates to acetate, ethanol, H₂ and CO₂, and sulfide was produced in the presence of sulfate, but in the absence of sulfate the fermentation products were acetate, ethanol, lactate, H₂ and CO₂ (Joubert and Britz 1987). SRP have also been used in glucose acidification reactors (Joubert and Britz 1988) and in sucrose acidification at low pH (Lopez et al. 2007).

Plant material and cellulosic compounds

SRP have not been reported to grow with polymers such as cellulose (Hansen 1994). Only some *Archaeoglobus* species have been reported to be able to utilize starch and peptides (Rabus et al. 2006). The ability to directly use hemicellulose has not been observed for SRP (Hansen et al. 1994). Polymeric substances are rarely direct substrates of SRP in nature, therefore they are dependent on the degradation products excreted by fermentative organisms for the electron donors (Muyzer and Stams 2008). *Desulfovibrio termitidis* isolated from termite gut has been shown to degrade carbohydrates (Trinkerl et al. 1990). *Desulfovibrio salexigens* was shown to be able to oxidize benzoic acids (Zellner et al. 1989). The benzoic acids are produced during lignin degradation (Zeikus 1981), therefore, these compounds may serve as electron donors for SRP in nature (Zellner et al. 1989).

1.2.4 Incomplete and complete acetate oxidizers

The genera of *Desulfovibrio*, *Desulfomicrobium*, *Desulfobulbus*, *Desulfococcus* and *Thermodesulfovibrio* contain species that are not able to oxidize acetate (Rabus et al. 2006, Barton and Fauque 2009). The selected SRP species able to oxidize acetate and to perform facultative autotrophy (to grow either autotrophically or heterotrophically) are listed in Table 3.

Table 3. The ability of selected SRP species to oxidize acetate and facultative autotrophy according to Brysch et al. (1987). The ability to oxidize acetate is labelled with (+) and inability with (-). The ability for facultative autotrophy is labelled also with (+/-).

Species	Acetate oxidation	Facultative autotrophy
<i>Desulfobacter hydrogenophilus</i>	+	+
<i>Desulfobacterium vacuolatum</i>	+	+
<i>Desulfococcus multivorans</i>	+	+
<i>Desulfococcus niacini</i>	+	+
<i>Desulfonema limicola</i>	+	+
<i>Desulfovibrio baarsii</i>	+	+

1.2.5 Carbon dioxide fixation and homoacetogenesis by sulfate reducing prokaryotes

Some SRP are able to grow autotrophically with CO₂ as the carbon source (Klemps et al. 1985, Brysch et al. 1987). CO₂ can also be used as electron acceptor in homoacetogenic growth, which has been shown to occur in some SRP species (Klemps et al. 1985, Brysch et al. 1987). Anaerobic homoacetogenic bacteria use CO₂ (as dissolved carbonic acid) and H₂ to synthesize acetate (Drake et al. 2006). *Desulfotomaculum orientis* was shown to be able to grow via homoacetogenesis with H₂ in the absence of sulfate, but the growth rate was very

slow (Klemps et al 1985). Also slow acetogenic growth was observed with formate, methanol and ethanol in the absence of sulfate (Klemps et al. 1985). The production of acetate as a by-product from H₂/CO₂ or formate under sulfate limitation has been observed for SRP species *Desulfobacterium autotrophicum*, *Desulfonema limicola*, *Desulfosarcina variabilis*, *Desulfotomaculum orientis*, *Desulfovibrio baarsii* and *Desulfococcus multivorans* (Brysch et al. 1987).

1.2.6 Physiology of genus *Desulfomicrobium*

Because the dominant species of the sulfate reducing enrichment culture used in the low temperature studies of the present work was *Desulfomicrobium* sp., therefore the characteristics of this genus are described in detail below. The genus *Desulfomicrobium* consists of eight species, and belongs to phylum of Deltaproteobacteria (Garrity et al. 2004, Dias et al. 2008). Seven of the members of this genus are mesophiles, one of them is thermophile (for a review, see Dias et al. 2008). The members of this genus are commonly found in anaerobic marine and freshwater sediments, anaerobic ponds and saturated mineral and organic deposits (Dias et al. 2008). The species of the genus *Desulfomicrobium* are gram-negative, non-sporulating, incomplete acetate oxidizers and require acetate as carbon source when grown with H₂ (Dias et al. 2008). The *Desulfomicrobium* species are not able to reduce nitrate (Dias et al. 2008). Desulfoviridin is absent and hydrogenases present (Dias et al. 2008). The optimum growth temperature is 25-55°C (Dias et al. 2008). *Desulfomicrobium norvegicum* (type strain Norway 4) was reported to be able to reduce elemental sulfur as alternative electron acceptor (Biebl and Pfenning 1977). The physiological characteristics of the species belonging to the genus *Desulfomicrobium* are listed in Table 4.

Table 4. Species and the properties of the species belonging to the genus *Desulfomicrobium*. The utilization of following electron donors: ethanol, lactate, acetate, formate and H₂ (+ acetate) are indicated with growth (+) and no growth (-).

Species	Growth range (°C) (T _{opt} , °C)	Optimal pH	Ethanol	Lactate	Acetate	Formate	H ₂ (+acetate)	T _d (h) with lactate	Reference
<i>D. norvegicum</i>	25-30	nr	+	+	-	+	+	nr	Sharak Genthner et al. (1997)
<i>D. hypogeium</i>	23-37 (30)	6.8	+	+	-	+	+	nr	Krumholtz et al. (1999)
<i>D. salsuginis</i>	10-40 (35)	7.2	+	+	-	+	+	9.2	Dias et al. (2008)
<i>D. aestuarii</i>	10-40 (35)	7.2	-	+	-	+	+	8.6	Dias et al. (2008)
<i>D. escambiense</i>	25-30	nr	+	+	-	+	+	nr	Sharak Genthner et al. (1994; 1997)
<i>D. macestii</i>	15-40 (35)	7.2	+	+	-	+	+	nr	Hippe et al. (2003)
<i>D. apsheronum</i>	25-30	nr	+	+	-	+	+	nr	Rozanova et al. (1988)
<i>D. baculatum</i>	28-37	nr	+	+	-	+	+	nr	Rozanova et al. (1988)
<i>D. orale</i>	25-39 (37)	nr	+	+	-	+	+	11	Langendijk et al. (2001)
<i>D. thermophilum</i>	37-60 (55)	6.5	+	+	-	nr	+	3.3	Thevenieau et al. (2007)

nr = not reported, T_d= doubling time

2 Factors affecting activity of sulfate reducing prokaryotes

2.1 Temperature

The cold areas cover around three quarters of Earth's surface. Most of this is deep ocean with a constant temperature around 4°C (Levitus and Boyer 1994). The temperate and permanently cold environments support prokaryotes that are able to adapt to the effects caused by cold (Morita 1975). Prokaryotes are classified according to their optimal temperature (T_{opt}), at which the growth is the highest. The microbes that are able to live at low temperature are either psychrophilic or psychrotolerant mesophiles. Obligate psychrophilic prokaryotes have their T_{opt} below 15°C (Morita 1975) and the maximum growth temperature (T_{max}) is below 20°C and minimum growth temperature (T_{min}) at 0 °C degrees or below (Scherer and Neuhaus 2006). Psychrotolerant species have T_{opt} above 20°C, T_{max} 25°C and T_{min} 7°C (Scherer and Neuhaus 2006). Mesophiles have T_{opt} above 25°C, T_{max} above 35°C and T_{min} of 10°C (Scherer and Neuhaus 2006). For a review, see Scherer and Neuhaus (2006). The optimal growth temperature may not reflect the optimal temperature of the activity, psychrophilic SRB (sulfate-reducing bacteria) have the highest sulfate reduction rate at a temperature that is several degrees higher than the T_{opt} (Knoblauch and Jørgensen 1999).

The psychrophilic species differ from psychrotolerant species with the specialized characteristics of cell components (e.g. cell wall, enzymes) that are adapted to life at low temperature. For mesophilic species metabolic activity and growth rate decrease with temperature, and at the lower temperature limit these functions become inhibited; as the temperature is increased, the metabolic and growth activities are resumed. Psychrophilic species may increase their activity and have higher growth rate at decreasing temperatures. Alternatively, even a small temperature increase may inhibit the activity.

2.1.1 General effects of low temperature on bacteria

The general effects of low temperature on chemical properties of molecules are opposite to those at elevated temperature. The kinetics of the chemical reactions slows down with decreasing temperature, while the solubility of many gases increases (for a review, see Amend and Shock 2001). Also the pH neutrality value increases from pH 7 (at 25°C) to pH 7.5 at 0°C (Amend and Shock 2001). The low activity of compounds at low temperatures is a challenge for psychrophilic and psychrotolerant prokaryotes (Amend and Shock 2001). Characteristic to low temperature is low enthalpy, reduced amplitudes and frequencies at the atomic and molecular scale (Tehei et. al 2004). At subzero temperatures the activity of water is low, and the solute concentration in the water needs to be high to keep water in its liquid form. The low water activity further increases the effects of low temperature (Tehei et al. 2004).

Life at low temperatures requires several features from biomolecules: the proteins and enzymes have to retain their activity and stability at low temperatures and the cytoplasmic membrane has to maintain functionality. The effects of temperature decrease and cold shock are: 1) catalysis and transport rates are extremely slow, 2) membrane fluidity is lost, 3) secondary structures of nucleic acids are destabilized, which inhibits protein synthesis and 4) at sub zero temperatures ice crystals may form inside the cells, damaging the cell structure (Cavicchioli *et al.* 2000). Because the temperature decrease affects the whole cell instantly, the cold shock response of the bacteria should be as fast as possible (Scherer and Neuhaus 2006). The cell responses to cold shock and adaptation to low temperature are discussed below.

2.1.2 Cell membrane

The cellular membrane is responsible for many key cellular processes, such as nutrient uptake (via membrane and transport proteins), ATP synthesis and electron flow (Scherer and Neuhaus 2006). Therefore it is vital to maintain the membrane functions via modification of the membrane properties (Scherer and Neuhaus 2006). The ability to maintain a functional cell membrane defines the lower growth temperature of the species, because the affinity for substrates, and thus the ability to transport them decreases simultaneously with the temperature (Nedwell 1999). This in turn causes that the critical concentration from which the bacteria can uptake a necessary amount of the substrate increases as the temperature decreases (Nedwell 1999). Maintenance of the cell membrane in a liquid state is necessary as cells acclimate to low temperature (Tarpgaard et al. 2005). Decreased temperature makes the cell membrane fatty acids waxy, therefore solidifying the membrane (Tarpgaard et al. 2005) and the membrane functionality is restored and stabilized by increasing the proportion of saturated membrane lipids that are able to maintain their viscosity at lower temperatures (Cavicchioli et al. 2000).

The membranes of psychrophilic SRB contain high amounts of unsaturated and cis-saturated fatty acids (Könneke and Widdel 2003). This reflects the adaptation of psychrophilic SRB to low temperatures (Könneke and Widdel 2003). Some psychrophilic SRB species examined by Könneke and Widdel (2003) did not show significant changes in the cell membrane fatty acid composition due to temperature change, thus the maintenance of the membrane function with the same fatty acid composition seems to be possible for a wider temperature range than expected. On the other hand, for mesophilic *Desulfobacterium autotrophicum* the proportion of unsaturated fatty acids in the cell membrane increased to 70% when grown at low temperature (4-10°C) (Rabus et al. 2002).

2.1.3 Proteins and compatible solutes

A rapid temperature decrease causes synthesis of cold inducible proteins (CIP) in the cell. The role of the CIPs is to adapt the organism to changes caused by low temperature. CIPs include cold shock proteins (CSP) and cold acclimation proteins (CAP) (Cavicchioli *et al.* 2000, Bakermans et al. 2007). CSPs are present immediately after a temperature decrease and function in the repairing of cold affected cellular processes, for example protein synthesis (Cavicchioli et al. 2000, Bakermans et al. 2007). CAPs are present when organisms are acclimatizing to growth at decreased temperature and also in organisms that live constantly at cold temperatures (Cavicchioli et al. 2000, Bakermans et al. 2007).

The temperature decrease from 37°C to 0°C may result in a 20-250 fold decrease in the activity of mesophilic enzymes (Feller and Gerday 2003). This is caused by the loss of (catalytic) enzyme structure, and further temperature decrease may even cause protein denaturation (Feller and Gerday 2003). Enzymes studied from psychrophilic bacteria are able to maintain high activity even at low temperature, but these enzymes are also very heat-labile (for a review, see Feller and Gerday 2003).

Microbes also produce compatible solutes to protect against the effects of the cold temperature (Scherer and Neuhaus 2006). These solutes, such as glycine, protect the cellular components (enzymes, membrane etc.) and prevent the cytoplasm from freezing (Scherer and Neuhaus 2006).

2.1.4 Cell growth

The cold stress caused by temperature decrease may change the cellular metabolism by inducing alternative pathways or increasing activity of key enzymes (Scherer and Neuhaus

2006). Psychrophilic SRB persist in low temperature and low nutrient concentration in the cold sea sediments (Knoblauch and Jørgensen 1999). In theory, the lowest growth temperature is just above the freezing point of the cytosol (Scherer and Neuhaus 2006). The availability of the substrate affects the growth rate of psychrophilic bacteria at temperatures around 0°C; increased growth rates have been measured at high substrate concentrations at low temperatures (Wiebe et al. 1992). The growth rate and the activity of the bacteria may also have a different optimal temperature, as has been shown by Isaksen and Jørgensen (1996) and Knoblauch and Jørgensen (1999). In their study SRB had the highest sulfate reduction activity several degrees above the optimal growth temperature, indicating that the temperature response of the activity is dependent on a small number of enzymes (Knoblauch and Jørgensen 1999).

2.2 Psychrophilic and psychrotolerant SRB

Three psychrophilic SRB species have been described so far, and fifteen mesophilic SRB species can be classified as psychrotolerant (Table 5). Although some species listed in Table 5 are able to oxidize acetate, it may not have been tested for all the species at low temperatures, e.g. these species oxidize acetate at their optimal temperature.

2.2.1 Growth rate

The growth rates of psychrotolerant and psychrophilic SRB are not necessarily low at their optimal temperatures, as shown in Table 5, which lists the growth parameters of selected psychrophilic and psychrotolerant SRB species with different electron donors. The growth rates of SRB pure cultures have been measured under optimal conditions; therefore they may not reflect the growth rates in natural environments. The growth rates of psychrophilic SRB are relatively high at low temperatures when compared to psychrotolerant species (Knoblauch and Jørgensen 1999).

The psychrotolerant and psychrophilic SRB described by Knoblauch and Jørgensen (1999) and Knoblauch et al. (1999) were able to grow at the freezing point of seawater (-1.8°C). The ability of the psychrophilic and psychrotolerant SRB to grow at *in situ* temperatures is more important than the T_{opt} of the species (Knoblauch and Jørgensen 1999). Rabus et al. (2002) reported a linear growth rate for *Desulfobacterium autotrophicum* over a temperature range of 4-29°C. Knoblauch and Jørgensen (1999) reported the highest growth yields of psychrophilic SRB to be around 0°C. Table 5 also lists doubling times for some SRB species below their optimal temperature, at -1.8 -10°C. The doubling times at the freezing point of seawater vary from a few days to 1-2 months. The growth rate with a specific substrate can be also presented as biomass yield per mol of substrate. The growth yields reported for psychrophilic and psychrotolerant SRB with tested electron donors are given in Table 5; the growth yields with lactate were generally higher than with fatty acids (acetate, propionate).

Table 5. The growth range, optimal growth temperature (T_{opt}), biomass doubling time (T_d) at T_{opt} , biomass doubling time below T_{opt} , growth yield (Y) with specific substrates and ability to oxidize acetate (Ac) (oxidation +, no oxidation -) of the described psychrotolerant and psychrophilic SRB species.

Species	Growth range (°C)	T_{opt} (°C)	T_d at T_{opt} (h) substrate)	T_d (h) below T_{opt} (°C)	Y (g VSS mol ⁻¹ d ⁻¹ substrate, °C)	Ac	Reference
Psychrotolerant mesophilic SRB							
<i>Desulfobulbus propionicus</i>	10-43	39	nr	nr	nr	-	Widdel and Pfenning (1984)
<i>Desulfovibrio alaskensis</i>	10-45	37	nr	nr	nr	-	Feio et al. (2004)
<i>Desulfomicrobium salsuginis</i>	10-40	35	nr	nr	nr	-	Dias et al. (2008)
<i>Desulfomicrobium aestuarii</i>	10-40	35	nr	nr	nr	-	Dias et al. (2008)
<i>Desulfobacter vibrioformis</i>	5-38	33	nr	nr	nr	+	Lien and Beeder (1997)
<i>Desulfobacter hydrogenophilus</i>	0-35	29-32	18 (acetate)	nr	nr	+	Widdel (1987)
<i>Desulfobacter postgatei</i>	10-37	28-32	nr	nr	nr	+	Widdel and Pfenning (1984)
<i>Desulfovibrio psychrotolerans</i>	10-50	28-30	nr	nr	nr	-	Sasi Jyothsna et al. (2008)
<i>Desulfovibrio aespoensis</i>	4-35	25-30	nr	nr	nr	-	Motamedi and Pedersen (1998)
<i>Desulfofaba fastidiosa</i>	5-33	28	4.3 (propionate)	nr	nr	-	Abildgaard et al. (2004)
<i>Desulfobacterium autotrophicum</i>	0-31	25-28	16.5 (H ₂)	nr	nr	+	Brysch (1987), Rabus et al. (2002)
<i>Desulfofaba hansenii</i>	8-30	20	nr	nr	nr	-	Finster et al. (2001), Abildgaard et al. (2004)
<i>Desulforhopalus vacuolatus</i>	0-24	18-19	22.5 (lactate)	80 (10°C) 200 (5°C)	10 (lactate, 10°C) 3 (acetate, 11°C)	-	Isaksen and Teske (1996), Isaksen and Jørgensen (1996)
<i>Desulfofrigus fragile</i>	-1.8-27	18	19 (acetate)	134 (-1.8°C)	7.2 (lactate, 4°C)	-	Knoblauch et al. (1999)
<i>Desulfotalea arctica</i>	-1.8-26	18	33 (lactate)	192 (-1.8°C)	5.6-6 (lactate, 0-4°C)	-	Knoblauch et al. (1999)
<i>Desulfobacter psychrotolerans</i>	-6-26	20	nr	267 (5°C) 1680 (-3.6°C)	4.3-4.5 (lactate, 5-15°C) 3.5 (acetate, 20°C)	-	Tarpgaard et al. (2005)
Psychrophilic SRB							
<i>Desulfofrigus oceanense</i>	-1.8-16	10	169 (acetate)	1176 (-1.8°C)	4.9 (acetate, 7°C) 3.7 (acetate, -1.8°C)	+	Knoblauch et al. (1999)
<i>Desulfotalea psychrophila</i>	-1.8-19	10	27 (lactate)	96 (-1.8°C)	4.2 (lactate, -1.8 -10°C)	-	Knoblauch et al. (1999)
<i>Desulfofaba gelida</i>	-1.8-10	7	144 (propionate)	840 (-1.8°C)	3.8 (propionate, 5°C)	-	Knoblauch et al. (1999)

T_{opt} = optimal growth temperature, T_d = doubling time, Y = growth yield (dry weight), Ac = Acetate oxidation (+/-), nr = not reported

2.2.2 Sulfate reduction rate

The sulfate reduction rate (SRR) of the SRB increases above the T_{opt} , and this property is common for psychrotolerant and psychrophilic SRB (Knoblauch and Jørgensen 1999). The ability to reduce sulfate above the T_{opt} of the organisms indicates that the cellular activity related to growth and energy production are uncoupled (Tarpgaard et al. 2005). Isaksen and Jørgensen (1996) studied the growth of psychrotolerant SRB *Desulforhopalus vacuolatus*, that had its highest growth rate at 0-12°C, and observed that this strain had highest sulfate reduction activity at 28°C, but the upper limit of growth was 24°C (T_{opt} 18-19°C). The ability to reduce sulfate above the temperature for growth is necessary in order to generate energy for repair of the heat denatured proteins (Isaksen and Jørgensen 1996). Table 6 lists the temperature ranges and rates of sulfate reduction measured for some psychrotolerant and psychrophilic SRB pure cultures and sediment incubations.

Table 6. Temperature ranges of sulfate reduction and highest sulfate reduction rates (SRR) reported for psychrotolerant and psychrophilic SRB pure cultures and sediment incubations.

Biomass type	SRR range (°C)	Highest SSR (mmol SO ₄ ²⁻ d ⁻¹) (°C)	Highest SRR (nmol cell d ⁻¹) (°C)	Reference
<i>Desulforhopalus vacuolatus</i>	-2.5 - 38	1.12 (28°C)	4.5*10 ⁴ (28°C)	Isaksen and Jørgensen (1996)
strain ak30	0-40	56.2 (35°C)	1.05*10 ³ (35°C)	Isaksen and Jørgensen (1996)
Sediment slurry	2-30	8*10 ⁻⁶ (12.5°C)	nr	Isaksen and Jørgensen (1996)
<i>Desulfofrigus oceanense</i>	-4 - 29	nr	12*10 ⁻⁶ (17°C)	Knoblauch and Jørgensen (1999)
<i>Desulfofrigus fragile</i>	-4 - 37	nr	33*10 ⁻⁶ (27°C)	Knoblauch and Jørgensen (1999)
<i>Desulfotalea psychrophila</i>	-4 - 25	nr	2.1*10 ⁻⁶ (12°C)	Knoblauch and Jørgensen (1999)
<i>Desulfotalea arctica</i>	-3 - 34	nr	31*10 ⁻⁶ (23°C)	Knoblauch and Jørgensen (1999)
<i>Desulfobacter psychrotolerans</i>	-3.6 - 31	nr	6.2 *10 ⁻⁶ (26°C)	Tarpgaard et al. (2005)
Sediment from Svalbard	-3.6 - 30	0.88*10 ⁻⁴ . ^a (18°C)	nr	Finke and Jørgensen (2008)
Sediment from Wadden sea	-3.6 - 40	1.8*10 ⁻³ . ^a (34°C)	nr	Finke and Jørgensen (2008)
Norilsk smelter site, <i>in situ</i> ^b	nr	10-30*10 ⁻⁶ . ^a (0°C)	nr	Karnachuck et al. (2005)

^a unit mmol cm⁻³ d⁻¹, ^b mean annual of temperature 0°C

SRB can be acclimated to tolerate lower temperatures, as was shown in a study by Rabus et al. (2002). In the study by Rabus et al. (2002), cells grown at 4 and 10°C had a lower upper temperature limit of sulfate reduction with lactate, e.g. the cells grown at 28°C were able to reduce sulfate at higher temperatures than low temperature acclimated cells (Rabus et al. 2002). This was explained by a decreased turnover rate of dissimilatory sulfate reduction enzymes, a reduced affinity towards substrate and a decreased efficiency in coupling of the substrate oxidation, electron transfer and sulfate reduction at lower temperature (Rabus et al. 2002). The decrease of the SRR of some psychrotolerant mesophilic SRB, “moderate psychrophiles” (psychrotolerant SRB with lower T_{opt} than mesophiles in general) and psychrophilic SRB at different temperatures are shown in Figure 1. The psychrophilic SRB maintained 50% of the maximum SRR at temperatures 0-5°C. Moderate psychrophiles had 50% of the SRR at 13-20°C and psychrotolerant mesophiles at 25°C.

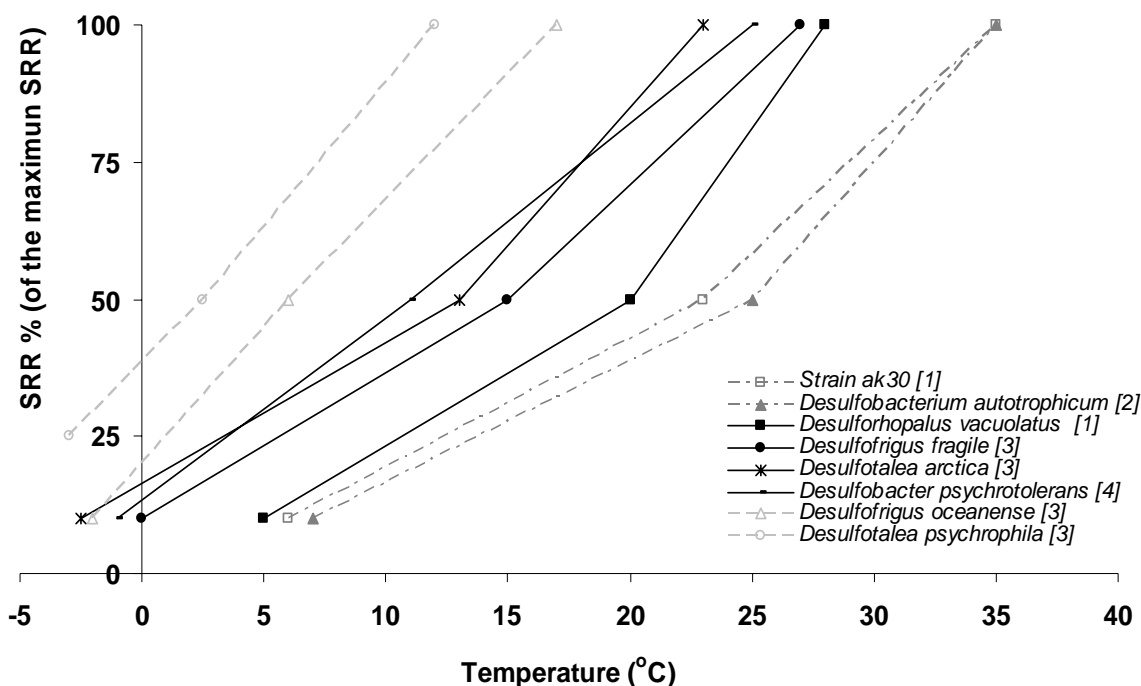


Figure 1. Percentage of the maximum sulfate reduction rate (SRR) obtained at several temperatures for psychrotolerant mesophiles *Strain ak30* and *Desulfobacterium autotrophicum* (grey dashed line with dots), moderate psychrophilic *Desulforhopalus vacuolatus*, *Desulfofrigus fragile*, *Desulfotalea arctica* and *Desulfobacter psychrotolerans* (black line) and psychrophilic *Desulfofrigus oceanense* and *Desulfotalea psychrophila* (light grey dashed line). The references are [1] Isaksen and Jørgensen (1996), [2] Rabus et al. (2002), [3] Knoblauch and Jørgensen (1999) and [4] Tarpgaard et al. (2005).

2.2.3 Thermodynamics of sulfidogenic electron donor oxidation at low temperature

Temperature affects the energy gained from sulfidogenic electron donor oxidation (Lettinga et al. 2001). The Gibbs free energy yield from fatty acid (acetate, propionate and butyrate) oxidation decreases as the temperature is decreased, but for hydrogen the energy yield increases with decreasing temperature (Lettinga et al. 2001, Table 7). This indicates that there is no thermodynamic limitation for biological sulfate reduction with H₂ at low temperature.

Table 7. The Gibbs free energy yield from sulfidogenic oxidation of butyrate, propionate, acetate and hydrogen at 37°C and 10°C according to Lettinga et al. (2001).

Reaction	ΔG' kJ reaction ⁻¹	
	37°C	10°C
CH ₃ CH ₂ CH ₂ COO ⁻ + 2.5 SO ₄ ²⁻ → 4 HCO ₃ ⁻ + 2.5 HS ⁻ + 0.5 H ⁺	-128.3	-116.4
CH ₃ CH ₂ COO ⁻ + 0.75 SO ₄ ²⁻ → CH ₃ COO ⁻ + CHO ₃ ⁻ + 0.75 HS ⁻ + 0.25 H ⁺	-34.9	-35.4
CH ₃ COO ⁻ + SO ₄ ²⁻ → 2 HCO ₃ ⁻ + HS ⁻	-49.5	-45.3
4 H ₂ + SO ₄ ²⁻ + H ⁺ → HS ⁻ + 4 H ₂ O	-148.2	-157.1

Only a few of the psychrotolerant and psychrophilic SRB species listed in Table 5 are able to oxidize acetate. The growth yields reported in Table 5 show that acetate does not support growth of SRB as well as lactate. For example, the growth yields of *Desulforhopalus vacuolatus* at 11°C were 10 g (dw) mol⁻¹ and 3 g (dw) mol⁻¹ for lactate and acetate, respectively (Isaksen and Jørgensen 1996). The bacteria require more substrate as temperature decreases (Wiebe et al. 1992). Therefore, growth at low temperature could be supported to some extent by addition of extra electron donor. On the other hand, in

bioreactors fed with organic electron donors, this tends to result in accumulation of acetate, which inhibits the SRB (Sahinkaya et al. 2007). Also the affinity towards substrates tends to decrease with the temperature, so the transport of substrates to the cell becomes the limiting factor and has a major effect on the competition between microbes (Nedwell 1999).

2.3 The effects of pH, salinity, oxygen, sulfide and metals on sulfate reducing prokaryotes

Most of the SRP are neutrophilic (Widdel 1988), but acid tolerant SRP have been shown to reduce sulfate at pH values as low as pH 3.8 (Kimura et al. 2003). High-rate sulfate reducing bioreactors (30-55°C) fed with formate, H₂/CO₂ mixture and sucrose have been successfully operated at pH 4-6 (Lopez et al. 2007, Bijmans et al. 2008a, Bijmans et al. 2008b). Some SRP species are alkaliphilic, and the highest pH reported for SRP species is 10 (Pikuta et al. 2003). SRP species from hypersaline alkalic lakes tolerating high salinity (up to 340 g L⁻¹) have also been described (Ollivier et al. 1991). Although the SRP were originally regarded as obligate anaerobes, some species (for example *Desulfovibrio aerotolerans*) are able to tolerate exposure to oxygen via oxidation of extracellular polyglucose (Mogensen et al. 2005).

The toxic effect of low pH is due to acidification of the cytoplasm, which inhibits the formation of a proton motive force (Thauer et al. 1977). The accumulation of fatty acids cause a similar effect, as the neutral, undissociated forms of the fatty acids (e.g. acetic acid) permeate easily via the cell membrane, and dissociate in the neutral cytoplasmic pH, causing acidification (Thauer et al. 1977). The pH affects the form of hydrogen sulfide, e.g. H₂S, HS⁻ or S²⁻ (Kawazuishi and Prausnitz 1987). The toxic form of sulfide is H₂S, because it passes through the cell membrane and inhibits cell function (Thauer et al. 1977, Reis et al. 1992). The toxicity of H₂S on SRB is direct, and independent on the availability of precipitation metals (Reis et al. 1992). The inhibitory effects of H₂S are reversible, and SRB can recover from shock concentrations (Okabe et al. 1995). H₂S concentrations of 2.6-16.1 mmol L⁻¹ (Reis et al. 1992, O'Flaherty et al. 1998, Kaksonen et al. 2004a) have been shown to be toxic to SRB. The H₂S toxicity in sulfidogenic bioreactor processes is discussed more in detail in Papers I and IV. Temperature also affects the major form of sulfide at neutral pH: the pK_a of sulfide at 9°C is 7.28 and 6.96 at 30°C (Kawazuishi and Prausnitz 1987). Therefore, the proportion of the H₂S form of sulfide increases as temperature decreases, and toxic H₂S concentration is reached at lower total dissolved sulfide concentration. This phenomenon is illustrated in Figures 2A-B, where the proportion of H₂S as a function of temperature and pH is shown.

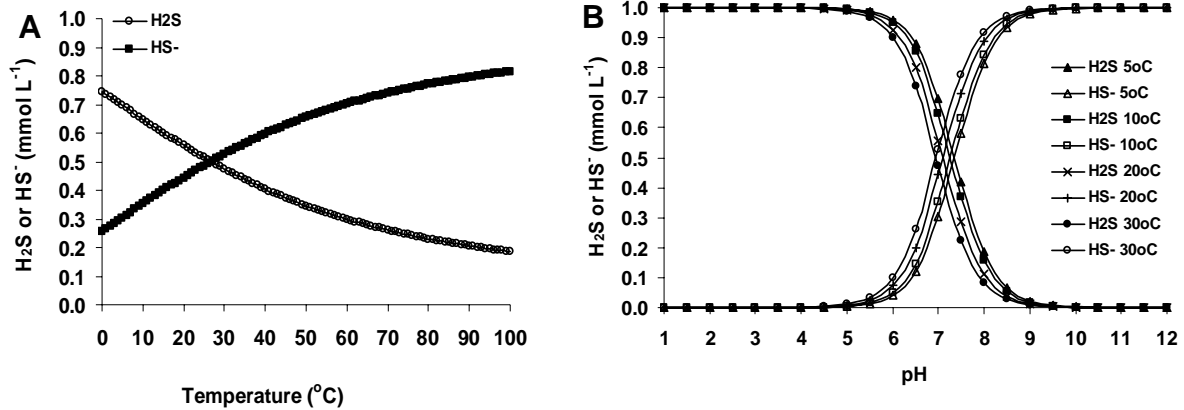


Figure 2. A: The relative concentrations of H₂S and HS⁻ at temperatures 0-100°C for 1 mmol L⁻¹ total dissolved sulfide concentration at pH 7. **B:** The relative concentrations of H₂S and HS⁻ at pH 1-12 for 1 mmol L⁻¹ total dissolved sulfide concentration at 5°C, 10°C, 20°C and 30°C.

A high concentration of most metals can be toxic to microbes, because they can damage the cell membrane and DNA and affect the metabolism via inhibiting the enzymes (Bruins et al. 2000). Bacteria have several methods to ameliorate the toxic effects of the metals. The cell membrane prevents the uptake of metal ions. The metal ions can be pumped out of the cell via active transport, the metals are sequestered inside or outside the cell, enzymes can detoxify the metals and the cell components can become less sensitive to metal (Bruins et al. 2000). The metal oxyanions molybdate (MoO₄²⁻), chromate (CrO₄²⁻), selenate (SeO₄²⁻) and wolframate (WO₄²⁻) inhibit SRB specifically, because they are sulfate analogs and are transported into the cell via the sulfate transport pathway (Patidar and Tare 2005). Inhibiting concentrations of Al, Cd, Cr³⁺, Cr⁶⁺, Cu, Fe, Hg, Ni, Pb and Zn reported for SRB are listed in Table 8. In sulfidogenic bioreactors, the toxic effects of metals and H₂S are controlled via metal precipitation of metals sulfides, thereby limiting the toxic effects of metals and H₂S on SRB in the bioreactors are limited (Kaksonen et al. 2004a; Bijmans et al. 2009a). This issue is discussed more in detail in Chapter 4.

Table 8. Metal concentrations (mg L⁻¹) that are reported to be toxic for SRB.

Biomass type	Al	Cd	Cr(III)	Cr (VI)	Cu	Fe	Hg	Ni	Pb	Zn	Reference
<i>Desulfovibrio desulfuricans</i>	27 ^a	nr	nr	nr	nr	nr	nr	nr	nr	nr	Amonette et al. 2003
Sulfate reducing enrichment culture from wastewater	nr	nr	60 ^b	nr	4 ^b	nr	nr	10 ^b	> 80 ^b	nr	Hao et al. 1994
Cu-adapted mixed sulfate reducing culture	nr	nr	nr	nr	170 ^c	nr	nr	nr	nr	nr	Jalali and Baldwin 2000
Sulfate reducing batch and continuous flow reactors	nr	nr	23 ^b	nr	6 ^b	nr	nr	13 ^b	25 ^b	25 ^b	Morton et al. 1991
Wastewater treatment sludge	nr	nr	nr	nr	80	> 400	nr	nr	nr	150	Martins et al. 2009
<i>Desulfovibrio desulfuricans</i>	nr	nr	nr	nr	nr	nr	nr	10	nr	13	Poulson et al. 1997
Mixed SRB culture	nr	nr	nr	nr	12 ^b	nr	nr	nr	nr	20 ^b	Utgikar et al.2001
Sulfate reducing strain L-60	nr	40	nr	nr	nr	nr	74	nr	80	nr	Loka Brathi et al. 1990
SRB enrichment	nr	nr	nr	nr	100 ^c	nr	nr	nr	nr	nr	Song et al. 1997
<i>Desulfobacterium sp.</i>	nr	nr	nr	35-70	nr	nr	nr	nr	nr	nr	Karnachuk et al. 1995
SRB culture from manure digester	nr	112 ^b	nr	nr	64 ^b	nr	nr	59 ^b	nr	65 ^b	Ueki et al. 1991
<i>Desulfotomaculum</i> strain DF-1	nr	nr	nr	nr	nr	nr	nr	nr	> 100	nr	Fortin et al. 1994

^a inhibition with total Al concentration, ^b total inhibition in batch bottle assay, ^c 50% inhibition of sulfate reduction in batch bottle assay with copper adapted inocula. nr = not reported

3 Role of sulfate-reducing bacteria in natural and engineered environments

3.1 Sulfate-reducing bacteria in natural environments

In nature, SRB can be found in any anaerobic environment which contains sulfate and organic material, for example sea and freshwater sediments, flooded soils and wetlands (Widdel 1988). SRB have also been isolated from hydrothermal vents (Jeanthon et al. 2002), microbial mats (Minz et al. 1999), AMD (Sen 2001), soda lakes (Brandt and Ingvorsen 1997) and the deep sub-surface (Kovacik et al. 2006). SRB have an important role as terminal degraders in the anaerobic degradation of organic matter (Barton and Fauque 2009). The capability to produce sulfide and to precipitate and reduce metals makes SRB important participants in the biogeochemical cycle of sulfur and metals (Barton and Fauque 2009). The SRB participation in the formation of metal sulfide, sulfur and carbonate deposits has been demonstrated (Widdel 1988).

3.1.1 Competition of sulfate-reducing bacteria, methanogenic archaea and homoacetogenic bacteria

The SRB compete for electron donors with methanogenic archaea and homoacetogenic bacteria in anaerobic environments (Gibson 1990, Muyzer and Stams 2008) as shown in Figure 3. SRB use a wide variety of substrate in comparison to methanogens, which use only some fatty acids (e.g. acetate) and H_2 (Gibson 1990, Muyzer and Stams 2008). In addition to acetate and H_2 , methanogens are able to utilize formate, methanol, ethanol, isopropanol, methylated amines and pyruvate (Stams et al. 2005).

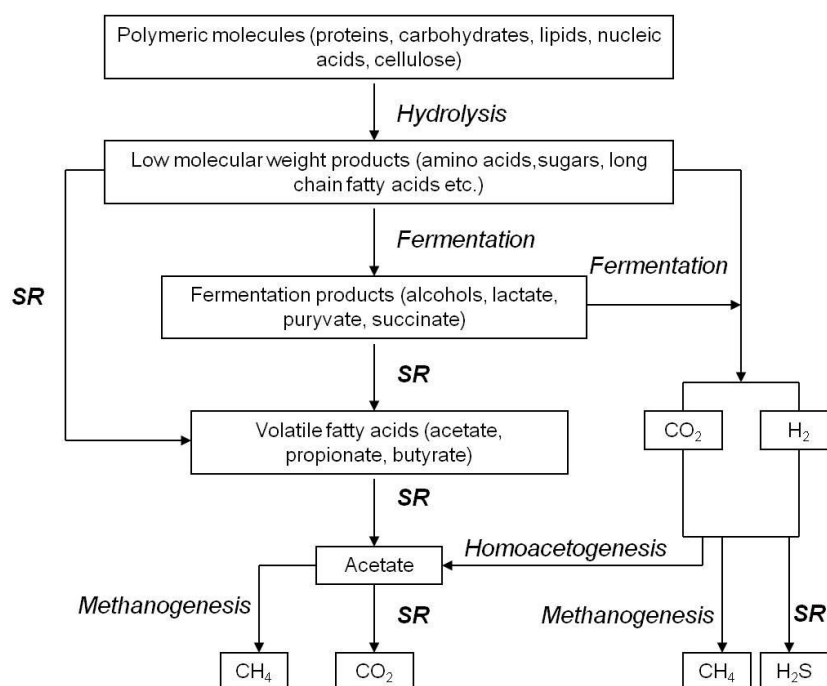


Figure 3. The steps included in anaerobic degradation of organic matter. The complex molecules are stepwise degraded to smaller molecules, and these serve as substrates for the terminal oxidation processes, methanogenesis and sulfate reduction (adapted from Gibson 1990). SR = sulfate reduction

When H_2 is the major electron donor, the SRB outcompete methanogens and homoacetogens for the electron donor, because SRB have a higher affinity for H_2 and also a lower threshold

value of H₂ utilization (Muyzer and Stams 2008). The competition of methanogens and SRB for acetate also favors SRB, but if sulfate limitation is applied, methanogens succeed (Bhattacharya et al. 1996). The competition for acetate is dependent also on the availability of other electron donors, and methanogens may also efficiently compete for acetate in sulfate rich conditions (Stams et al. 2005). In sea sediments, SRB are the major acetate consumers (Stams et al. 2005). Table 9 summarizes specific kinetic parameters for hydrogen and acetate utilization by some SRB and methanogens. These parameters are indicative of the competition between SRB and methanogens.

Table 9. Kinetic parameters for hydrogen and acetate utilization by some SRB and methanogens according to Oude-Elferink et al. (1994) and Stams et al. (2003).

Species	Hydrogen				
	K _m (μ M)	K _s (μ M)	V _{max} (μ mol min ⁻¹ g ⁻¹)	μ _{max} (d ⁻¹)	Y (g mol ⁻¹)
<i>Desulfovibrio desulfuricans</i>	1.8-4.0	nr	88	1.6-4.3	1.9
<i>Desulfovibrio vulgaris</i>	1.3-4.0	nr	30	0.7-5.5	0.6-3.1
<i>Desulfovibrio G11</i>	1.1	2.4-4.2	65	1.2-1.6	1.4-2.0
<i>Desulfomicrobium escambium</i>	nr	nr	nr	1.4	nr
<i>Methanobacterium bryantii</i>	nr	nr	nr	0.3-1.9	0.6
<i>Methanobacterium formicicum</i>	2	nr	nr	1.2-3.1	0.9
<i>Methanobacterium ivanovii</i>	14	nr	nr	0.8-1.7	1.1
<i>Methanobrevibacter arboriphilus</i>	6.6	nr	nr	0.7-3.4	0.6-1.3
<i>Methanospirillum hungatei</i>	5.0	5.8-7.3	70	1.2-1.8	0.3-0.5
Species	Acetate				
	K _m (mM)	K _s (mM)	V _{max} (μ mol min ⁻¹ g ⁻¹)	μ _{max} (d ⁻¹)	Y (g mol ⁻¹)
<i>Desulfobacter postgatei</i>	0.07-0.2	nr	53	0.7-1.1	4.3-4.8
<i>Desulfotomaculum acetoxidans</i>	nr	nr	nr	0.7-1.4	5.6
<i>Desulfohabdus amnigena</i>	0.6	nr	28	0.14-0.2	nr
<i>Desulfobacca acetoxidans</i>	0.6	nr	43	0.3-0.4	nr
<i>Methanosarcina barkeri</i>	3.0	5.0	nr	0.5-0.7	1.6-3.4
<i>Methanosarcina mazei</i>	nr	nr	nr	0.5	1.9
<i>Methanosaeta soehgenii</i>	0.4-0.7	0.5	38	0.08-0.3	1.1-1.4
<i>Methanosaeta concilii</i>	nr	0.8-1.2	16	0.2-0.7	1.1-1.2

μ M = μ mol L⁻¹, mM = mmol L⁻¹, nr = not reported

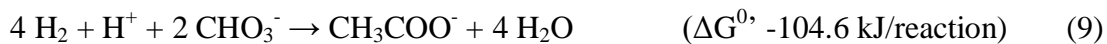
3.1.2 Role of sulfate-reducing bacteria in low temperature sea sediments

Although sulfate reduction may proceed at slow rates in the sediments, it is a significant part of the carbon cycle as sulfate reduction accounts for 6-62% of the carbon mineralization in the sea sediments (Jørgensen 1982, Alongi et al. 2007). According to Finke and Jørgensen (2008) the rate of sulfate reduction in Arctic sea sediment is dependent on the rate of fermentation and the rate of both of these processes are temperature dependent. The sulfate reduction rate of the Arctic sea sediment incubations above *in situ* temperature decreased over time, and did not increase as was expected (Finke and Jørgensen 2008). In temperate North Sea sediment the fermentative species tolerated higher temperatures than SRB (Finke and Jørgensen 2008). Vandieken et al. (2006a) reported that sulfate reduction was the major anaerobic mineralization process in Arctic sediment samples contributing to 57 % of the

organic matter degradation, the rest being the result of Fe (III) reduction. The carbon turnover rate in the arctic coastal sediments was in the same range with the rates measured in temperate sediments (Arnosti et al. 1998). Vandieken et al. (2006a) reported that *Desulfuromonas*, *Desulfuromusa*, *Shewanella* and *Desulfovibrio* reduced Fe (III) in arctic sediment samples. Another study showed that sulfate reduction was the dominating carbon mineralization process in Antarctic sediment samples (Purdy et al. 2003). The main sulfate reducing species in these samples belonged to group *Desulfotalea-Desulfohopalus* (Purdy et al. 2003). These psychrophilic SRB species have been isolated from Arctic sediment by Knoblauch et al. (1999), and their identification from Antarctic sediments illustrates their importance in the low- temperature deep-sea sediments (Purdy et al. 2003).

3.1.3 Homoacetogenic bacteria

Anaerobic homoacetogenic bacteria use CO₂ (as dissolved carbonic acid) and H₂ to synthesize acetate via the reductive acetyl-CoA pathway (for a review, see Drake et al. 2006) as shown in Equation 9 (Thauer et al. 1977):



Homoacetogenic bacteria commonly belong to the genus *Acetobacterium*, *Acetoanearobium*, *Acetogenium* and *Clostridium*. Also *Desulfotomaculum orientis* and *Desulfobacterium autotrophicum*, for example, have been shown to grow via homoacetogenesis (Klempes et al. 1985, Brysch et al. 1987, Drake et al. 2006). The products of homoacetogenic bacteria depend on the cultivation conditions and substrates, thus these bacteria can also produce acetate by fermentation from a wide range of organic molecules (Drake et al. 2006).

In Tundra soils, where the only inorganic electron acceptor is carbonate, methanogens and homoacetogens are responsible for H₂ consumption (Kotsyurbenko et al. 2001). The competition is defined by H₂ uptake kinetics because H₂ concentrations are low in soils (Kotsyurbenko et al. 2001). The threshold pressure for H₂ is the minimal partial pressure in which a specific microbe is still able to take up H₂, and this characteristic is temperature dependent (Kotsyurbenko et al. 2001). The psychrotolerant homoacetogenic species had decreasing H₂ threshold pressures when the temperature was decreased, thus they lost the competition for H₂ to methanogens, as methanogens were able to maintain lower H₂ threshold pressures (Kotsyurbenko et al. 2001). Kotsyurbenko et al. (1996) reported that during incubations of tundra soil samples, the homoacetogens had higher growth yields than methanogens with H₂/CO₂ at temperatures 6-28°C, and the difference between the yields increased as temperature was decreased. This demonstrated the competitiveness of homoacetogens over methanogens at non-H₂ limiting conditions at temperatures below 10°C (Kotsyurbenko et al. 1996).

3.1.4 Interactions of sulfate reducing prokaryotes with other species

If the availability of sulfate is limited, syntrophic communities are responsible for the oxidation of organic acids (Muyzer and Stams 2008). Brysch et al. (1987) reported that when sulfidogenic enrichment was grown with H₂ and CO₂, the presence of *Acetobacterium* sp. in the enrichment was identified, indicating a commensalistic relationship between acetate producing *Acetobacterium* and heterotrophic SRBs. In this mixed culture of *Desulfovibrio* sp. and *Acetobacterium* sp. the *Desulfovibrio* sp. grew faster than the *Acetobacterium* sp., therefore the *Acetobacterium* sp. did not take over the mixed culture (Brysch et al. 1987). Acetogenic bacteria are present in anaerobic environments and are able to consume H₂ and CO₂ at fast rate (Krumholz et al. 1999). The species in the study by Krumholz et al. (1999) were *Desulfomicrobium hypogeium* and *Acetobacterium psammolithicum*. Over a pH range

of 6.8-7.9 the pH did not control the flow of electrons to sulfate reduction and acetogenesis in H₂/CO₂ supplemented enrichment cultures at 30°C (Krumholz et al. 1999). Under laboratory conditions the electron flow from H₂ to sulfate reduction was 23-30% and the autotrophic acetogenesis was favored as electron sink in this enrichment culture (Krumholz et al. 1999). The K_m (affinity to substrate) values for H₂ are higher for acetogens than for SRB, but SRB possess higher V_{max} (specific oxidation rate) with H₂, and therefore SRB have a competitive advantage (Krumholz et al. 1999). The threshold value for H₂ was 100 times higher for *A. psammolithicum* than for *D. hypogeiium*, but the authors suggested that in natural environments, where H₂ is present at threshold concentrations, the SRB dominate as H₂ consumers (Krumholz et al. 1999).

Anaerobic methane oxidation, in which sulfate reduction is coupled to methane oxidation, has been shown to occur in marine sediments (Nauhaus et al. 2007) and also in bioreactor conditions (Meulepas et al. 2009). Anaerobic methane oxidation coupled to sulfate reduction requires syntrophic interaction between species, but the mechanism of this relationship is still somewhat unclear, although several mechanisms have been proposed (Valentine and Reeburgh 2000, Nauhaus et al. 2002).

3.2 Sulfate-reducing bacteria in engineered environments

SRB have been found in paper industry process waters, oil fields and anaerobic digesters (Widdel 1988, Gibson 1990). SRB cause economic damage due to the toxic effects of H₂S, corrosion of metals (e.g. pipes), concrete and stonework, spoilage of food products and the souring of oil and gas reservoirs (Barton and Fauque 2009). H₂S also causes problems due to malodour (Bhattacharya et al. 1996). Anaerobic digesters treating organic wastewaters may suffer from high sulfide concentrations that inhibit methane production and contaminate the produced biogas (Muyzer and Stams 2008). The H₂S produced by SRB causes souring of oil and gas reservoirs (Barton and Fauque 2009). The latter is due to the practice of filling the emptied reservoirs with seawater, bringing together sulfate and organic material in crude oil, which SRB oxidize (Barton and Fauque 2009). Apart from reservoir souring, H₂S corrodes piping and causes blockages in the pipes due to formation of metal precipitates (Barton and Fauque 2009). The activities of SRB in industrial processes have been controlled with biocides, nitrate or by limiting the availability of nutrients, sulfate or electron donors, but also by varying pressure and pH (Widdel 1988).

In anaerobic bioreactors, the competition between methanogens and SRB is affected by the kinetic properties of the species, adherence, the ability to utilize mixed substrates, the relative number of the microbes, pH, temperature and concentrations of inhibitors, e.g. H₂S or ammonia (Oude Elferink et al. 1994). In these anaerobic reactors, SRB and methanogens compete directly only for H₂ and acetate, because these are the common substrates for both groups (Stams et al. 2005). Oude Elferink et al. (1998) reported that in a full-scale anaerobic reactor treating wastewater from a paper mill the methanogens consumed acetate, formate and hydrogen. Propionate was consumed by SRB, which competed with *Synthrobacter*-like bacteria for this substrate (Oude Elferink et al. 1998). Theoretically, all the COD (chemical oxygen demand) can be oxidized by SRB, if the molar COD to sulfate ratio is below 0.66-0.5 (Oude Elferink et al. 1998). In hydrogen fed reactors the SRB, methanogenic archaea and homoacetogenic bacteria (HB) compete for H₂ (Weijma et al. 2002). The thermodynamics of H₂ utilization favor the SRB over methanogens, which in turn utilize H₂ more efficiently than homoacetogens (Stams et al. 2005). Excessive acetate production results in loss of electron donor in full-scale reactor operation, but at low concentrations the produced acetate nevertheless supports the growth of heterotrophic SRB (Weijma et al. 2002).

4 Sulfate reducing bioprocesses for metal precipitation and recovery

The sulfate reducing processes can be applied for sulfate removal from wastewaters, selective metal recovery, SO₂ removal from flue gas (Buisman et al. 2007), and treatment of paper mill wastewater (Janssen et al. 2009), but also to treat sulfuric acid waste, AMD and acid rock drainage (ARD), lime plant feed, metallurgical process streams (smelters, refiners and metal processing plants) and bioleaching liquors (Lawrence et al. 2003, Huisman et al. 2006). The formation and effects of AMD have been reviewed by Johnson and Hallberg (2005). AMD and mine wastewaters cause severe damage to the biota of the receiving water bodies (Tabak et al. 2003). AMD usually contains more than 3 g L⁻¹ of sulfate, a high concentration of dissolved solids and several heavy metals (Tabak et al. 2003). Table 10 shows examples of the pH, metal and sulfate concentrations reported for AMD, mine wastewaters and metal contaminated ground water.

Table 10. AMD, mine- and metallurgical industry wastewater compositions, the unit is mg L⁻¹ except for pH.

Site	pH	SO ₄ ²⁻	Al	As	Cd	Co	Cu	Fe	Mg	Mn	Ni	Zn	Reference
Kennecott Copper mine	nr	25000	2412	nr	nr	nr	44	512	2640	200	nr	82	Buisman et al. (1999)
Berkley Pit AMD	2.2	2400	293	0.51	1.38	1.23	223	514	nr	223	2.1	630	Tabak et al. (2003)
Zinc mine AMD	3.7	3800	nr	nr	nr	nr	19	337	nr	nr	nr	660	Huisman et al. (2006)
Berkley Pit AMD	2.3	8150	24	nr	1.96	1.51	178	879	nr	150	1.0	nr	Hammack and Dijkman (1999)
Kennecott Copper mine AMD	nr	30000	2200	nr	nr	nr	nr	675	4500	350	nr	nr	van Houten et al. (2006)
Budelco zinc smelter wastewater	nr	nr	nr	nr	nr	nr	nr	nr	10- 300	nr	nr	3000- 5000	van Houten et al. (2006)
Copper Queen Mine wastewater	2.2- 2.4	nr	3950	nr	nr	nr	nr	1800 - 2500	2890	1620	nr	930	Ashe et al. (2008)
Narembec metal contaminated groundwater	3.1	2260	156	0.01	nr	0.06	0.15	3.2	1620	1.3	0.1	0.8	Franzmann et al. (2008)
Leviatham Mine, CA, USA	2.9- 3.2	nr	nr	20- 30	nr	nr	0.7	90- 220	nr	nr	0.5	0.9	Tsakamoto et al. (2004)

nr = not reported

The most common treatment for metal and sulfate containing wastewaters is currently precipitation with hydrated lime (Ca(OH)₂) or limestone (CaCO₃) (Huisman et al. 2006, Buisman et al. 2007). The metals precipitate as hydroxides and sulfate as gypsum (Buisman et al. 2007, Huisman et al. 2006). Sulfate precipitation with lime results in effluent sulfate concentration of 1600-2000 mg L⁻¹, because of the solubility of gypsum (Lopez et al. 2009, Lawrence et al. 2003). The removal of residual sulfate is difficult by chemical or physical techniques (Buisman et al. 2007). The hydroxide precipitation is also relatively easy to control based on pH (Veeken et al. 2003). When AMD is treated with hydroxide precipitation, the end product is a mixture of metal hydroxides with no re-use potential, and this sludge requires safe disposal (Tabak et al. 2003). Lime treatment plants may produce either low density metal hydroxide sludge (5% solids) or high density sludge (more than 30% solids) (Lawrence et al. 2003). Removal of the residual metals may require the operation of the lime plant at a higher pH, which results in high lime consumption, especially if the concentration of Mg, Mn and Zn in the treated stream are high (Lawrence et al. 2003).

Alternative physico-chemical techniques for sulfate removal are anion exchange resins (regeneration required) (Lopez et al. 2009), reverse osmosis (Kratochvil et al. 2008), precipitation with barium (Kratochvil et al. 2008) and precipitation as ettringite-mineral (Kratochvil et al. 2008).

4.1 Biological hydrogen sulfide production

4.1.1 Sulfide production

There are a limited number of publications on commercial hydrogen sulfide production. Biologically produced sulfide is claimed to have a lower cost than the sulfide chemicals on the market (Huisman et al. 2006). The sulfide chemicals used in chemical sulfide precipitation are Na_2S , NaHS , CaS , FeS and H_2S (Huisman et al. 2006). The advantage of using biologically produced H_2S is that no extra Na, Ca or Fe is introduced to the process (Buisman et al. 1999). The capacity of biological sulfide production is limited by the capacity of the SRB, and if large volumes of H_2S are needed in the precipitation process, chemical production of H_2S has to be chosen.

The benefits of a biological hydrogen sulfide production process are: 1) production rate can be controlled via dosage of the electron donor, 2) on-site process can be operated on demand and 3) the transport and storage safety issues and costs can be excluded (Huisman et al. 2006). When the production scale of biologically produced H_2S is more than 2 tons of H_2S d^{-1} , the electron donor is H_2 from steam reformation of natural gas or cracking of methanol (Buisman et al. 1999). For example, Paques B.V. has been operating a 500 m^3 H_2 -fed gas-lift reactor in Budelco zinc refinery for the treatment of sulfate and zinc polluted ground water (Buisman et al. 1999) for more than 10 years. At the moment the largest sulfate reducing reactor systems utilize waste products (organic waste concentrate, cellulose wastewater, fermentation effluent) as electron donors (Buisman et al. 2007). The sulfide production rates obtained in the plants are rarely reported, but for example the sulfide production rate of the mine wastewater treatment plant in a North American zinc mine was 0.26-0.43 kg m^{-3} reactor volume (Huisman et al. 2006). The industrial scale sulfate reducing bioreactors operating in 2010 are summarized in Table 11. Sulfur-reducing bioreactors are also included, as they are used for biological hydrogen sulfide production. Table 11 provides an overview of the capacity of the biological sulfate reduction technology. Since the process conditions and capacities of these industrial scale bioreactors have not been reported in similar manner for each case, the comparison becomes difficult.

Table 11. Capacities and process parameters of industrial scale and pilot scale sulfidogenic treatment plants.

Reactor type, site	Electron donor	pH, T (°C)	Influent Q (m ³ h ⁻¹)	Treatment capacity	Products	Reference
Gas-lift, Budelco	H ₂ (from natural gas reformation)	nr	400	500 kg SO ₄ ²⁻ /h	ZnS	Buisman et al. (1999)
Gas-lift, at US zinc mine	nr	Nr	700	0.26-0.43 kg S m ⁻³ AMD	nr	Huisman et al. (2006)
UASB	Ethanol	7.0 26.0	0.01	9.9 g SO ₄ ²⁻ L ⁻¹ d ⁻¹ ^a	CuS, ZnS	Hammack and Dijkmann (1999)
UASB, Zinifex 1	Ethanol	nr	nr	1.2 ton S d ⁻¹	ZnS, FeS, S ⁰	Buisman et al. (2007)
UASB, Emmetec	Ethanol	nr	nr	1.2 ton S d ⁻¹	S ⁰	Buisman et al. (2007)
Gas-lift, Zinifex 2	H ₂	nr	40 m ³ h ⁻¹	3.2 ton S d ⁻¹	ZnS (10 tons d ⁻¹)	Boonstra and Buisman (2003), Buisman et al. (2007)
UASB, Landau colliery	Concentrated organic waste	nr	nr	2.5 ton S d ⁻¹	CaCO ₃ , S ⁰	Buisman et al. (2007)
UASB, Lenzig	Cellulose waste water	nr	nr	6 ton S d ⁻¹	ZnS, S ⁰	Buisman et al. (2007)
UASB, BioDeSOx ^b	Fermentation effluent	nr	nr	12.5 ton S d ⁻¹	S ⁰	Buisman et al. (2007)
Gas-lift	Synthesis gas ^c	7-7.5 30-35	7-21	15 kg SO ₄ ²⁻ m ⁻³ d ⁻¹	ZnS	van Houten et al. (2006)
UASB, Eerbeek	Paper mill effluent	7.1 nr	480-1200	nr ^d	S ⁰ , CH ₄	Janssen et al. (2009)
Stirred-tank ^e Bisbee Mine	Acetate	nr 25	10900	3.5 ton H ₂ S d ⁻¹	CuS	Lawrence et al. (2003), Ashe et al. (2008)
Stirred-tank ^e Caribou zinc mine	nr	nr	700	nr	ZnS, CuS, CdS, PbS	Lawrence et al. (2003)
Stirred-tank ^e Caribou zinc mine	nr	nr	2100	2100 ton d ⁻¹ contaminated tailings	ZnS, CuS, CdS, PbS	Lawrence et al. (2003)

nr = not reported, UASB = up-flow anaerobic sludge blanket reactor, T = Temperature, influent Q = influent flow rate, ^a HRT 3.2 h, ^b flue-gas treatment plant, ^c the composition of the synthesis gas 76 % H₂, 20 % CO₂, 3 % N₂ 1% CO and CH₄, ^d simultaneous methane production and sulfate reduction in which the CH₄ production is the major process, ^e Sulfide production via sulfur reduction.

4.1.2 Electron donors

The various electron donors used by SRB were discussed in Chapter 1. In this Chapter, only the commercially used electron donors are discussed. Gaseous electron donors, such as hydrogen (van Houten et al. 1994) and synthesis gas (van Houten et al. 1996, van Houten et al. 2006) are used in industrial scale reactors, but direct use of methane (Meulepas et al. 2009) is of high interest. Alcohols, for example, ethanol (Kaksonen et al. 2004a) or methanol (Weijma et al. 2000), and also lactate (Kaksonen et al. 2003b) are good substrates for SRB, but their limitation is the accumulation of acetate as intermediate oxidation product (Kaksonen et al. 2003a) and the high market price of pure substrates. Organic waste materials would be a feasible option, but their weakness is varying quality and quantity (Bijmans 2008), and SRB are not able to directly use very complex organic material. The electron donor also affects of the microbial ecology of the system, as other anaerobic microbes, e.g. methanogenic archaea and homoacetogenic or fermentative bacteria compete for the substrate with SRB in mixed cultures. Van Houten et al. (2006) reported remarkable acetate production by homoacetogenic bacteria in a synthesis gas fed sulfate reducing bioreactor. The highest acetate concentration was 4 g L⁻¹ (van Houten et al. 2006). The acetogenesis ceased during the reactor operation, because the acetogens were not able to efficiently compete with SRB and methanogens (van Houten et al. 2006). Also a significant part of the hydrogen was directed to methane production (28-47 %) (van Houten et al. 2006). Methanogenesis was controlled by limiting the CO₂ supply, but it could not be completely suppressed (van Houten et al. 2006).

Many low cost materials have been tested as substrates for SRB in bioremediation of mine waste waters and AMD. The solid substrates are suitable for passive systems, but for active bioreactor treatment, liquid or gaseous substrates are required. The low cost substrates include wine waste (Costa et al. 2008), sewage sludge (Waybrant et al. 1998), leaf mulch (Waybrant et al. 1998), wood chips (Waybrant et al. 1998, Zagury 2006), manure (Waybrant et al. 1998, Zagury et al. 2006) and sawdust (Waybrant et al. 1998), leaf compost (Zagury et al. 2006) and cheese whey (Drury 1999). These solid substrates can be utilized in semi-passive barriers (Waybrant et al. 1998) or column-type reactors (Zamsov et al. 2006) and anaerobic filters (Tsukamoto et al. 2004). Soluble substrates, for example molasses (Maree and Strydom 1987) and biodiesel manufacturing waste (glycerol) (Zamsov et al. 2006) are suitable for bioreactors.

The electron donor is the largest single operational cost of a sulfate reducing bioreactor (Buisman et al. 2007). The largest operating sulfate reducing plants (see Table 11) utilize waste products as electron donors (Buisman et al. 2007). Therefore, the research for low-cost electron donors has generated a lot of interest. Also the use of methane has commercial interest, because the electron donor costs would decrease by 50 % when compared to hydrogen and ethanol (Buisman et al. 2007). This would make a sulfate reducing plant a lower-cost option than a chemical lime treatment plant (Buisman et al. 2007). The energy yield of methane oxidation coupled to sulfate reduction is -17 kJ mol^{-1} , which results in extremely slow growth rates of these organisms, as doubling times can extend up to 7 months (Nauhaus et al. 2006).

The local availability and the market price of pure electron donors (ethanol, H_2 etc.) for sulfate reducing plants have to be considered case specifically, because the price and availability of electron donors varies geographically. The calculation of electron donor costs are often not reported. For these reasons, it is difficult to make an economical comparison. Hydrogen is the most energetic electron donor for SRB (Thauer et al. 1977), and is, therefore, preferred either as pure substrate or in synthesis gas, which also contains other gases (synthesis gas composition: 76 % H_2 , 20 % CO_2 , 3 % N_2 1% CO , van Houten et al. 2006). Hydrogen fed to sulfate reducing bioreactors is usually produced by reforming natural gas, and this requires building a separate process unit (van Houten et al. 1996, Hammack and Dijkman 1999). The study of the economics of treatment of the Berkley Pit AMD by Hammack and Dijkman (1999) compared the use of ethanol and H_2 , and indicated that H_2 is more feasible option as electron donor as shown in Table 12. The economics of H_2 and ethanol use are also dependent on the reactor size.

Table 12. Comparison of the economics of ethanol and natural gas (as H_2) as electron donors for treatment of Berkley Pit AMD with inflow of $19\,000 \text{ m}^3 \text{ d}^{-1}$ and including recovery of Cu and Zn (Hammack and Dijkman 1999). The unit is USD per m^3 treated AMD.

	Ethanol fed UASB	Gas-lift reactor fed with H_2
Operational costs	1.60	0.55
Capital costs (USD)	1.00	0.67
Combined costs	2.60	1.22
Value of recovered metals	0.57	0.57
Actual costs	2.03	0.65

A more recent comparison of the commercially used electron donor cost to reduce 1 kg of biological sulfate reduction is shown in Table 13 (Bijmans 2008). Table 13 shows that synthesis gas is the most economic commercial electron donor at the moment, but as will be discussed below, the largest sulfate reducing plants are operated with waste materials as electron donors (Buisman et al. 2007).

Table 13. Electron donor costs for reduction of 1 kg sulfate (Bijmans 2008).

Electron donor	Market price (01/2008)	Volume needed to reduce 1 kg SO ₄ ²⁻	Price of electron donor (USD kg ⁻¹ SO ₄ ²⁻)
Ethanol	0.6 USD L ⁻¹ ^{a, b}	0.4 L	0.24
H ₂ ^c	0.21 USD m ⁻³ ^d	0.934 m ⁻³	0.20
Synthesis gas ^e	± 0.15 USD m ⁻³ ^f	0.934 m ⁻³	± 0.14
Natural gas ^g	0.24 USD m ⁻³ ^h	0.292 m ⁻³	0.07

^a Ethanol market <http://ethanolmarket.aghost.net> (Jan 2008), ^b California Energy Commission, http://www.energy.ca.gov/gasoline/graphs/ethanol_10-year.html (Jan 2008), ^c produced from natural gas, ^d Mueller-Langler et al. (2007), ^e 76% H₂, 16% CO₂ (produced from natural gas), ^f the cost will be between hydrogen and natural gas, ^g 80% CH₄, ^h Energy Information Administration http://tonto.eia.doe.gov/dnav/ng/ng_pri_sum_dcu_nus_m.htm

Franzmann et al. (2008) estimated that treatment of metal contaminated, highly saline Narembee groundwater with ethanol-fed sulfidogenic FBR would cost 3.72 USD m⁻³ treated water (includes electron donor, capital costs and disposal of metal sulfides), but this value was calculated based on lower sulfate reduction rate than that estimated by Bijmans (2008).

Hydrogen sulfide can be produced from elemental sulfur by sulfur-reducing bacteria in a site without sulfate containing waste stream (Huisman et al. 2006). For biological H₂S production via sulfur reduction, less electron donor is required, thus the electron donor costs are only 25% of a similar sulfate reduction process (Huisman et al. 2006).

4.2 Metal sulfides

When sulfidogenic treatment is applied to AMD, the produced metal sulfide sludge has low risk of metal re-solubilisation at neutral and acidic conditions, but sludge storage is preferably under anoxic flooded open pit or similar conditions (Lawrence et al. 2003). Sulfide precipitation allows selective recovery of specific metals (Huisman et al. 2006, Bijmans et al. 2009a). For example Cu, Zn, Ni and Mn can be selectively recovered as sulfides by varying the precipitation pH (Lawrence et al. 2003). Metal sulfides have 6-10 times lower sludge volume than hydroxide sludges, and also the effluent metal concentration is lower (0.10-1 mg L⁻¹) when metals are precipitated as sulfides (Huisman et al. 2006). The qualities of metals sulfide sludge, e.g. higher density and stability and better thickening and dewatering properties improve their processing in the refinery (Peters et al. 1985, Huisman et al. 2006). The use of sulfide precipitation also decreases the lime consumption in the effluent treatment (Lawrence et al. 2003, Huisman et al. 2006). The corrosiveness of hydrogen sulfide on the process equipment can be avoided via using plastic containers (Huisman et al. 2006).

Metal precipitation as sulfides and hydroxides are the most common strategies for mine wastewater treatment, but the qualities of these products differ. Table 14 summarizes the solubilities of selected metal sulfides and hydroxides. A lower solubility product is associated with a higher precipitation tendency of the product.

Table 14. The solubility products of selected metal sulfides and hydroxides (mol L⁻¹) (Dean 1999).

	Ag	Al	Cu	Co	Fe	Mg	Mn	Ni ^a	Pb	Zn
MeS	6.3*10 ⁻⁵⁰	2.0*10 ⁻⁷	6.3*10 ⁻³⁶	4.0*10 ⁻²¹	6.3*10 ⁻¹⁸	-	3.2*10 ⁻¹⁹	2.0*10 ⁻²⁶	8.0*10 ⁻²⁸	6.6*10 ⁻⁵⁰
MeOH	2.0*10 ⁻⁸	1.3*10 ⁻³³	2.2*10 ⁻²²	5.9*10 ⁻¹⁵	4.9*10 ⁻¹⁷	5.6*10 ⁻¹²	1.9*10 ⁻¹³	5.5*10 ⁻¹⁶	1.4*10 ⁻¹⁵	3.0*10 ⁻¹⁷

MeS = metal sulfide, MeOH = metal hydroxide, ^a the value for NiS is for γ-NiS, the values for α-NiS β-NiS are 3.20*10⁻¹⁹ and 1.0*10⁻²⁴, respectively.

The concentration of soluble metals of selected metal sulfides vs. pH is shown in Figure 4, which demonstrates the low solubility of metal sulfides over a wide pH range.

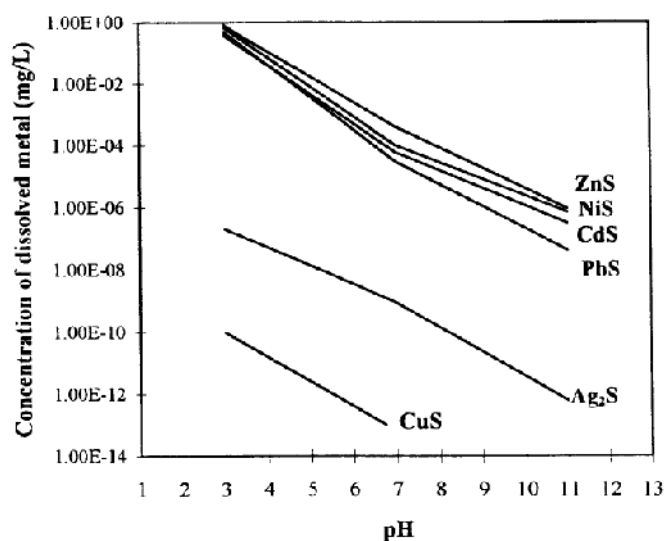


Figure 4. The concentration of dissolved metal of CuS, Ag₂S, PbS, CdS, NiS and ZnS at pH 3-11 (US EPA 1985).

The precipitation of metals as sulfides or hydroxides is also dependent on pH and the concentration of dissolved sulfide in solution. Table 15 shows some examples of pH values reported for selective precipitation of metals as sulfides.

Table 15. The pH range for sulfide precipitation of selected metals. The precipitation tendency is dependent on temperature, concentration and activity of the species in the solution, therefore the values given in different references vary.

CuS	CoS	FeS	MnS	NiS	ZnS	Reference
-1.5 - -2 ^a	4.5-5.8 ^a	5-7.4 ^a	7.7-9.7 ^a	5.6-8.6 ^a	2.2-4.1 ^a	Hammack and Dijkman (1999)
2.2-3	-	4.5-6.6	8-10.3	-	2.2-4.5	Tabak et al. (2003)
6-10.2	-	-	-	-	-	Burkin (2001)

^a P_{H₂S} = 0.5 %, above the upper pH limit the effluent metal concentration may exceed 0.1 mg L⁻¹.

Besides pH, the precipitation tendency of metal sulfides is dependent on ORP (oxidation or reduction potential). Pourbaix diagrams (pH-Eh diagrams) are used to visualize the prevalence of the different species. The boundary lines between species are defined by temperature, activity and concentration of the species in the solution (Burkin 2001). Figure 5A (Burkin 2001) shows the different sulfur species in water at 25°C, for example at pH 7, H₂S is the major form of sulfur below ORP -0.2 mV. When metals are included to the S-H₂O system, the figures become more complex. The concentration of the species affects on the determination of the boundary lines between chemical species, therefore the Pourbaix diagrams are indicative in the estimation of the precipitation product, and the product quality should always be analysed with X-ray diffraction (XRD) or equivalent analysis. Figure 5B shows that a Cu-S-H₂O system, demonstrating that in a sulfidogenic bioreactor operating in neutral pH region (pH 6.5-8) with an ORP of -400 mV, Cu will be expected to precipitate as Cu₂S.

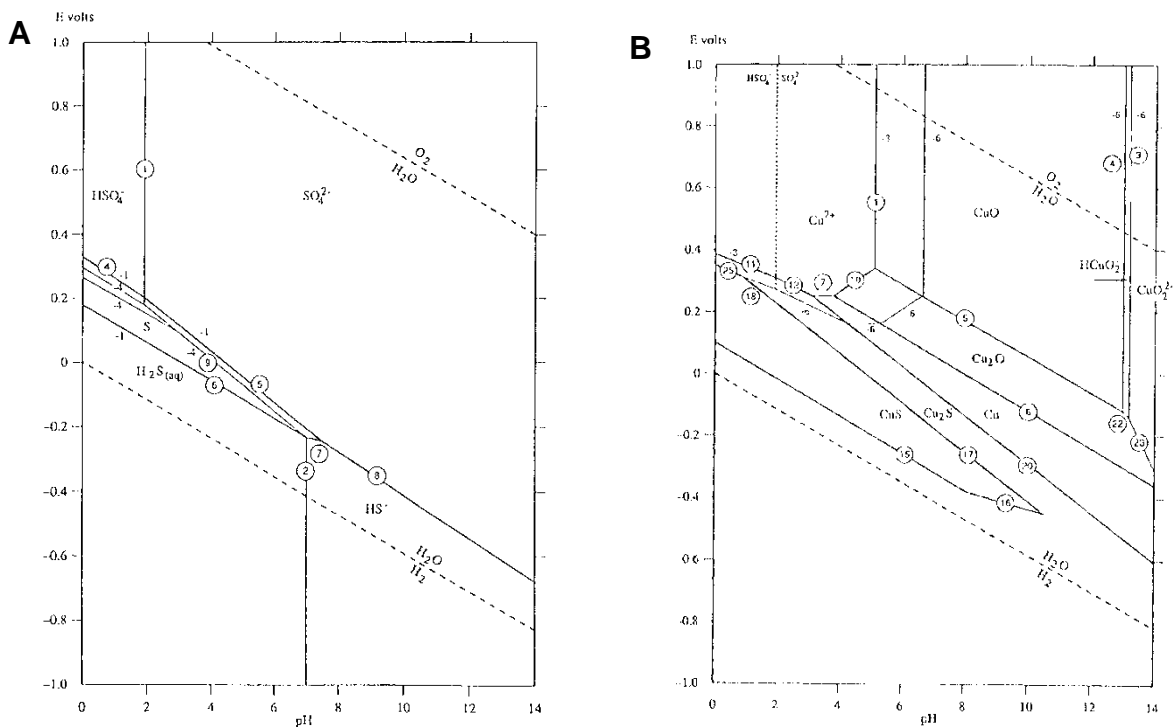


Figure 5. A: The Pourbaix-diagram of S-H₂O system at 25°C, the concentration of S ionic species and H₂S (aq) are 10⁻¹ and 10⁻⁴ mol L⁻¹, respectively (from: Burkin 2001). **B:** The Pourbaix-diagram of Cu-S-H₂O system at 25°C, the concentration of S ionic species and H₂S (aq) is 10⁻¹ and Cu ionic species 10⁻³ and 10⁻⁶ mol L⁻¹ (from: Burkin 2001). The circled numbers in the figures refer to the calculation of the respective boundary lines.

Figure 6A shows that a Fe-S-H₂O system, showing that in a sulfidogenic bioreactor operating in neutral pH region (pH 6.5-8) with an ORP of -400 mV, Fe is expected to precipitate as FeS or Fe₂S. Figure 6B shows the Pourbaix diagram of a Ni-S-H₂O system, demonstrating that in a sulfidogenic bioreactor operating in neutral pH region (pH 6.5-8) with an ORP of -400 mV, Ni will be expected to precipitate as γ-NiS.

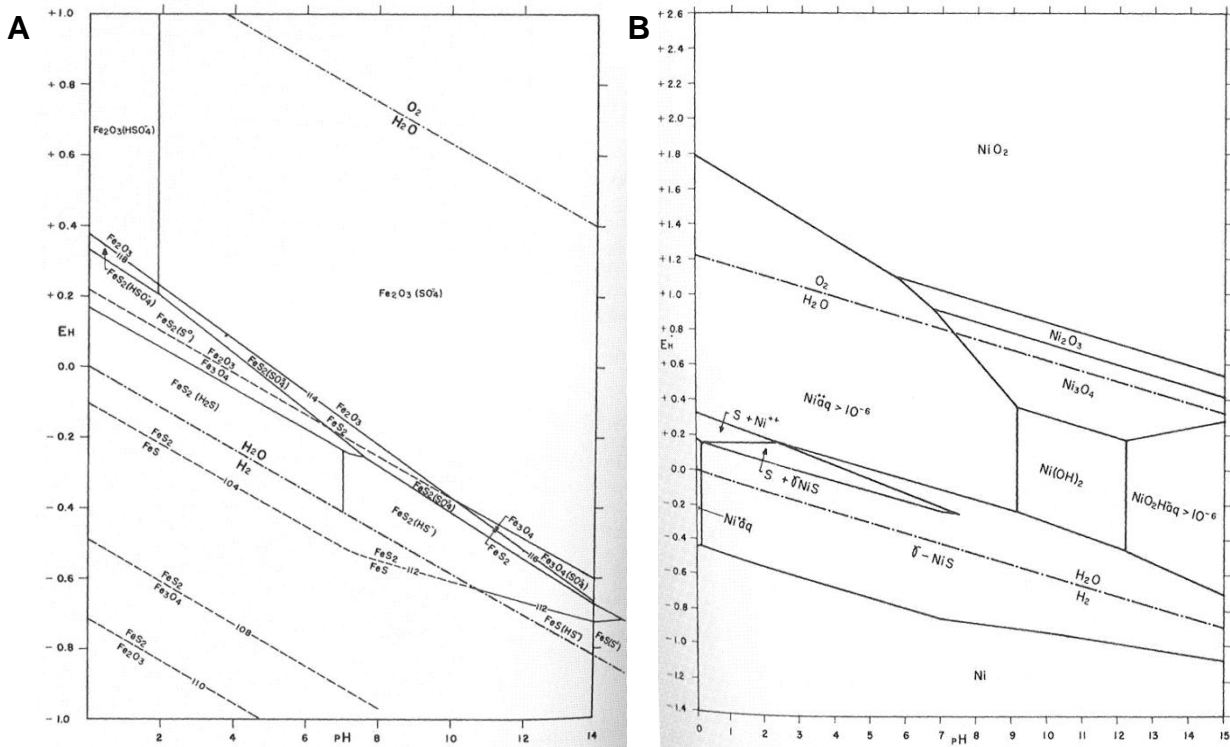


Figure 6. A: The Pourbaix-diagram of Fe-S-H₂O system at 25°C (1 atm), the concentration of total dissolved S is 10⁻¹ (from: Garrels and Christ 1965). **B:** The Pourbaix-diagram of Ni-S-H₂O system at 25°C (1 atm), the concentration of total dissolved S is 10⁻³ (from: Garrels and Christ 1965).

In Figure 7A the Pourbaix diagram of a Mn-S-H₂O system shows that the window for MnS precipitation is very narrow, and only when a sulfidogenic bioreactor operates above pH 8, MnS might be obtained with ORP of -400 mV. Figure 7B presents that of a Co-S-H₂O system, which shows that in a sulfidogenic bioreactor operating in neutral pH region (pH 6.5-8) with an ORP of -400 mV, Co is expected to precipitate as CoS.

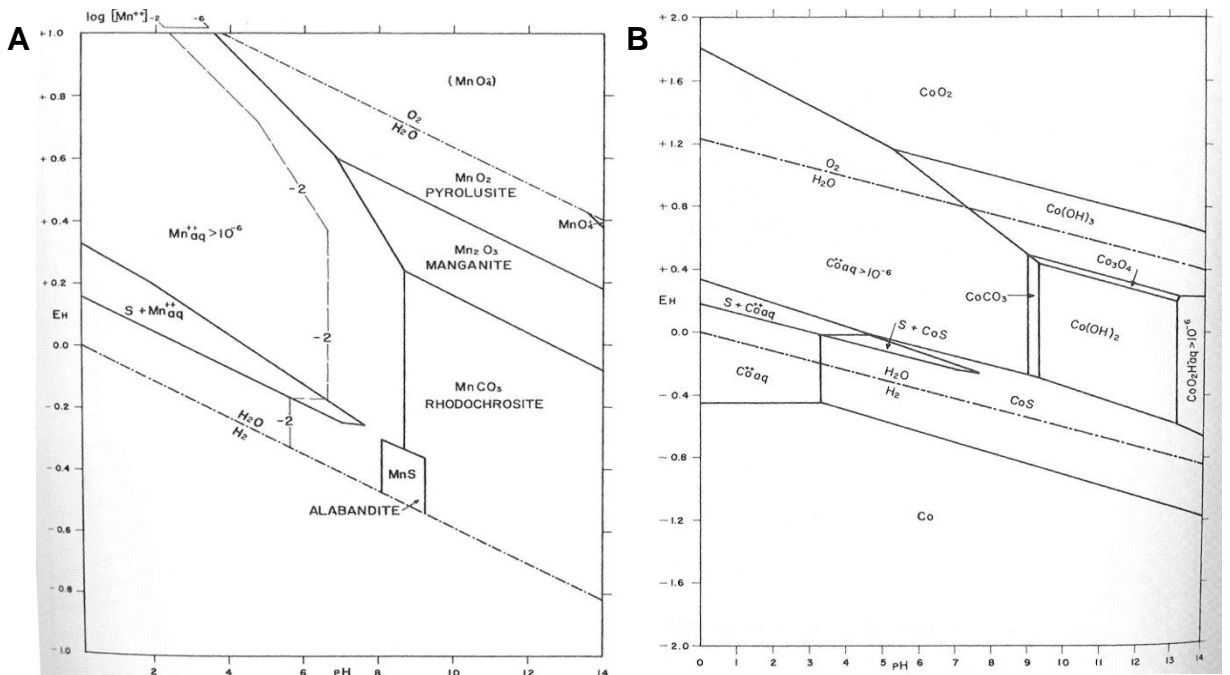


Figure 7. A: The Pourbaix-diagram of Mn-S-H₂O system at 25°C (1 atm), the concentration of total dissolved S is 10⁻¹ (from: Garrels and Christ 1965). **B:** The Pourbaix-diagram of Co-S-H₂O system at 25°C (1 atm), the concentration of total dissolved S is 10⁻¹ and carbonate species 10^{-4.9} (from: Garrels and Christ 1965).

4.2.1 Metal sulfide formation

The stoichiometry of sulfide addition to the metal solution should be carefully controlled, because the unreacted sulfide will remain in the solution and needs to be removed (Veeken et al. 2003). In a bioreactor the hydrogen sulfide production and distribution is homogeneous, which improves the precipitation in a single-stage process when compared to injection of the H_2S from a point source (Bijmans et al. 2009a).

The metal sulfides with low solubility products tend to initially form very small particles and later on small crystals in the solution (Mersmann 1999). This causes problems in the separation of the metal sulfide sludge from the solution (Veeken et al. 2003). On the other hand, Veeken et al. (2003) calculated that mean particle size of $10\ \mu m$ should be sufficient for separating the metal sludge by settling. When smaller particles of $3\ \mu m$ mean particle size are separated by settling, an order of magnitude larger sedimentation units are required (Veeken et al. 2003). Therefore, the agglomeration of the particles has a major role in the metal sulfide particle formation (Veeken et al. 2003). The particle size distribution is dependent on the kinetics of the crystallization (the rate of crystal growth and nucleation), which is in turn controlled by supersaturation, and should be an optimized parameter in the precipitation process (Mersmann 1999). Supersaturation conditions are defined by the ability of the solvent to dissolve the soluble components, e.g. if there are more soluble compounds in the solution than can be dissolved, the solution is regarded as supersaturated (Laidler and Meiser 1999). The supersaturation is a result of energy barrier that has to be overcome before precipitation can occur (Burkin 2001). The level of supersaturation can be controlled by a low concentration of reactants, efficient mixing and by use of precipitation seed (for example recycled sludge) that improves the crystal growth (Mersmann 1999). The crystal size decreases as the supersaturation increases (Mersmann 1999). When the precipitating component is added at a slow rate and the precipitating species has time to form on existing particles, large crystals can be formed (Burkin 2001). Fast addition of the reagent results in homogeneous supersaturation and causes formation of small particles, which tend to aggregate (Burkin 2001).

The initial forms of iron sulfide (FeS) formed by SRB are amorphous with poor crystalline structure (Herbert et al. 1998), but the more stable crystals of mackinawite ($Fe_{1+x}S$) are formed within a few days (Rickard 1995). The formation of highly crystalline mackinawite requires up to 2 years in aqueous solution at $25^\circ C$ (Rickard 1995). Mackinawite also serves as precursor in pyrite (FeS_2) formation, but this process has a very slow rate at ambient temperatures (Schoonen and Barnes 1991). Remoundaki et al. (2008) reported the formation of mainly amorphous Zn and Fe sulfides in a sulfate reducing fixed-bed reactor, which are not suitable for the recovery of metals. Because of these reasons, the particle growth and crystallization of metal sulfides in biological metal precipitation processes have to be optimized. On the other hand, formation of crystalline sphalerite, pyrite and wurtzite has been reported for fluidized-bed reactors and gas-lift reactors (Kaksonen et al. 2003b, Bijmans et al. 2009a; 2009b). Thus the production of recoverable metal sludge is possible in reactor configurations where sulfate reduction and metal precipitation occur simultaneously.

4.2.2 Sulfide precipitation processes

The sulfate reducing processes can be single or two-stage processes. In a single-stage process the biological sulfate reduction and metal precipitation occur in the same unit, but in a two-stage process the sulfate reduction and metal precipitation occur in separate units (Hao 2000). In a two-stage process the H_2S is directed to the metal precipitation unit either in dissolved form or as gas, which is stripped from the bioreactor liquid (Hao 2000, Huisman et al. 2006).

When treating AMD, the metal precipitation stage can be placed ahead of the bioreactor, so the SRB will be less exposed to the toxic effects of metals of the AMD (Hao 2000). If the sulfate and metals are in separate streams, the process configuration is simplified (Hao 2000). When the sulfide production and metal precipitation occur at separate stages, the size of the bioreactor and the sulfide production rate can be optimized (Huisman et al 2006). The process charts of metal precipitation are discussed in the next chapter.

The problem with the single-stage sulfate reduction and metal precipitation process is that high metal concentrations may have inhibitory effects on SRB (Tabak et al. 2003, see Table 8). On the other hand, no toxic effect of free or precipitated Zn, Fe and Ni on SRB were observed in single-stage sulfidogenic reactor processes studied by Kaksonen et al. (2003a; 2003b, 2004a) and Bijmans et al. (2009a; 2009b) because of efficient precipitation, mixing and dilution of the influent in the reactor. Van Houten et al. (2006) reported that high zinc loading to a sulfate reducing gas-lift reactor (at Budelco site, the Netherlands) caused inhibition of sulfate reduction, but that the reactor operation recovered within a week. The ZnS precipitation in the gas-lift reactor did not have inhibitory effect on sulfate reduction rate, even though the total solids (TS) concentration in the reactor was high, 129 g L^{-1} , of which 95 % was ZnS (van Houten et al. 2006).

4.2.3 Metal recovery

The economics of the biologically produced metal sulfide precipitates are dependent on the ability to selectively recover metals and to produce pure metal sludges (Veeken et al. 2003). The quality of the biologically precipitated metal sulfide is high, for example sludge containing 90 % CuS can be obtained (Huisman et al. 2006).

Single-stage processes

Production of crystalline, recoverable metal sulfides in single-stage bioreactors has been shown in many studies, but this requires careful control of the reactor operation. A single-stage bioreactor has low investment costs because of simple reactor design (Bijmans et al. 2009a). The operation of a sulfate reducing bioreactor at low pH enables the selective recovery of specific metal sulfides, and the acidic metal solution does not need to be neutralized to reach the optimal pH of neutrophilic SRB (Bijmans et al. 2009a). The selective recovery of nickel sulfide at pH 5 in a sulfate reducing gas-lift bioreactor has been shown by Bijmans et al. (2009a). In this process the nickel sulfide was selectively precipitated from a nickel-iron solution by controlling the pH (Bijmans et al. 2009). This was possible because the solubilities of the iron and nickel sulfides are dependent on pH, and below pH 5 only NiS precipitates, while no FeS is precipitated, but if the pH increases to 5.5, FeS precipitates as well, and the end product becomes a mixture of Ni and Fe sulfides (Bijmans et al. 2009a). In this experiment nickel precipitated as millerite (NiS), Ni_4S_3 and Ni_3S_4 , and iron as mackinawite (FeS) (Bijmans et al. 2009a). The precipitate contained 63 % Ni, whereas pure nickel sulfide contains 65% Ni (Bijmans et al. 2009a). In a similar experiment, efficient ZnS precipitation as crystalline sphalerite was obtained at pH 5.5 (Bijmans 2009b). The Zn precipitation rate was $7.2 \text{ mmol L}^{-1} \text{ d}^{-1}$ and the effluent Zn concentration was $0.9 \text{ } \mu\text{mol L}^{-1}$ (Bijmans et al. 2009b). Kaksonen et al. (2003b) reported formation of pyrite (FeS_2), iron sulfide (FeS), crystalline sphalerite (ZnS) and wurtzite (ZnS) in a sulfidogenic fluidized-bed bioreactor treating Fe and Zn containing acidic wastewater.

Multiple-stage processes

Tabak et al. (2003) operated a sulfide precipitation process with biologically produced sulfide, in which the selective precipitation of Cu, Zn, Al and Fe was followed by co-precipitation of minor metals to single sludge. In the four-stage process, Cu and Zn were precipitated simultaneously as CuS-ZnS sludge in the first stage, then Al was precipitated as hydroxide, FeS was precipitated in the third unit process, and the minor metal components (that had concentration below 3 mg L^{-1}) of the AMD (Cd, Ni, As, Co) were precipitated in the last stage (Tabak et al. 2003). The process resulted in 95% metal purity of Cu-Zn sludge, 90% for Al, and 60-90% for Fe with some Mn as co-precipitate (Tabak et al. 2003). While selective precipitation of Cu and Zn was studied by Sampaio et al. (2008), they observed that the selective precipitation of copper required high pS (low sulfide potential in a sulfide selective electrode), and 100% selective precipitation of Cu over Zn was obtained at pH 2 and 3 (Sampaio et al. 2008).

In a two-stage process, in which H_2S containing gas from bioreactor is recycled, adjustment of the optimal precipitation pH may need alkali addition, as shown in Figures 7 and 8 (Huisman et al. 2006). If non-gaseous electron donor is used, the H_2S can be separated from the solution by a stripping unit, and on average 23 % of the dissolved sulfide of the UASB was transferred to the gas phase in the stripping unit (Hammack and Dijkman 1999). The recycled gas from a gas-lift bioreactor (GLB) used for metal precipitation in a separate contactor contained typically 3-15 % H_2S (Buisman et al. 1999). Figures 8 and 9 show flow charts of a two-stage process recovering one or two metals.

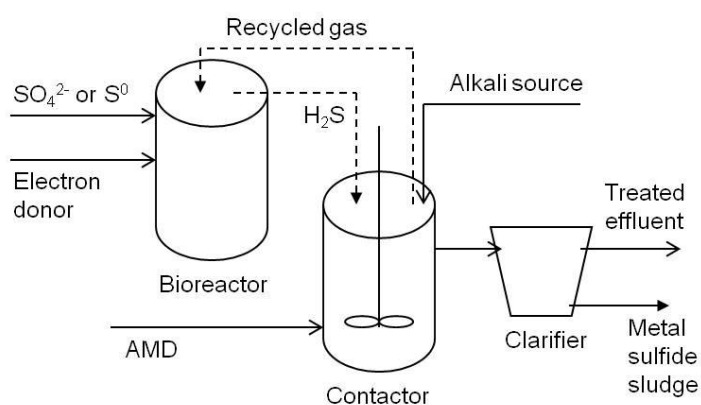


Figure 8. The process configuration of single metal recovery process with a two-stage process, in which hydrogen sulfide is produced in a bioreactor, and the metal is precipitated with gaseous H_2S in a separate contactor unit (Huisman et al. 2006).

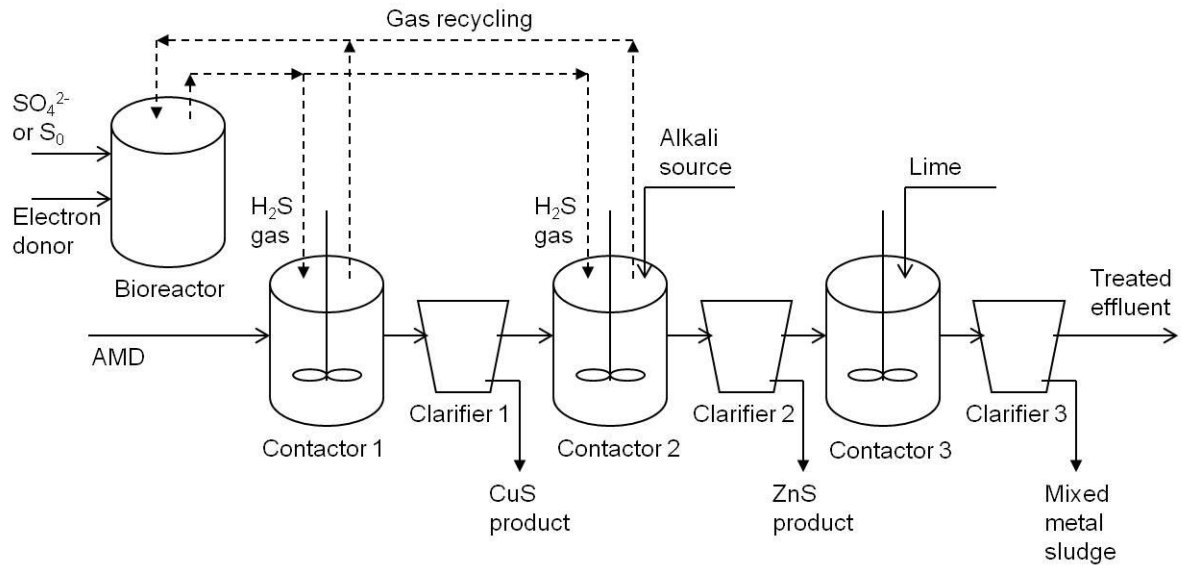


Figure 9. The process configuration of Cu and Zn metal recovery process from AMD with a two-stage process, in which hydrogen sulfide is produced in a bioreactor, and the metals are precipitated with gaseous H_2S in a separate contactor units, and the other metals in the AMD are precipitated with lime in the final stage (Huisman et al. 2006).

The process configuration may include also iron precipitation as ferric hydroxide prior to metal sulfide precipitation; this will reduce FeS precipitation in the sulfide precipitation and decrease consumption of H_2S (Lawrence et al. 2003). Also, aluminum removal as hydroxide can be added to the process prior to sulfide precipitation (Lawrence et al. 2003, Tabak et al. 2003). An interesting alternative to aluminum precipitation as $\text{Al}(\text{OH})_3$ is aluminum removal from AMD as alunite ($\text{KAl}_3(\text{OH})_6(\text{SO}_4)_2$), which has been reported for a sulfidogenic systems by Gusek (2002).

Ashe et al. (2008) reported that CuS precipitation could be optimized via control of the redox potential. The Bisbee Biosulphide plant treats on average 9500 m^3 of copper containing AMD per day (Ashe et al. 2008). The copper recovery in the Bisbee plant is 64 tons per month (Ashe et al. 2008). The acidity produced in the CuS precipitation is recycled to the heap leaching process (Ashe et al. 2008), thus the bioprocess for AMD treatment also benefits the primary metal recovery process.

Low temperature NiS recovery has been applied at Raglan Mine, Northern Quebec (Canada) (Lawrence et al. 2003). The process included Fe removal as ferric hydroxide prior to NiS precipitation with biologically produced sulfide (Lawrence et al. 2003). The NiS precipitation occurred at temperatures as low as $1.4\text{--}16^\circ\text{C}$. The product was saleable granular NiS with good settling properties, although the settling of the precipitate was occasionally disturbed by the low temperature (Lawrence et al. 2003). A sand filter as an effluent polishing step was suggested to further improve the effluent quality (Lawrence et al. 2003).

4.3 Reactor processes

4.3.1 Reactor types

In the present study, bioreactors with efficient biomass retention (fluidized-bed bioreactor, gas-lift bioreactor, sub-merged membrane bioreactor) were used. Besides these reactors, various bioreactor configurations have been used, and a more detailed comparison of the properties of sulfidogenic bioreactor types has been made by Kaksonen and Puhakka (2007). The technical benefits of the three reactor types used in the present study are discussed briefly below and the reactor characteristics are summarized in Table 16.

Fluidized-bed bioreactor

The introduction of granular and fluidized-bed reactor (FBR) had a major impact on the conversion capacity of anaerobic treatment, because these reactors allow the uncoupling of hydraulic retention time (HRT) and sludge retention time (SRT) (Mulder et al. 2001). The factors determining the conversion capacity are biological conversion rate, mass transport and biomass concentration (Mulder et al. 2001). The maximum growth rate of anaerobic biomass is in general 0.008-0.015 h⁻¹, but the growth rate decreases for aggregated biomass due to diffusion limitations (Mulder et al. 2001). In an FBR, the biomass grows as biofilm on carrier material, which is fluidized by high recycle flow of the reactor liquid (Speece 1983). The benefits of FBR are efficient mass transfer and dilution of the treated influent (Rittmann, 1982, Márin et al. 1999). The liquid recycling in the FBR requires remarkably high energy input (Speece 1983) and the shear forces are large, which affects on the biofilm quality (Rittmann 1982). Also the high shear forces between the carrier particles affect the carrier material durability, and have a remarkable effect on the bioreactor performance and biomass retention (Leenen et al. 1996). The configuration of a FBR is shown in Figure 10A.

Gas-lift bioreactor

The gas-lift bioreactors (GLB) fed with synthesis gas or H₂/CO₂ mixture as electron donor have already been proven to be industrially applicable (van Houten et al. 1994, van Houten et al. 2006). Gas-lift reactors have very efficient mixing and mass transfer due to high gas recycling rate (van Houten et al. 1996). GLBs can be operated with a carrier material, for example pumice (van Houten et al. 1996), or with suspended biomass (Bijmans et al. 2009a; 2009b), and an internal settler provides the retention of the biomass within in the reactor. The configuration of a GLB is shown in Figure 10B.

Sub-merged membrane bioreactor

The use of a suspended biomass reactor requires an adequate biomass retention system, such as a settler or a membrane, especially if low HRTs are applied (Mulder et al. 2001). In a sub-merged membrane bioreactor (MBR) SRT and HRT are independent from each other (Brindle and Stephenson 1996). Membrane bioreactors produce high quality effluent, as the membrane retains colloidal material, but so far this reactor type had been demonstrated only for niche applications, and it is not suitable for wastewaters with high solids content (e.g. metal sulfides) that may block the membrane (Mulder et al. 2001). The configuration of a MBR is shown in Figure 10C.

Table 16. The technical characteristics of FBRs, MBRs and GLBs.

FBR	MBR	GLB
+ Efficient mixing (Speece 1983) + Dilution of the treated influent protects microbes from toxic concentrations (Márin et al. 1999) + Small diffusion limitation in the biofilm (Márin et al. 1999, Mulder et al. 2001) ^a - High energy costs of pumping (Speece 1983) +/- Liquid substrates (only) can be used (Kaksonen et al. 2003a)	+ Almost complete biomass retention (Mulder et al. 2001) + Biomass retention is independent on liquid retention (Brindle and Stephenson 1996) + Suspended biomass (Brindle and Stephenson 1996) + Gaseous or liquid substrates (Bijmans et al. 2008a, Paper III) - High solid content of the influent or biomass can block the membrane (Mulder et al. 2001)	+ Applied successfully in industrial scale (van Houten et al. 2006) + Suspended biomass or carrier bound biomass (van Houten et al. 1996, Bijmans et al. 2009a) + Efficient mixing (van Houten et al. 1994) + Sulfide can be stripped from the recycled gas (Bijmans et al. 2008b) + Direct metal recovery is possible (Bijmans et al. 2009a; 2009b) +/- Gaseous substrates ^b (Bijmans et al. 2009a)

^aWhen compared to granular biomass, ^b nitrogen can be used for mixing with liquid substrates

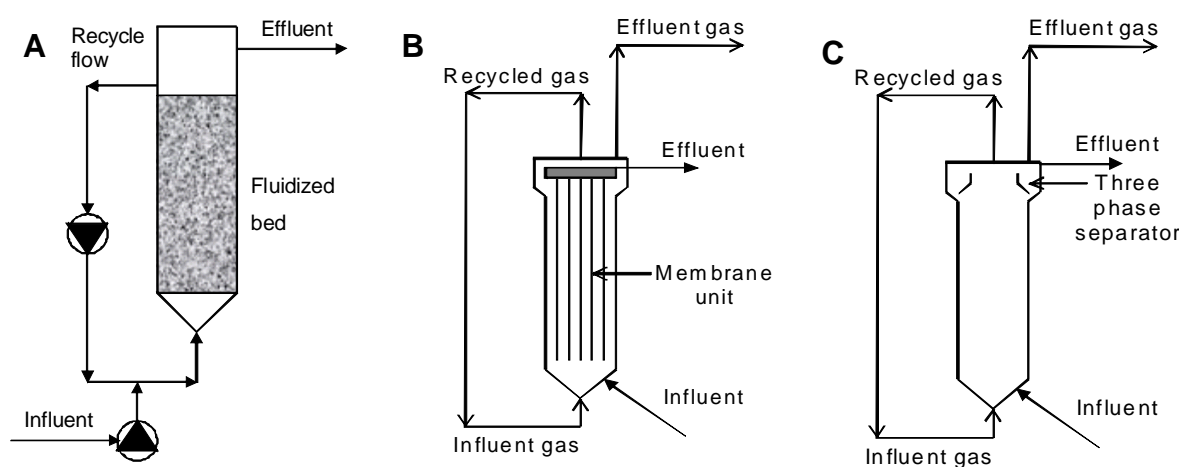


Figure 10. The configuration of a fluidized-bed bioreactor (A), sub-merged membrane bioreactor with gas feeding (B) and gas-lift bioreactor with internal settler for biomass retention (C).

In laboratory scale various reactor types, reactor configurations and electron donors have been used. Table 17 summarizes reactor types, operational parameters, electron donors, sulfate reduction rates and sulfidogenic biomass activities reported for laboratory (bench) scale reactors. The highest sulfate reduction rates ($104\text{--}288 \text{ mmol SO}_4^{2-} \text{ L}^{-1} \text{ d}^{-1}$) and specific sulfidogenic activities of the biomass have been reported for gas-lift reactors and sub-merged membrane bioreactors fed with either hydrogen or formate. Table 17 presents also results of the low temperature sulfate reducing bioreactors, and the rates of sulfate reduction in these reactors are low when compared to reactors operated at $35\text{--}35^\circ\text{C}$. The highest sulfate reduction rates obtained so far at low temperature were obtained with anaerobic filters containing solid substrates supplemented with ethanol and methanol (Tsukamoto et al. 2004).

Table 17. Summary of sulfate reduction rates (SRR) and specific sulfidogenic activities (SSA) reported for various sulfidogenic bioreactors.

Reactor type	T (°C)	pH	Electron donor (and carbon source)	SRR (mmol SO ₄ ²⁻ L ⁻¹ d ⁻¹)	SSA (mmol SO ₄ ²⁻ g VSS ⁻¹ d ⁻¹)	Reference
Fluidized-bed	8	6-7	Ethanol	2.6	0.8	Sahinkaya et al. (2006)
Fluidized-bed	8	7	Ethanol	2.7	0.5 ^a	Sahinkaya et al. (2007)
Fluidized-bed	35	8	Lactate	24	nr	Kaksonen et al. (2003b)
Fluidized-bed ^c	28-32	7.6	Ethanol	31	nr	Franzmann et al. (2008)
Fluidized-bed	35	8	Ethanol	42	nr	Kaksonen et al. (2003a)
Fluidized-bed	35	8	Ethanol	44.8	3.7 ^a	Kaksonen et al. (2004a)
Fluidized-bed	30	7	Ethanol	66	nr	Nagpal et al. (2000b)
Gas-lift	30	5	H ₂ , (CO ₂)	51	395	Bijmans et al. (2008b)
Gas-lift	30	7	Synthesis gas	104	nr	van Houten et al. (1996)
Gas-lift	30	6	H ₂ , (CO ₂)	135	39	van Houten et al. (1995a)
Gas-lift ^b	30	7	H ₂ , (CO ₂)	288	nr	van Houten et al. (1994)
MBR	30	4	H ₂ , (CO ₂)	6	85	Bijmans (2008)
MBR ^c	33	7.3	Ethanol, Acetate	69	57	Vallero et al. (2005)
MBR	30	4.5	H ₂ , (CO ₂)	111	79	Bijmans (2008)
MBR	30	6	Formate	143	110	Bijmans et al. (2008a)
MBR	30	4	Formate	151	64	Bijmans (2008)
MBR	30	5	Formate	188	81	Bijmans (2008)
EGSB	33	7.8-8.4	Ethanol	73	nr	de Smul et al. (1999)
EGSB	33	8	Acetate	65	nr	Stucki et al. (1993)
EGSB	35	8	Acetate	105	4	Dries et al. (1998)
UASB	30	7-8	Acetate	5.6	5.6	Omil et al. (1997)
Up-flow packed bed reactor	25	8.1	Molasses	23	nr	Maree and Strydom (1987)
Packed-bed reactor	35	nr	Synthesis gas	25	nr	du Preez and Maree (1994)
Packed-bed reactor	30	7.2	Acetate	4.8	nr	Steed et al (2000)
SBR	30	7	H ₂ , (CO ₂)	21	nr	Herrera et al. (1997)
Stirred tank	20-22	7.2	Primary sewage sludge	8.3	nr	Whiteley et al. (2003)
Anaerobic filter	5	6.5	Methanol, spent manure	10-14	nr	Tsukamoto et al. (2004)
Anaerobic filter	5	6.5	Ethanol, spent manure	11-15	nr	Tsukamoto et al. (2004)
Column bioreactor	nr	6-8	Biodiesel manufacturing waste	4.7	nr	Zamsov et al. (2006)
Column bioreactor	14-24	6.5-7.5	Mixture of cheese whey, sawdust and cow manure	120-250	nr	Drury (1999)

^a Calculated based on the given biomass of the FBR carrier material, ^b Bench scale reactor fed with 80% H₂ and 20% CO₂, ^c High salinity (50 g L⁻¹), nr = not reported, EGSB = Expanded-granular-sludge blanket, MBR = (sub-merged) membrane bioreactor, UASB = up-flow anaerobic sludge blanket reactor, SBR= sequencing batch reactor

4.3.2 Process options for biological sulfate reduction

The effects of pH, temperature and the toxic effects of metals and H₂S on SRP were discussed in Chapter 2. The effect of these factors on industrial scale reactor operation is briefly discussed here. Also the microbial ecology of the reactors has to be controlled to direct the electron donor flow for use in sulfate reduction. The treated bioreactor effluent usually has satisfactory pH, metal and sulfate concentrations (Huisman et al. 2006, Kaksonen et al. 2003a, Kaksonen et al. 2004a), while acetate accumulates in the effluent if the organic electron donor oxidation is incomplete (Kaksonen et al. 2004a) or acetate is produced from H₂ and CO₂ by homoacetogenic bacteria (van Houten et al. 2006). If the effluent of the treatment process contains residual chemical oxygen demand (COD, e.g. acetate), it can be removed in an oxidative trickling filter (Hammack and Dijkman 1999).

Sulfide and metal toxicity

The toxicity of metals and sulfide on SRB can be controlled in bioreactors even at low pH as was shown by Bijmans et al. (2009a; 2009b). High sulfate reduction rates and efficient metal

precipitation were obtained at low pH (4.5-5), when toxic H₂S is the main sulfide form, but the H₂S was consumed in metal sulfide precipitation (Bijmans et al. 2009a; 2009b). Also Kaksonen et al. (2003a; 2003b; 2004a) demonstrated that efficient sulfate reduction combined with Fe and Zn precipitation occurred at a total dissolved sulfide concentration of 12-16 mmol L⁻¹ at pH 7-8. The above described results are contradictory to results obtained by Reis et al. (1992), who reported that H₂S toxicity is direct and independent of the availability of precipitating metals. The metal precipitation in the bioreactor has an important role in the control of H₂S toxicity. This should be considered when the reactor configuration is selected, as efficient mixing improves the metal precipitation. Also the effect of mixing to the quality and size of the produced metal sulfide particles has to be taken into account when metal recovery is considered. The dilution rate is also important because it decreases the metal concentrations that the bacteria have to tolerate. Gas-lift bioreactors and fluidized-bed bioreactors have good mixing and dilution characteristics (Márin et al. 1999).

H₂S toxicity varies depending on the substrate used for sulfate reduction. For example inhibitory H₂S concentrations were 2.6 and 3.9 mmol L⁻¹ for sulfidogenic ethanol and acetate oxidation, respectively (Kaksonen et al. 2004a), indicating that ethanol oxidation is more prone to H₂S toxicity than acetate oxidation. When organic electron donors are used and the bioreactor is operated at neutral pH, the optimization of sulfidogenic acetate oxidation has an important role in the control of H₂S toxicity (Kaksonen et al. 2004a). Acetate oxidation produces the majority of the alkalinity, thus it controls the reactor pH and also the proportion of toxic H₂S. A higher reactor pH is obtained with complete acetate oxidation, resulting in lower H₂S concentration due to effect of pH on sulfide forms (Kaksonen et al. 2004a). Hammack and Dijkman (1999) reported that a pilot scale sulfidogenic reactor treating Berkley Pit AMD was operated below 600 mg L⁻¹ dissolved sulfide concentration due to the toxicity of H₂S on SRB. When extra H₂S is produced, the process can be expanded with a sulfide oxidation unit, in which the H₂S is oxidized to elemental sulfur by sulfide oxidizing bacteria (Hammack and Dijkman 1999, Huisman et al. 2006).

pH and salinity

The industrial scale sulfidogenic bioreactors listed in Table 11 are operated at a neutral pH. If sulfide is recycled in gaseous form to the metal precipitation stage, the alkalinity generation in the biological stage cannot be utilized. Therefore, additional neutralization with lime is required for treatment (Huisman et al. 2006). The pH tolerance of SRP has recently been studied in detail (Kimura et al. 2006, Bijmans 2008) in attempt to increase the operational pH range of sulfidogenic bioreactors. As discussed above, the operation of a sulfate reducing bioreactor at low pH allows selective metal precipitation (Buisman et al. 2007), which improves possibilities to use SRP for direct metal recovery (Bijmans 2008). Operation at a pH above 8.5 would allow the direct use of sulfidogenic bioreactors for treatment of brine waters from washing of natural gas (Buisman et al. 2007). The pH also affects the relative toxicity of H₂S and acetate: at low pH acetic acid is more inhibitory than H₂S, but at a higher pH H₂S toxicity plays has the major role (Reis et al. 1992).

High salinity has been shown to inhibit the operation of sulfidogenic bioreactor (Vallero et al. 2003). Salinity tolerance of SRP would improve the anaerobic treatment of wastewaters from various food industries (Vallero et al. 2005). Sulfate reduction in high salinity conditions has been studied by Vallero et al. (2003; 2005) and Franzmann et al. (2008). With a MBR configuration inoculated with salt tolerant SRB, sulfate reduction was efficient (69 mmol L⁻¹ d⁻¹) and the specific sulfidogenic activity (57 mmol SO₄⁻² g VSS⁻¹ d⁻¹) was high with ethanol and acetate as electron donors (Vallero et al. 2005). The sulfate reduction rate obtained with

FBR configuration was somewhat lower ($31 \text{ mmol L}^{-1} \text{ d}^{-1}$) than with a MBR due to problems in maintenance of biomass in the FBR (Franzmann et al. 2008).

Temperature

Low operation temperature affects on the activity of SRB as seen on Table 17. There are no reports available on the operation of industrial scale reactors at decreased temperatures and on the activities of mesophilic SRB in reactors at sub-optimal temperatures. Temperature affects the ability and rate of substrate utilization by SRB, which is an important issue regarding low temperature sulfidogenic treatment. Heating of a wastewater stream to the optimal temperature of mesophilic SRB is not cost-effective. Therefore, the lack of heating costs in a low temperature reactor may make the process economically feasible, although the rate of sulfate reduction is slower and therefore the required reactor size increases. The application of low temperature sulfate reduction is a compromise between the microbial activity, reactor size and operational temperature.

Engineering of bioprocess to support sulfate reduction

The engineering of bioprocesses to support sulfate reduction has generated a lot of interest because in anaerobic reactors other microbial groups (e.g. acetogens and methanogens) will also compete for the electron donor. Therefore, the composition of microbial populations and the flow of electrons to various processes have to be studied.

Diverse sulfate-reducing bacterial communities have been identified from ethanol-fed lab-scale sulfidogenic FBRs (Kaksonen 2004b; 2004c), and a full-scale UASB reactor (Dar et al. 2007), although different SRB species compete for the same electron donor. Competition of SRB and homoacetogens was observed in a H_2 -fed gas-lift reactor at pH 5 (Bijmans et al. 2009a). The molecular analysis of the biomass in this GLB revealed the presence of several *Desulfovibrio* sp. and *Clostridium* sp. (Bijmans et al. 2009a). Dar et al. (2008) reported that sulfate limiting conditions caused a change in the major metabolic pathway in a lactate-fed CSTR. Acetogens and methanogens were the dominating populations and SRB and methanogens were dependent on the fermentation products. The majority of the SRB identified from the lactate-fed continuously stirred tank reactor (CSTR) were incomplete oxidizing species, which did not oxidize acetate (Dar et al. 2008). Van Houten et al. (2006) identified *Desulfomicrobium* sp., *Desulfovibrio* sp. and several methanogenic species from a synthesis gas-fed industrial scale gas-lift reactor. In a formate-fed MBR, thermodynamics clearly defined the occurrence and order of biological reactions (Bijmans et al. 2008a). The acetogenesis and sulfate reduction started only after formate had been converted into hydrogen and the formation of hydrogen made the sulfate reduction and acetogenesis thermodynamically feasible (Bijmans et al. 2008a).

A sulfidogenic gas-lift reactor treating zinc containing wastewater (located at Budel in The Netherlands) had a remarkable proportion of electron donor directed to methane production and homoacetogenesis observed as high methane production and high acetate concentration in the reactor, respectively (van Houten et al. 2006). The strategy to direct the process towards sulfate reduction was to limit the availability of CO_2 , whereupon the carbon source of methanogens and homoacetogens became depleted (van Houten et al. 2006). The highest acetate concentration was observed right after reactor start-up. Acetate concentration decreased after this, indicating that homoacetogens were no longer able to efficiently compete for H_2 while also acetate utilization by other species in the reactor increased (van Houten et al. 2006). Methanogenesis did not cease during the reactor run (van Houten et al. 2006). This was explained by the fact that the reactor sludge retention time was 4-7 days, thus

the competition between species was defined by Monod kinetics rather than H₂ threshold pressure, and SRB and methanogens have relatively similar kinetic properties (van Houten et al. 2006). Weijma et al. (2002) reported that in a lab-scale gas-lift reactor, the competition between SRB and methanogens was not dependent on kinetics, but rather by better retention of SRB biomass in the reactor. In this study, the SRB were not able to outcompete homoacetogenic bacteria for H₂ (Weijma et al. 2002). Also, Dar et al. (2008) reported that acetogens were not outcompeted by SRB in a lab-scale stirred-tank reactor. Thus the electron donor affects on the competition and composition of microbial communities in sulfate reducing bioreactors besides retention time, pH, and operational temperature and wastewater composition.

Several methods have been used to direct the electron flow of anaerobic bioprocess to sulfate reduction. Sufficient sulfate supplementation is necessary to produce high sulfidogenic activity (Oude Elferink et al. 1998), but the control of electron donor utilization by microbial groups is more problematic. In gas-lift bioreactors, the activity of methanogens and acetogens can be controlled by limiting the availability of CO₂ (van Houten et al. 2006). The control of temperature and pH may not result in inhibition of methanogenic and acetogenic species, as active methanogenesis and acetogenesis have been show to occur at high temperature (Weijma et al. 2000) and low pH (Bijmans et a. 2009a; 2009b), thus these parameters may not be sufficient to control other anaerobic processes. H₂S may not be very inhibitory to homoacetogenic bacteria: Bijmans et al. (2008b) reported that at H₂S concentration of 18.2 mmol L⁻¹, an acetate concentration of 18 mmol L⁻¹ was constantly produced in a H₂-fed gas-lift bioreactor at pH 5. Careful control of enrichment conditions supports the dominance of SRB in anaerobic bioreactors. For example Kaksonen et al. (2004a) showed high electron (80%) flow to sulfate reduction in an ethanol-fed fluidized-bed bioreactor, although the bacterial community was shown to be versatile (Kaksonen et al. 2004b; 2004c). Therefore, when organic electron donors that yield acetate as an intermediate oxidation product are used, the enrichment conditions should be optimized for the slow growing acetate oxidizing SRB by providing sufficient biomass retention and neutral pH.

4.4 Capital and operational costs of a sulfidogenic bioreactor

Reports in the literature discussing the economics of sulfate reducing reactors are scarce and difficult to compare. Therefore, a few examples are presented here. The costs of electron donor have been discussed above. Here, the capital costs (capex) and operational costs (opex) of sulfidogenic reactors are compared to chemical precipitation process utilizing lime. The capital cost for building a Biosulphide H₂S production plant at the Bisbee mine site (USA) was 3.2 million USD (Ashe et al. 2008), but the capital costs were defined locally. The capital costs of the Bisbee plant are summarized in Table 18.

Table 18. The capital costs of Bisbee Biosulphide plant. The profit from copper sale will cover the capital costs within 3 years (Ashe et al. 2008).

	USD
Plant equipment	1100000
Direct construction costs ^a	1453000
Indirect construction costs ^b	1865000
Total costs	3218000

^aIncludes site preparation, tank erection, electricity, construction equipment, concrete and steel etc., ^bEngineering work, construction management, insurance etc.

The economics of selective metal precipitation with biologically produced sulfide compared to lime precipitation for treatment of Berkley Pit AMD are shown in Table 19 (Tabak et al. 2003). Table 19 shows that the metal recovery option makes the process economically feasible when compared to treatment with hydroxide precipitation, and the investment costs are covered within one year. The economics are case-specific, and depend on the availability and cost of the electron donor for SRB, and also the market price of the metals and the price of the construction.

Table 19. Economics of treatment of Berkley pit AMD (Tabak et al. 2003).

Process	Capital costs (million dollars)	Cash flow (million dollars year ⁻¹)	Cost savings (million dollars year ⁻¹)	Payment time (years)
Two-stage lime precipitation ^a	9.4	-4.5	0	Infinity
Selective recovery of Cu and Zn ^b	14.4	-2.1	2.4	2.1
Selective recovery of Cu, Zn, Al, Fe and Mn ^c	19.5	10.5-24.8	15-26.9	0.37-0.67

^a Two-stage lime precipitation plant with waste disposal, ^b Selective precipitation of Cu and Zn only with disposal of other metal sludges, ^c recovery of these metals.

Another comparison of lime treatment and sulfide precipitation costs for mine wastewater treatment was made by Lopez (2009). Table 20 summarizes the economic assessment made for these processes, showing again that the metal recovery would make the sulfide precipitation a more feasible option than lime precipitation. The landfill sludge disposal for hydroxide sludge costs is 10-100 USD m⁻³, while for hazardous waste it will be more than 100 USD m⁻³ (Buisman et al. 1999).

Table 20. Comparison of treatment options for Wellington Oro (Colorado, US) mine wastewater treatment (Lopez et al. 2009).

	Lime treatment	Sulfide precipitation
Plant capacity	35 m ³ h ⁻¹	35 m ³ h ⁻¹
Produced sludge volume	1500 m ³ year ⁻¹	0
Zinc recovery for sale	0	81000 kg year ⁻¹
Zinc content of the product	8.5 %	61 %
Zinc revenue	0	50000 USD year ⁻¹
Capital costs ^a	2.6 million USD	1.7 million USD
Annual operating costs	150 000 USD	132 000 USD
Metal sludge storage costs	variable	0

^a estimate

Besides lime treatment and sulfide precipitation, the costs of chemical and biogenic sulfide should be compared. Table 21 summarizes the comparison of these options (Buisman et al. 1999). Values in Table 21 indicate that if lime treatment is used as a polishing step in the process, the alkalinity generated in the biological sulfide production will decrease the costs of biogenic sulfide when compared to chemical H₂S sources, but this depends on the chosen process configuration. It should be taken into account that lime treatment does not remove all the sulfate, because, due to the high gypsum solubility, 1600-2000 mg L⁻¹ SO₄²⁻ will remain in solution (Lawrence et al. 2003, Lopez et al. 2009). The costs to decrease the effluent sulfate concentration below 500 mg L⁻¹ after lime treatment varies from 0.6 to 6 USD m⁻³ treated water (Lopez et al. 2009).

Table 21. Economics of biogenic and chemical H₂S production compared to use of NaSH and liquefied H₂S according to (Buisman et al. 1999).

Process	Total costs (USD ton ⁻¹ S)	Savings in lime treatment chemical utilities (USD ton ⁻¹ S)	Savings in lime treatment sludge disposal (USD ton ⁻¹ S)	Total costs including savings (USD ton ⁻¹ S)
45 % NaHS solution	1000	-	-	1000
Liquefied H ₂ S	400-600	-	-	400-600
Chemical production on site	400-600	-	-	400-600
Biological production on site	500-800	300	10-100	200-500

The costs include depreciation with 15% annuity, the costs savings in lime treatment include chemicals and utilities, and the saving on the sludge disposal depends on the installation type and disposal cost m⁻³, the disposal cost of metal hydroxide sludge is 1-4 USD m⁻³ (Buisman et al. 1999).

The costs of biologically produced sulfide also depend on the production scale. Figure 11 shows the generation of the operational costs versus the H₂S production scale (Buisman et al. 1999).

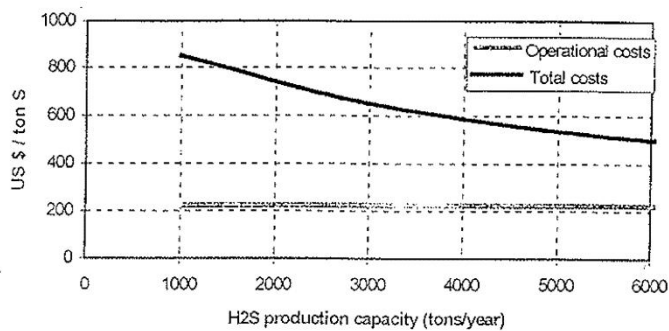


Figure 11. The development of operational and total cost of biological H₂S production (Buisman et al. 1999).

Figure 11 shows that the total costs will decrease as the production scale increases (Buisman et al. 1999). This is due to fact that capital costs related to reactor size do not have a remarkable effect on the total costs, e.g. building a larger reactor is not proportionally more expensive than building a smaller one.

5 Hypotheses and objectives

The biological sulfate reduction technology for mine wastewater treatment, biological hydrogen sulfide production and metal recovery are process options that can be integrated to the metallurgical processes currently in operation. The major drawbacks of the sulfate reduction technology are cost of the pure electron donors (such as ethanol, lactate and hydrogen) for SRB (Buisman et al. 2007), bioreactor operation costs (mainly heating) and the tolerance of the SRB to inhibitory compounds of the treated solution, e.g. high metal concentrations and acidity. The ability of the SRB to use the electron donor completely and to compete for the electron donor with other species, for example methanogenic archaea and homoacetogenic bacteria, define the efficiency of the biological process. With organic electron donors the sulfidogenic oxidation rate of acetate is the limiting factor. When hydrogen is used, methanogenic species compete efficiently for this electron donor. The breakthroughs for sulfate reduction technology would be a decrease of the electron donor costs and an extension of the tolerance of SRB to inhibitory effects of pH, temperature and metals, but also to control the biological process to support the growth of SRB over other species. The currently operated small scale industrial sulfate reducing reactors are fed with pure electron donors, but the largest industrial sulfate reducing reactors are fed with waste products as electron donors (Buisman et al. 2007). Therefore, the decrease of electron donor costs would allow use of larger process units. For example methane as electron donor would decrease the costs of the whole sulfate reduction process below the costs of lime treatment (Buisman et al. 2007). Besides methane, other low cost electron donors have to be studied, since anaerobic methane oxidizers grow extremely slow, with doubling times around 7 months (Nauhaus et al. 2007). The tolerance of SRB to inhibitory effects of low pH and high metal concentrations can be resolved to some extent by reactor technology and design, as has been shown by Kaksonen et al. (2003a; 2003b; 2004a) and by Bijmans et al. (2008a; 2008b; 2009a; 2009b). The control of the biological process to support SRB and especially sulfidogenic acetate oxidation requires still more research, as these define the efficiency of electron donor utilization by SRB.

The main objective of this thesis was to develop bioprocess technological solutions for treatment of mine wastewaters, AMD and biological H₂S production. The experimental work was performed with bioreactors inoculated with two mesophilic sulfate reducing enrichment cultures, thus the characteristics of these enrichment cultures could be compared. The bioprocess technological objectives were to study the sulfate reduction rates, the electron donor utilization efficiency for sulfate reduction, especially acetate oxidation, and the metal precipitation capacities. These factors were studied at the optimal temperature of mesophilic SRB (30-35°C), but also at sub-optimal temperatures (9-15°C). Also, the suitability and characteristics of three reactor types including fluidized-bed bioreactors, membrane bioreactors and gas-lift bioreactors were compared. The bioreactors were fed with metal- and sulfate containing acidic wastewater and various electron donors (ethanol, lactate, plant material hydrolyzate, formate, hydrogen), and the ability of the enrichment cultures to use the electron donors and to reduce sulfate, precipitate metals and to neutralize the wastewater were compared. The potential of pre-treated plant material as low cost electron donor for mesophilic SRB for mine wastewater treatment was also studied.

The objective of microbiological research was to improve understanding of the microbial communities and the microbial ecology of sulfate reduction at low temperatures. Profiling of microbial communities was done by using denaturing gel gradient electrophoresis (DGGE)

with universal bacterial primers and specific primers for SRB. The activity of the species in the low temperature FBR was studied by extracting RNA from reactor biomass and analyzing the cDNA corresponding to this RNA with DGGE. The characteristics of the low temperature enrichment culture in respect to temperature were studied by determining the temperature range of sulfate reduction, the utilization of formate and hydrogen versus temperature, and the electron flow to sulfate reduction at sub-optimal temperatures.

The objectives and hypotheses of the present study are listed below according to the publications they are presented in:

- Sulfate can be reduced at 9°C in a sulfidogenic fluidized-bed bioreactor with a non-acetate yielding electron donor formate. This hypothesis was tested in experiments presented in Paper I.
- Synthetic and real mine wastewater can be treated at low temperature formate-fed sulfidogenic fluidized-bed bioreactor at 9°C. This hypothesis was tested in experiments presented in Paper I.
- The analysis microbial community and the active species of the formate-fed low temperature fluidized-bed bioreactor (Paper I).
- Determination of the formate utilization pathway in the formate-fed FBR operated at 9°C (Paper I).
- At sub-optimal temperatures, the sulfate reduction and specific sulfidogenic activity of mesophilic sulfidogenic enrichment cultures can be measured and studied under complete biomass retention in hydrogen-fed membrane bioreactors. This hypothesis was tested in experiments presented in Paper II.
- At sub-optimal temperatures, the electron flow to sulfate reduction and biomass growth rate of mesophilic sulfidogenic enrichment cultures can be measured and studied under complete biomass retention in hydrogen-fed membrane bioreactors. This hypothesis was tested in experiments presented in Paper II.
- The temperature range of sulfate reduction, the optimal temperature of the low temperature and the presence of psychrophilic SRB species in the sulfidogenic enrichment culture can be assessed with a temperature gradient assay. This hypothesis was tested in experiments presented in Paper III.
- Sulfate can be reduced at 9°C with industrially proven electron donor and reactor set-up, a hydrogen-fed gas-lift bioreactor. This hypothesis was tested in experiments presented in Paper III.
- Mine wastewater can be treated biologically with ethanol-lactate fed sulfidogenic fluidized-bed bioreactor operated at 35°C. This hypothesis was tested in experiments presented in Paper IV.
- Hydrogen sulfide can be produced biologically using mine wastewater as sulfate source in ethanol-lactate fed sulfidogenic fluidized-bed bioreactor operated at 35°C without sulfide removal by stripping or metal precipitation. This hypothesis was tested in experiments presented in Paper IV.
- Determination of the sulfidogenic acetate oxidation kinetics in FBR at 35°C (Paper IV).
- Reed Canary grass (*Phalaris arundinacea*) hydrolyzate can be used as low-cost electron donor for mine wastewater treatment in a sulfidogenic fluidized-bed bioreactor operated at 35°C. This hypothesis was tested in experiments presented in Paper V.
- Reed Canary grass dry plant material can be used as substrate for SRB at 35°C. This hypothesis was tested in experiments presented in Paper V.

6 Materials and methods

The bioreactor and batch bottle experiments of the present study and the publications they are presented in are summarized in Table 22.

Table 22. The bioreactor experiments and the objectives of the study, used reactor type, inocula, operational temperature electron donors, treated wastewater and the publications describing the results of the experiments.

Experiment and objective	Reactor type	Inocula	T (°C)	Electron donor	Treated wastewater	Reference
Determination of sulfate reduction rate at low temperature	FBR	Low temperature enrichment culture	9	Formate	Synthetic wastewater	Paper I
Low temperature sulfidogenic FBR treatment of real mine wastewater	FBR	Low temperature enrichment culture	9	Formate	Diluted barren bioleaching solution	Paper I
Analysis microbial community and the active species in the low temperature FBR	FBR	Low temperature enrichment culture	9	Formate	Synthetic and real mine wastewater	Paper I
Formate utilization pathway in the FBR	Batch bottle	Low temperature enrichment culture	9	Formate	Synthetic	Paper I
Specific sulfidogenic activity and sulfate reduction rate of mesophilic enrichment cultures at sub-optimal temperatures	MBR	Low temperature and mesophilic enrichment culture	9-35	H ₂	Synthetic	Paper II
Electron flow to sulfate reduction and biomass growth rate of mesophilic enrichment cultures at sub-optimal temperatures	MBR	Low temperature and mesophilic enrichment cultures	9-35	H ₂	Synthetic	Paper II
Temperature range of sulfate reduction and the optimal temperature of low temperature enrichment culture	Batch bottle	Low temperature enrichment culture	4-42	Formate	Synthetic	Paper III
Sulfate reduction rate at 9°C in gas-lift bioreactor with industrially proven reactor set-up and electron donor	GLB	Low temperature enrichment culture	9	H ₂	Synthetic	Paper III
Biological mine wastewater treatment at 35°C with FBR	FBR	Mesophilic enrichment culture	35	Ethanol, lactate	Synthetic	Paper IV
Biological hydrogen sulfide production in FBR	FBR	Mesophilic enrichment culture	35	Ethanol, lactate	Synthetic	Paper IV
Sulfidogenic acetate oxidation kinetics in FBR	FBR	Mesophilic enrichment culture	35	Acetate	Synthetic	Paper IV
Reed Canary grass hydrolyzate as low-cost electron donor for mine wastewater treatment	FBR	Mesophilic enrichment culture	35	Plant material hydrolyzate	Synthetic	Paper V
Potential of reed Canary grass dry plant material as substrate for SRB	Batch bottle	Mesophilic enrichment culture	35	Dry plant material	Synthetic	Paper V

T = Temperature, FBR = fluidized-bed bioreactor, MBR = membrane bioreactor, GLB = gas-lift bioreactor

6.1 Bioreactors and inocula

Sulfate reduction at 9-35°C was studied with three reactor types: fluidized-bed bioreactor (FBR), membrane bioreactor (MBR) and gas-lift bioreactor (GLB). Two inocula were used, one from an ethanol-fed FBR operated at 35°C described by Kaksonen et al. (2004b; 2004c) and one from low-temperature metal contaminated sediment (Karnachuck et al. 2005) and enriched in an ethanol-fed FBR at 8°C by Sahinkaya et al. (2006; 2007). The inocula from an ethanol-fed FBR operated at 35°C is referred in the text as mesophilic enrichment culture. The inocula from an ethanol-fed FBR at 8°C by is referred in the text as low temperature enrichment culture. The reactor experiments performed for the mesophilic enrichment culture are shown in Figure 12. Two reactors, one FBR 1 and one MBR 2 were operated with mesophilic enrichment culture (Papers II, IV and V). The reactor experiments for the low temperature enrichment culture were as shown in Figure 13. Three reactors were operated with low temperature enrichment culture, one FBR 2, one MBR 1 and one GLB (Papers I, II and III).

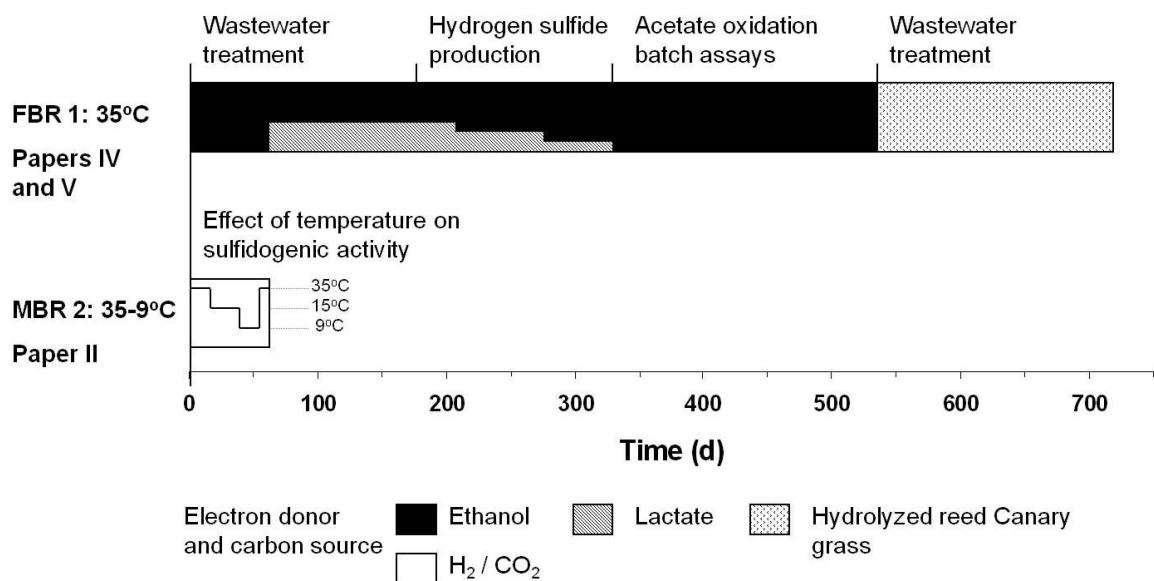


Figure 12. The operation periods, temperatures, electron donors and carbon sources of the fluidized-bed bioreactor 1 (FBR 1) and membrane bioreactor 2 (MBR 2) inoculated with mesophilic sulfidogenic enrichment culture. The FBR 1 was operated as mine wastewater treatment reactor during days 1-181 and 540-720, for biological hydrogen sulfide production during days 182-331 and for acetate oxidation kinetic batch assays during days 332-539. The MBR 2 was operated at 35°C during days 1-17, at 15°C on days 18-41, at 9°C on days 42-55 and at 35°C on days 56-60.

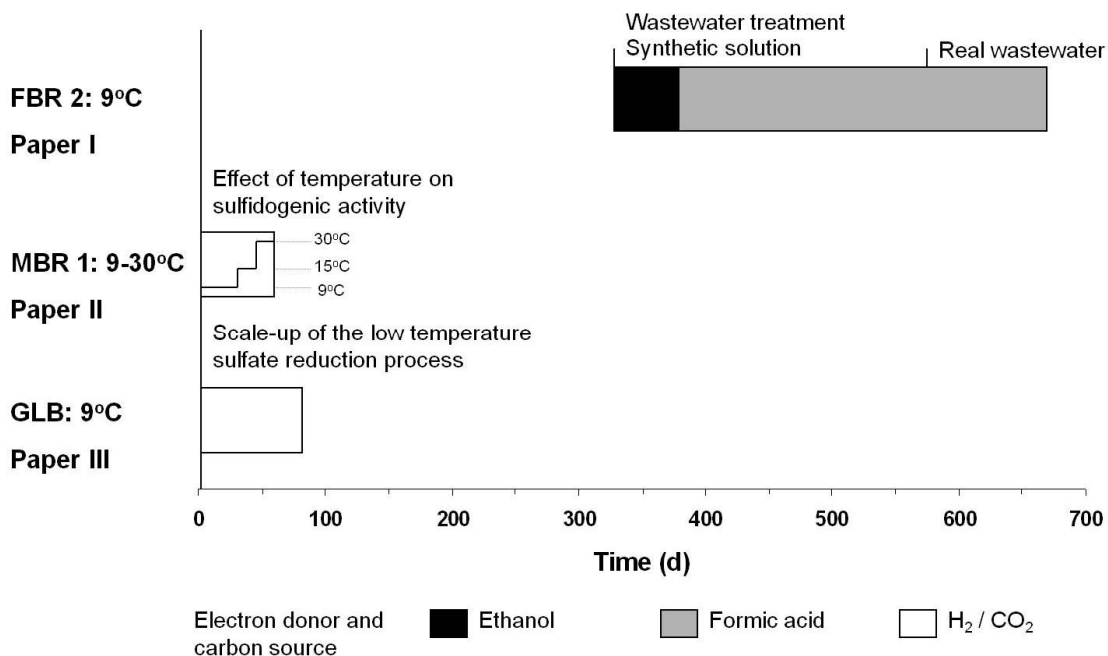


Figure 13. The operation periods, temperatures, electron donors and carbon sources of fluidized-bed bioreactor 2 (FBR 2), membrane bioreactor 1 (MBR 1) and gas-lift bioreactor (GLB) inoculated with psychrotolerant mesophilic sulfidogenic enrichment. The performance of the FBR 2 before day 328 has been described by Sahinkaya et al. 2007 and 2006. The FBR 2 was operated as mine wastewater treatment reactor during days 328-580 with synthetic waste water and on days 581-665 with diluted real mine wastewater. The MBR 1 was operated at 9°C during days 1-30, at 15°C on days 31-46 and at 30°C on days 47-56. The GLB experiment was operated at 9°C with synthetic waste water.

The configurations of the FBRs, MBRs and GLB were as shown in Figures 14-16. The reactor configurations have been described in detail by Kaksonen et al. (2004a) for FBR, Bijmans et al. (2008a) for MBR and Bijmans et al. (2008b) for GLB. The carrier material for the FBRs was ceramic particles (Silicate \varnothing 0.5-1 mm, bulk density 0.77 kg L^{-1} , Filtralite, Norway) and pumice for the GLB (\varnothing 0.2-0.5 mm, density ca. 2440 kg m^{-3} , Aquavolcano, Aquatech, Papendrecht, The Netherlands).

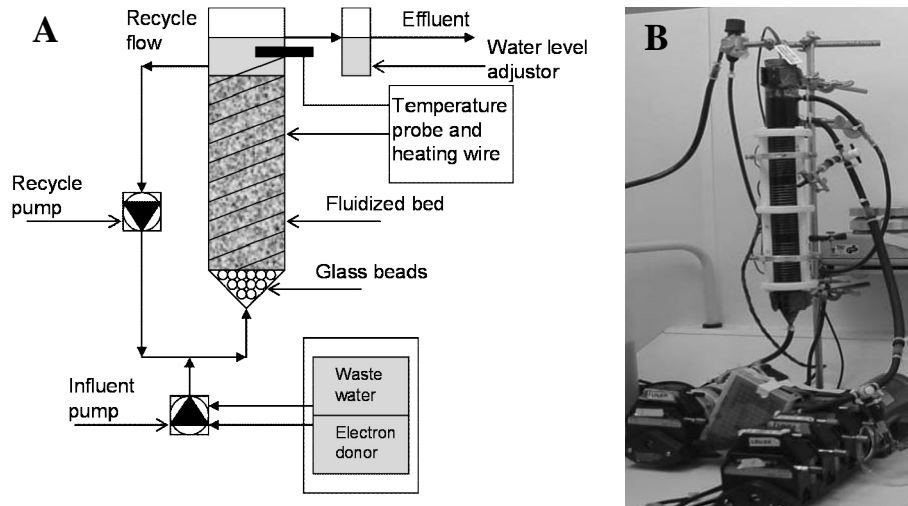


Figure 14. A) A schematic diagram of a fluidized-bed bioreactor (FBR) configuration and B) the FBR set-up in the laboratory.

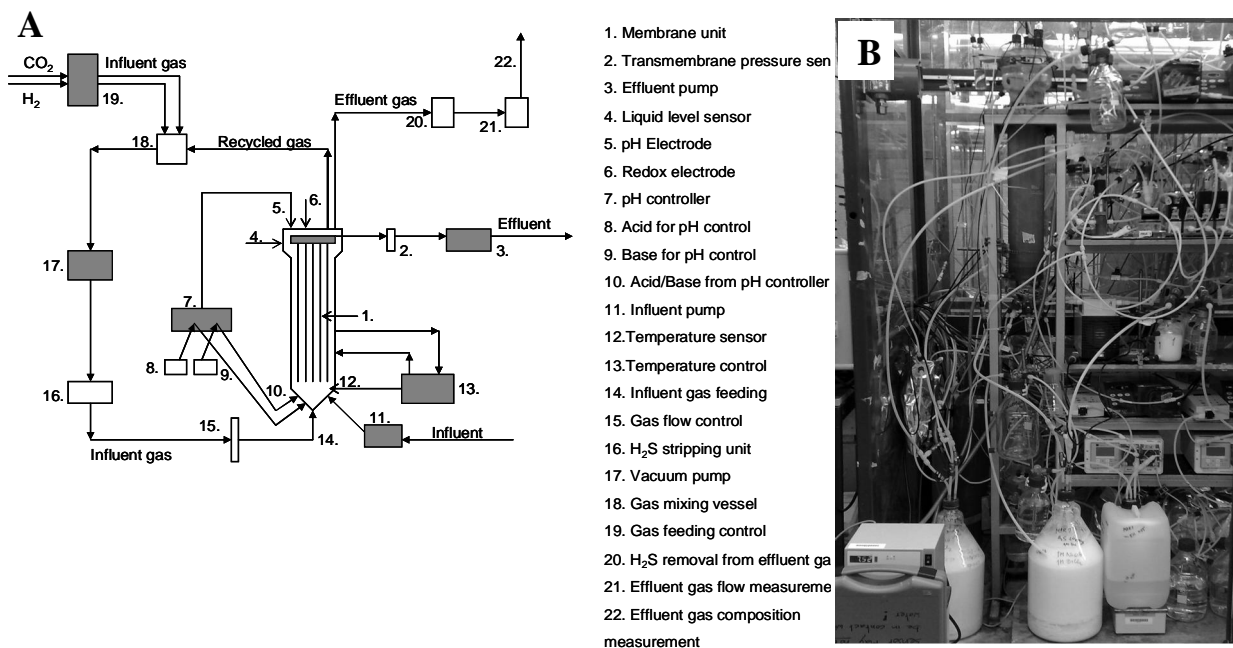


Figure 15. A) A schematic diagram of a membrane bioreactor configuration and B) the MBR set-up in the laboratory.

sulfide (DS) and H₂S concentrations were maintained below 2 and 1 mmol L⁻¹, respectively. The batch assays were performed as follows: the influent feeding was stopped, and then 50 mL of the FBR liquid was removed with a syringe and replaced with 50 mL of anoxic solution containing acetate, sulfate and nutrients. The sulfate-acetate ratio in the solution was 2:1. The FBR was operated using recycle flow only, sampled for acetate and DS at regular intervals, and for pH and sulfate in the beginning and the end of each assay.

6.2.2 Kinetic calculations

The results of the acetate kinetic batch assays were plotted according to Lineweaver-Burk (10) and Hanes-Woolf (11) plots, and the K_m (the affinity towards acetate) and V_{max} (acetate oxidation rate) values obtained from these plots were used to fit the measured data to the Michaelis-Menten equation (12) (Wilson and Walker 2001). In the equations 10-12 the [S] refers to the substrate concentration and the v₀ to the measured acetate oxidation rate.

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} * \frac{1}{[S]} + \frac{1}{V_{max}} \quad (10)$$

$$\frac{[S]}{v_0} = \frac{K_m}{V_{max}} + \frac{[S]}{V_{max}} \quad (11)$$

$$v_0 = \frac{V_{max} * [S]}{K_m + [S]} \quad (12)$$

6.3 Batch bottle experiments

Batch bottle experiments were performed to analyze the substrate utilization by the enrichment cultures. One batch bottle experiment was performed for the low temperature enrichment culture from FBR 2 to study whether formate is used by SRB directly, or as H₂ and CO₂ (Paper I). Also a temperature gradient assay was performed for the FBR 2 low temperature enrichment culture to study the temperature range of sulfate reduction with formate as electron donor (Paper III). Batch bottle assays were also performed with the mesophilic enrichment culture to study the utilization of plant material hydrolyzate and dry plant material as substrates for SRB at 35°C with the inoculum from FBR 1 (Paper V). The batch bottle experiment were made in modified Postgate B medium and inoculated with reactor biomass. The details of the batch bottle experiments are described in Papers I, III and V.

6.4 Physico-chemical analysis

The physico-chemical analyses carried out for reactor and batch bottle experiments were as shown in Table 23.

Table 23. The physico-chemical analyses used in the reactor and batch bottle experiments.

Analysis	Instrument(s)	Paper	Reference
Sulfate	Ion chromatograph, liquid chromatograph	I-V	I, II, SFS (1995), Sipma et al. (2004)
Dissolved sulfide	Spectrophotometer	I, IV, V	I, Cord-Ruwisch (1985)
Dissolved sulfide	Dr. Lange kit LCK-653 and spectrophotometer	II, III	II, Bijmans et al. (2008b)
Volatile carboxylic acids and alcohols	Gas chromatograph	I-V	I, II, V, Kaksonen et al. (2004a), Koskinen et al. (2006), Weijma et al. (2000)
Gas phase composition	Gas chromatograph	I-III	I, II Koskinen et al. (2006), Bijmans et al. (2008b)
Dissolved organic carbon (DOC)	Total organic carbon analyzer	I, IV, V	I, Kaksonen et al. (2004a)
Chemical oxygen demand (COD)	Titration	V	V, SFS (1988)
Soluble metals	Atomic absorption spectrometer	I, IV, V	I, SFS (1980a), SFS (1980b)
Soluble metals	ICP-AES	I	I
Alkalinity	Potentiometric titration	I, IV, V	I, SFS (1996)
Acidity	Potentiometric titration	I, IV, V	I, SFS (1981)
pH	pH electrode	I-V	I
Redox	Redox electrode	II, III	II
Biomass bound nitrogen	Dr. Lange kit LCK-238 and spectrophotometer	II, III	II, Bijmans et al. (2008b), Esener et al. (1983)
Total suspended solids (TSS)	Balance, oven	I-V	I, SFS (1990)
Volatile suspended solids (VSS)	Balance, furnace	I-V	I, SFS (1990)
Total solids (TS)	Balance, oven	I, III, IV, V	I, Kaksonen et al. (2004a)
Volatile solids (VS)	Balance, furnace	I, III, IV, V	I, Kaksonen et al. (2004a)

6.5 Analysis of the bacterial community

The composition of the FBR 2 microbial community was analyzed with polymerase chain reaction – denaturing gel gradient electrophoresis (PCR-DGGE). The bacterial community was analyzed using universal 16S rRNA primers. The presence of sulfate-reducing bacteria was analyzed using SRB specific *dsrB* primers (Geets et al. 2006). The active bacteria and SRB species in the FBR 2 were analyzed using RNA extraction followed by reverse transcription of the RNA to cDNA. The primers for the DGGE contained a GC-clamp attached to the 5' end of the forward primer (5'-GCG CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3'). The microbial community of FBR 1 was not analyzed, as it had already been analyzed in detail and described by Kaksonen et al. (2004b; 2004c). The methods used for bacterial community analysis were as listed in Table 24, the primers in Table 25 and the programs used to analyze sequence data in Table 26.

Table 24. Methods used in the microbial community analysis of the FBR 2 in Paper I.

Analysis
DNA extraction
RNA extraction
Polymerase chain reaction (PCR)
cDNA synthesis
Denaturing gel gradient electrophoresis
DNA sequencing

Table 25. Primers used in the analysis of the FBR microbial community for the affiliation of sulfate-reducing bacteria and their activity. The primers were used for both DNA and cDNA templates.

Primer	Target gene	<i>E. coli</i> 16S rRNA gene position	Sequence (5' to 3')	Reference
27F*	16S rRNA	8-27	AGA GTT TGA TCM TGG CTC AG	Lane 1991
1100R*	16S rRNA	1100-1114	GGG TTG CGC TCG TTG	Lane 1991
BAC-V3F**	16S rRNA	341-357	CCT ACG GGA GGC AGC AG	Muyzer et al. 1996
907R**	16S rRNA	907-926	CCG TCA ATT CMT TTG AGT TT	Lane 1991
DSRp2060F	<i>dsrB</i>		CAA CAT CGT YCA YCA CCA GGG	Modified from Geets et al. 2006
DSR4R	<i>dsrB</i>		GTA TAG CAG TTA CCG CA	Modified from Wagner et al. 1998

* Outer primers of the nested-PCR, ** Inner primers of the nested-PCR

Table 26. Programs used in the analysis of the DNA sequence data in Paper I.

Program	Reference(s)
BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi
BioEdit	http://www.mbio.ncsu.edu/BioEdit/BioEdit.html
Chimera check	Ribosomal database project http://rdp.cme.msu.edu/cgis/chimera.cgi?su=SSU
ARB	http://www.mikro.biologie.tu-muenchen.de
Treeview	http://taxonomy.zoology.gla.ac.uk/rod/treeview.html
MEGA	Molecular evolutionary genetic analysis http://www.megasoftware.net/

6.6 Calculations and modeling

The precipitation of metals in FBR 1 and FBR 2 (papers I, IV and V) was estimated using a Chemical Modeling Program OLI (OLI stream analyzer 2.0, OLI systems Inc. Morris Plains, NJ, USA). The results of the acetate kinetic batch assays performed for FBR 1 (Paper II) were analyzed using SPSS Sigma Plot 2001 Enzyme Kinetics Module 1.1 (SPSS Inc. Chicago, Illinois). The sulfide production and biomass growth measured in the temperature gradient assay (Paper III) performed for the low temperature enrichment culture were fitted to the Ratkowsky equation (Ratkowsky et al. 1983) using Matlab 8 (The Mathworks Inc., Natic, MA, USA) as described by Franzmann et al. (2005).

7 Results and discussion

7.1 Sulfate reduction

7.1.1 Sulfate reduction rates in the reactor experiments

The sulfate reduction rates (SRR), percent sulfate reduction and specific sulfidogenic activities (SSA) reported in the reactor experiments of the present study (Papers I-V) and in other studies are listed in Table 27. The sulfate reduction percent is dependent on the ratio of sulfate and electron donor loading, therefore, the sulfate reduction percent will remain low, if excess sulfate is fed to the reactor.

The sulfate reduction rates in Table 27 are illustrated in Figure 17. The highest SRRs have been reported for gas-lift bioreactors (with and without carrier material) and membrane bioreactors operated at 30°C with H₂ as electron donor and CO₂ as carbon source. Figure 17 shows a limited number of studies for bioreactors operated below 20°C. To show the SRRs below 20°C, these values are illustrated in Figure 18. Below 20°C the highest SRRs were those reported for anaerobic filters operated at 5°C, while the highest SRR reported in Paper I at 9°C was in the same range. This result indicates that FBRs are a suitable reactor type for low temperature mine wastewater treatment, and stable SRRs were achieved with high biomass concentration.

Table 27. Sulfate reduction rates (SRR) and specific sulfidogenic activities (SSA) obtained in the present study and other studies. Experiments with FBR 1 and MBR 2 were performed with the same inoculum originating from an ethanol-fed FBR operated at 35°C (Kaksonen et al. 2004a). The experiments with FBR 2, GLB and MBR 2 were performed with a low temperature sulfidogenic enrichment culture originating from an ethanol-fed FBR operated at 8°C (Sahinkaya et al. 2007). The percent sulfate reduction is given in parenthesis with the SRR.

Reactor	T (°C)	pH	Electron donor	SRR (mmol SO ₄ ²⁻ L ⁻¹ d ⁻¹)	SSA (mmol SO ₄ ²⁻ gVSS ⁻¹ d ⁻¹)	Reference
Fluidized-bed	8	7	Ethanol	2.7 (75 %)	0.5 ^a	Sahinkaya et al. (2007)
Fluidized-bed	8	6-7	Ethanol	2.6 (35%)	0.8	Sahinkaya et al. (2006)
Fluidized-bed ^c	28-32	7.6	Ethanol	31	nr	Franzmann et al. (2008)
Fluidized-bed	35	8	Ethanol	44.8	3.7 ^a	Kaksonen et al. (2004a)
Fluidized-bed	35	8	Ethanol	42 (60- 85 %)	nr	Kaksonen et al. (2003a)
Fluidized-bed	35	8	Lactate	24 (85 %)	nr	Kaksonen et al. (2003b)
Fluidized-bed	30	7	Ethanol	66	nr	Nagpal et al. (2000b)
FBR 1	35	7	Ethanol-lactate	100 (30-96 %)	21	Paper IV
FBR 1	35	7	Plant material hydrolyzate	21-34 (20-98 %)	nr	Paper V
FBR 2	9	8	formate	8-14 (63-96 %)	1.6-2.4	Paper I
Gas-lift ^b	30	7	H ₂	288 (59 %)	nr	van Houten et al. (1994)
Gas-lift	30	6	H ₂ ,	135 (49 %)	39	van Houten et al. (1995a)
Gas-lift	30	7	Synthesis gas	104	nr	van Houten et al. (1996)
Gas-lift	30	5	H ₂	51	395	Bijmans et al. (2008b)
Gas-lift	30-35	7.5	Synthesis gas	156	nr	van Houten et al (2006)
GLB	9	7.5	H ₂	6.2 (15-54 %)	1.8	Paper III
MBR	30	6	Formate	143 (100 %)	110	Bijmans et al. (2008a)
MBR	30	5	Formate	188	81	Bijmans (2008)
MBR	30	4	Formate	151	64	Bijmans (2008)
MBR	30	4.5	H ₂	111	79	Bijmans (2008)
MBR	30	4	H ₂	6	85	Bijmans (2008)
MBR ^c	33	7.3	Ethanol, Acetate	69 (62 %)	57	Vallero et al. (2005)
MBR 1	9	7.5	H ₂	6.9 (44 %)	33	Paper II
MBR 1	15	7.5	H ₂	12 (61 %)	52	Paper II
MBR 1	30	7.5	H ₂	15 (59 %)	57	Paper II
MBR 2	35	7.5	H ₂	23 (77 %)	92	Paper II
MBR 2	15	7.5	H ₂	13 (48 %)	53	Paper II
MBR 2	9	7.5	H ₂	4.7 (36 %)	21	Paper II
EGSB	33	7.8- 8.4	Ethanol	73 (80-90%)	nr	de Smul et al. (1999)
EGSB	33	8	Acetate	541 (80 %)	nr	Stucki et al. (1993)
EGSB	35	8	Acetate	105 (60-94%)	4	Dries et al. (1998)
UASB	30	7-8	Acetate	5.6 (23%)	5.6	Omil et al. (1997)
Up-flow packed bed reactor	25	8.1	Molasses	23	nr	Maree and Strydom (1987)
Packed-bed reactor	35	nr	Synthesis gas	25	nr	du Preez and Maree (1994)
Packed-bed reactor	30	7.2	Acetate	4.8 (77 %)	nr	Steed et al (2000)
SBR	30	7	H ₂	21	nr	Herrera et al. (1997)
Stirred tank	20-22	7.2	Primary sewage sludge	8.3	nr	Whiteley et al. (2003)
Anaerobic filter	5	6.5	Methanol, spent manure	10-14	nr	Tsukamoto et al. (2004)
Anaerobic filter	5	6.5	Ethanol, spent manure	11-15	nr	Tsukamoto et al. (2004)
Column bioreactor	nr	6-8	Biodiesel manufacturing waste	4.7	nr	Zamsov et al. (2006)
Column bioreactor	14-24	6.5- 7.5	Mixture of cheese whey, sawdust and cow manure	120-250	nr	Drury (1999)

^a Calculated based on the given biomass of the FBR carrier material, ^b Bench scale reactor fed with 80% H₂ and 20% CO₂, ^c High salinity (50 g L⁻¹), FBR = fluidized-bed bioreactor, MBR = membrane bioreactor, GLB = gas-lift bioreactor, EGSB = expanded sludge bed reactor, UASB = up-flow anaerobic sludge blanket reactor, SBR = sequencing batch reactor, nr = not reported

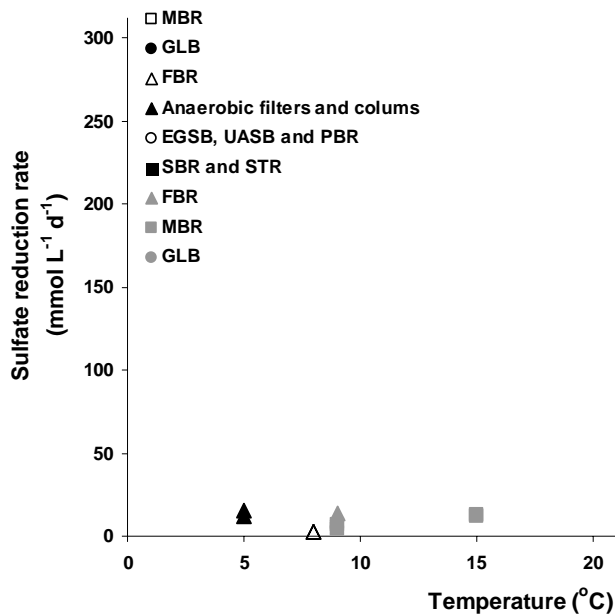


Figure 17. The sulfate reduction rates (SRR) for the various reactor types reported in Table 27. The data points of the present study are indicated with grey color. PBR = packed bed reactor, STR = stirred tank reactor.

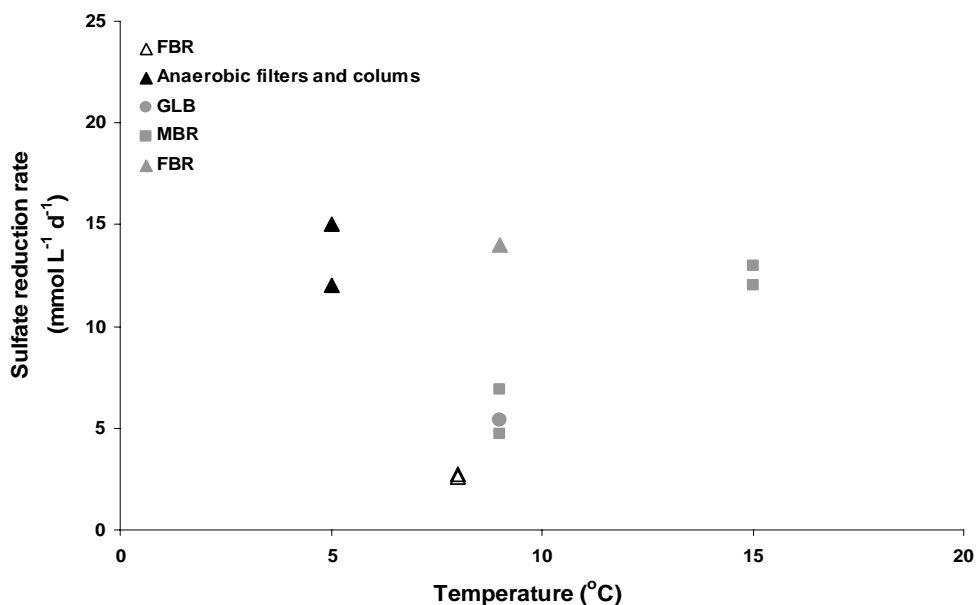


Figure 18. The sulfate reduction rates (SRR) for the various reactor types operated below 20°C reported in Table 27. The data points of the present study are indicated with grey color.

Apart from SRR, the efficiency of a sulfidogenic bioreactor can be evaluated based on specific sulfidogenic activity (SSA) of the biomass (unit $\text{mmol SO}_4^{2-} \text{ g VSS}^{-1} \text{ d}^{-1}$). These values are not often reported, but those values listed in Table 27 are illustrated in Figure 19. Again, the highest SSAs were obtained in H_2/CO_2 fed GLBs and MBRs. This is due to efficient mass transfer and biomass retention in these reactors. Although FBRs can support high SRR, the specific sulfidogenic activity remains low, which may be due to high biomass

concentration in these reactors. MBRs are not suitable for treatment of metal containing mine wastewater because the membrane can be blocked. However, MBRs are good devices to study the characteristics of the sulfidogenic enrichment cultures. Thus, this reactor type cannot be considered for practical application in mine wastewater treatment. The calculation of SSA is dependent also on the ability to quantify the total reactor biomass, and errors may arise from unquantified biomass growing on reactor walls especially in lab-scale reactors.

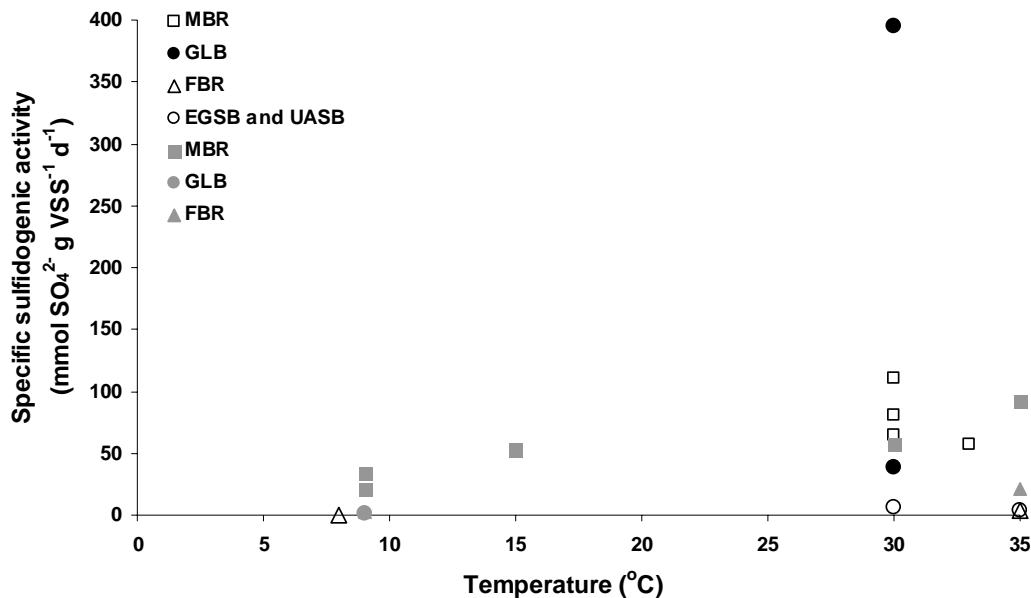


Figure 19. The specific sulfidogenic activities of the present study and the results reported in Table 27. The data points of the present study are indicated with grey color.

The choice of efficient reactor type should be defined based on the electron donor and the design SRR. The GLB should be chosen if H₂ or synthesis gas are used, and FBRs or UASB reactors are suitable for liquid electron donors, such as ethanol. Based on the present study, both GLB and FBR are suitable reactor types for low temperature mine wastewater treatment, as high SRR and biomass retention was achieved with these reactor types (see Chapter 7.3 for data on biomass growth). The SRRs reported for UASBs and FBRs are slightly lower than for GLBs, but the process design SRR will depend on the wastewater characteristics. Therefore lower SRR can be sufficient depending on inflow quantities and quality of the wastewater.

7.1.2 Biological sulfide production at 35°C and sulfide toxicity

Biological hydrogen sulfide production was studied with FBR 1 (Paper IV). In this study, the highest stable SRR was 100 mmol SO₄²⁻ L⁻¹ d⁻¹, corresponding to hydrogen sulfide production rate of 73.2 mmol H₂S L⁻¹ d⁻¹. Sulfide inhibition occurred during the biological H₂S production experiment, as the total dissolved sulfide and H₂S concentration were 19 and 18 mmol L⁻¹, respectively. The increase of the H₂S form of sulfide was due to pH decrease from 7.3 to 6.8 in the FBR 1. The high H₂S concentration inhibited especially the sulfidogenic acetate and lactate oxidation rates (Paper IV).

7.1.3 Temperature dependency of sulfate reduction of the low temperature enrichment culture

A temperature gradient assay was performed to assess temperature range of sulfate reduction and the cardinal temperatures of the low temperature sulfidogenic enrichment culture originating from FBR 2 (Paper III). The cardinal temperatures for sulfide production were: T_{opt} 30.9 (\pm 0.2) °C, T_{min} 7.2 (\pm 1.4) °C and T_{max} 40.9 (\pm 0.3) °C (Figure 20A). For the biomass growth the cardinal temperatures were: T_{opt} 27.0 (\pm 0.2) °C, T_{min} 3.0 (\pm 2.3) °C and T_{max} 41.7 (\pm 3.1) °C (Figure 20B). The results of the temperature gradient assay showed that the low temperature sulfidogenic enrichment culture was a psychrotolerant mesophilic enrichment culture with an optimal temperature for sulfate reduction at 31°C.

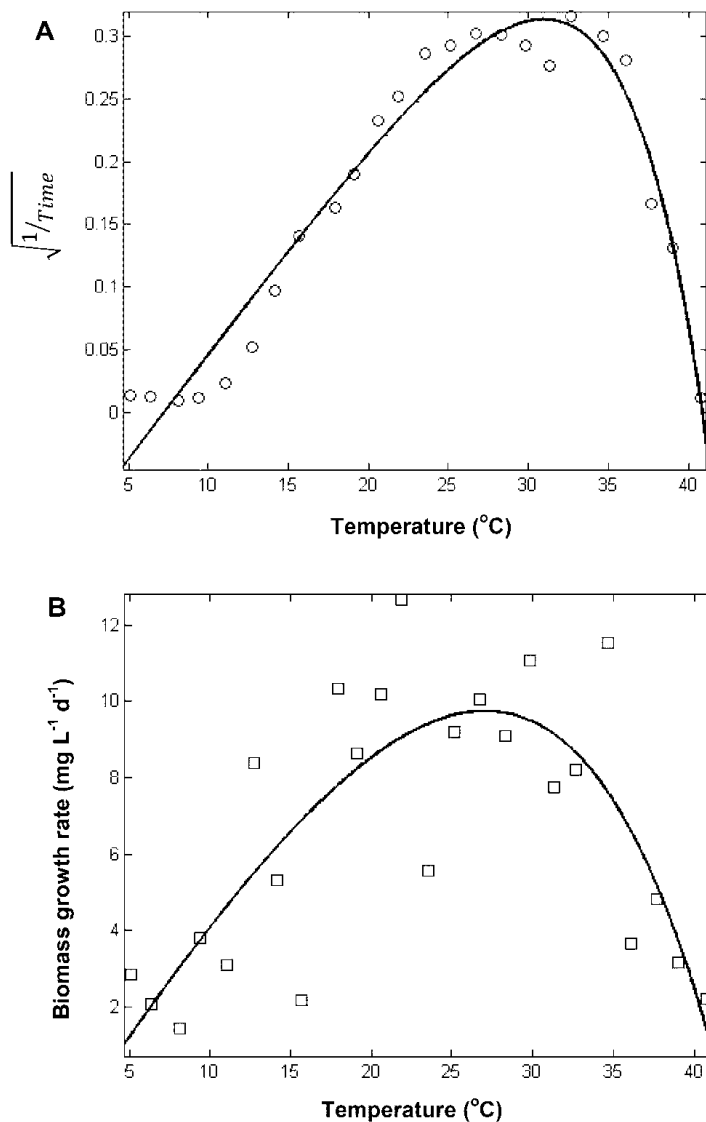


Figure 20. The results of the temperature gradient assay performed for the low temperature enrichment culture. A: The half-life of sulfate reduction calculated from sulfide production and B: biomass growth rate fitted to Ratkowsky Equation (Ratkowsky et al. 1983). This Figure has been published in Paper III.

7.2 Electron donor utilization

7.2.1 Electron donor utilization rates

The organic electron donor utilization rates in the FBR experiments of the present study (Papers I-V) and values reported in other reactor studies are listed in Table 28. When organic electron donors that yield acetate as intermediate product in sulfidogenic oxidation were used, the rate of acetate oxidation became the rate-limiting step. The reactor process should be optimized for retention and enrichment of the acetate oxidizing SRB. Kaksonen et al. (2004a) showed that efficient acetate oxidation was achieved in an ethanol-fed FBR treating mine wastewater. The sulfidogenic acetate oxidation produces majority of sulfide and alkalinity, which are necessary for metal precipitation and neutralization, and if the acetate oxidation is complete, there is no residual COD in the reactor effluent.

The maintenance and enrichment of the acetate oxidizing SRB requires careful control of the reactor conditions. Complete acetate oxidation in an FBR can be achieved only after a long operation period (Kaksonen et al 2004a, Paper IV). The heat sensitivity of acetate oxidizing SRB was demonstrated in Paper IV, as the unintentional short-term increase of FBR 1 temperature from 35°C to 55°C on day 253 did not significantly affect the ethanol oxidation capacity, while lactate- and acetate oxidation rates decreased significantly after the temperature increase (Paper IV). During the following 50 days the acetate oxidation capacity was resumed to 50 % of the original level, demonstrating the slow recovery of the acetate oxidation capacity (Paper IV).

Table 28. Electron donor oxidation rates and CODs (chemical oxygen demand of soluble compounds) removal rate measured in the FBR experiments in the present study and other studies. The percent oxidation is given inside parenthesis.

Reactor type	T (°C)	Electron donor	Ethanol (mmol L ⁻¹ d ⁻¹)	Lactate (mmol L ⁻¹ d ⁻¹)	Acetate (mmol L ⁻¹ d ⁻¹)	Formate (mmol L ⁻¹ d ⁻¹)	CODs (g L ⁻¹ d ⁻¹)	Reference
FBR	8	Ethanol	7.6 (99 %)	nr	1.1 (19 %)	nr	nr	Sahinkaya et al. (2006)
FBR	8	Ethanol	7.6 (99 %)	nr	1.9 (28 %)	nr	nr	Sahinkaya et al. (2007)
FBR	35	Ethanol	7.6 (80-97 %)	nr	nr	nr	nr	Kaksonen et al. (2003a)
FBR	35	Lactate	nr	8 (99 %)	nr	nr	nr	Kaksonen et al. (2003b)
FBR	35	Ethanol	60 (99 %)	nr	30-37	nr	nr	Kaksonen et al. (2004a)
FBR	28-32	Ethanol	15	nr	nr	nr	nr	Franzmann et al. (2008)
EGSB	33	Acetate	nr	nr	459 (95%)	nr	nr	Stucki et al. (1993)
EGSB	35	Acetate	nr	nr	389 (89 %)	nr	25.5 (81-89 %)	Dries et al. (1998)
Packed-bed reactor	30	Acetate	nr	nr	5.8 (98%)	nr	nr	Steed et al. (2000)
FBR 1	35	Ethanol-lactate	62 (90-99 %)	20 (74 %)	60 (50-99 %)	nr	nr	Paper IV
FBR 1	35	Plant material hydrolyzate	nr	nr	nr	nr	1.2-2.1 (40-90 %)	Paper V
FBR 2	9	formate	nr	nr	nr	52 (75-99 %)	nr	Paper I

FBR = fluidized-bed bioreactor, EGSB= expanded granular sludge blanket reactor, nr = not reported,

Toxicity of undissociated formic acid and acetic acid on SRB

Toxicity of undissociated formate on SRB was observed in FBR 2. The unstable FBR 2 operation during period I was characterized by simultaneous decrease of sulfate reduction, accumulation of acetate and drop of pH. The results indicated that unstable reactor operation resulted from the high formic acid loading when the influent pH was 3, i.e., below the pK_a (3.8) of formic acid. This caused formic acid toxicity, as the undissociated formic acid (and acetic acid) passed the cell membrane and dissociated in cytoplasm and, therefore, inhibited the SRB activity. The process recovered within a few days after the sulfate reduction had ceased. Similar toxic effects of undissociated acetate was observed during the first 90 days of the FBR 1 operation, when the undissociated acetic acid concentration was 0.1-3.6 mmol L⁻¹. Reis et al. (1990) reported that undissociated acetic acid concentration of 0.9 mmol L⁻¹ inhibited 50% of the activity of mixed SRB culture grown with lactate.

7.2.2 Plant material hydrolyzate as substrate for SRB at 35°C

A low cost substrate, *Phalaris arundinacea* was acid hydrolyzed (reed Canary grass hydrolyzate, RCGH) and used as substrate for sulfate reduction in FBR 1 (Paper V). The soluble sugars of the RCGH were presumably fermented into volatile fatty acids (VFA) and hydrogen, which served as electron donors for SRB. The highest sulfate reduction rate obtained with RCGH was 21-34 mmol L⁻¹ d⁻¹ in this FBR experiment. The major degradation product in the FBR was acetate, and acetate oxidation was also the limiting step in the FBR operation. RCHG has potential as low-cost electron donor, but complete oxidation of this complex substrate requires long reactor HRT when compared to use of electron donors with simple formula. Also the hydrolyzation process has to be optimized prior to large-scale application.

7.2.3 Electron flow to sulfate reduction in the reactor experiments

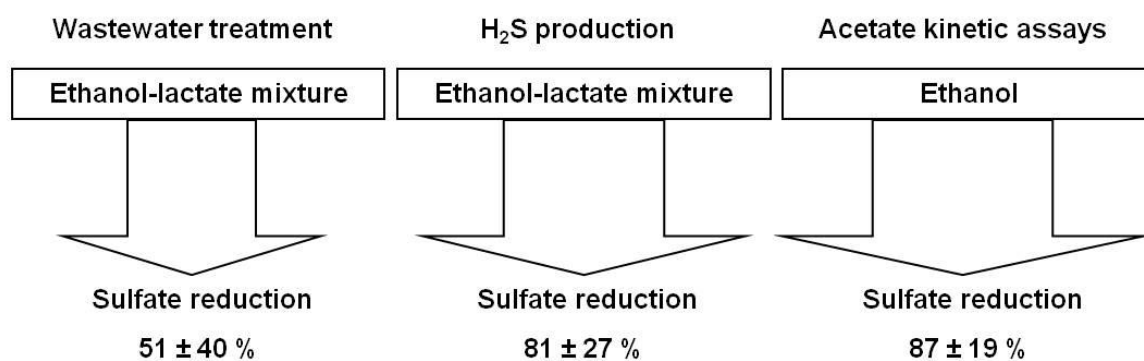
The ability of a given biomass to utilize electron donor for sulfate reduction can be reported as percent electron flow to sulfate reduction. The electron flows to sulfate reduction, acetate production (via homoacetogenesis) and methanogenesis in the reactor experiments of the present study are discussed in detail below and illustrated in Figures 21-25. For the studies with FBR 1, the electron flow only to sulfate reduction was quantified. Part of the electron donor left FBR 1 in the form of unoxidized acetate and the effluent contained 5-70 mmol L⁻¹ acetate, being on average only 8 mmol L⁻¹. With plant material hydrolyzate, the electron flows could not be chemically quantified due to complex pathways of the hydrolyzate degradation. The electron flow to biomass could only be quantified for MBRs, where the daily biomass quantification was possible. The electron flow to biomass growth in the MBRs was 0.4-2.9 %. For the GLB and MBR experiments, the H₂ feeding to the reactors was 50 % in excess to stoichiometric sulfate loading. The excess H₂ was necessary to provide sufficient system overpressure and required effluent gas composition and volume measurement. Therefore 100% electron flow to sulfate reduction could not be achieved in these experiments. Below, the electron flow studies are presented according to the used inocula, the mesophilic enrichment culture used in FBR 1 and MBR 2 or the psychrotolerant mesophilic enrichment culture used in FBR 2, MBR 1 and GLB.

Electron flow to sulfate reduction in the studies with mesophilic enrichment culture

The mesophilic enrichment culture was used to inoculate the reactors FBR 1 and MBR 2. The electron flow to sulfate reduction in the ethanol-lactate fed FBR 1 (35°C) is shown in Figure 21 (Paper IV). Part of the electron donor was not completely oxidized during the period of H₂S production, and left the reactor in the effluent as acetate. The incomplete electron donor oxidation was due to slow growth and enrichment rate of the acetate oxidizing SRB. Also

there was an unbalance in the acetate production from sulfidogenic ethanol and lactate oxidation and the acetate oxidation rate by SRB as discussed in Chapter 1. The electron flow to sulfate reduction increased to 99 % during the wastewater treatment period, although the average electron flow to sulfate reduction was 50 %. The low average electron flow to sulfate reduction was due to incomplete electron donor oxidation in the beginning of this experimental period as shown in Figure 21. Possible methane production was not quantified in this study.

FBR 1: 35°C



SYMBOLS:



Figure 21. The electron flow to sulfate reduction in the three operation periods of the ethanol-lactate fed FBR 1 (35°C). The electron flow to methanogenesis was not quantified, and part of the electrons left the FBR unused in the form of acetate, which was produced in the sulfidogenic ethanol and lactate oxidation.

The effect of temperature on electron flow with the mesophilic enrichment culture

The electron flow to sulfate reduction, acetate production (via homoacetogenesis) and methanogenesis in the experiment with H₂/CO₂-fed MBR 2 was as shown in Figure 22 (Paper II). The electron flow to sulfate reduction decreased as the temperature was decreased, indicating that the ability of the SRB to utilize this electron donor decreases as temperature is decreased. The energy gain (ΔG^0) from sulfidogenic H₂ oxidation increases as temperature decreases (Lettinga et al. 2001), thus the decreased ability to use substrate was due to lowered affinity and substrate transport rate of the cells (Nedwell 1999) rather than thermodynamic limitation of the sulfate reduction process due to temperature. In the end of this experiment the MBR temperature was increased from 9 to 35°C for a few days, and the activity of SRB was resumed, resulting in immediate increase of the electron flow to sulfate reduction from 24 % to 62 % (Paper II). The electron flow to biomass consumed 0.4-1.9 % (average 0.5 %) of the electron flow. The electron flow to acetate was calculated based on the measured acetate concentration in the MBR. Acetate production consumed only a minor proportion of the electrons, and electron flow to acetate production ceased with the decreasing temperature. The acetate may have served as carbon source for heterotrophic SRB, thus there might have been some acetate consumption in the reactor, which was not possible to quantify in this study. The inocula of this reactor originated from FBR 1, and as can be seen in the Figure 20, there was some methane production at 35 and 15°C, thus it can be assumed, that there might

also have been some methanogenic activity in the FBR 1 during the experiments, but as this was not quantified, the occurrence of methanogenesis in FBR 1 is only speculative. However, these results show that the methanogenic species may have survived in the enrichment as the microbial community of this enrichment has been shown to be diverse by Kaksonen et al. (2004b; 2004c), or the methanogens may have originated from the MBR equipment, which was previously operated with a different enrichment culture, which had methanogenic activity (Bijmans 2008). The methane production ceased as the operational temperature was decreased from 35 to 15°C and stayed at this level. Altogether methanogenesis and acetate production were not significant electron sinks for this reactor and sulfate reduction consumed the majority of the electrons.

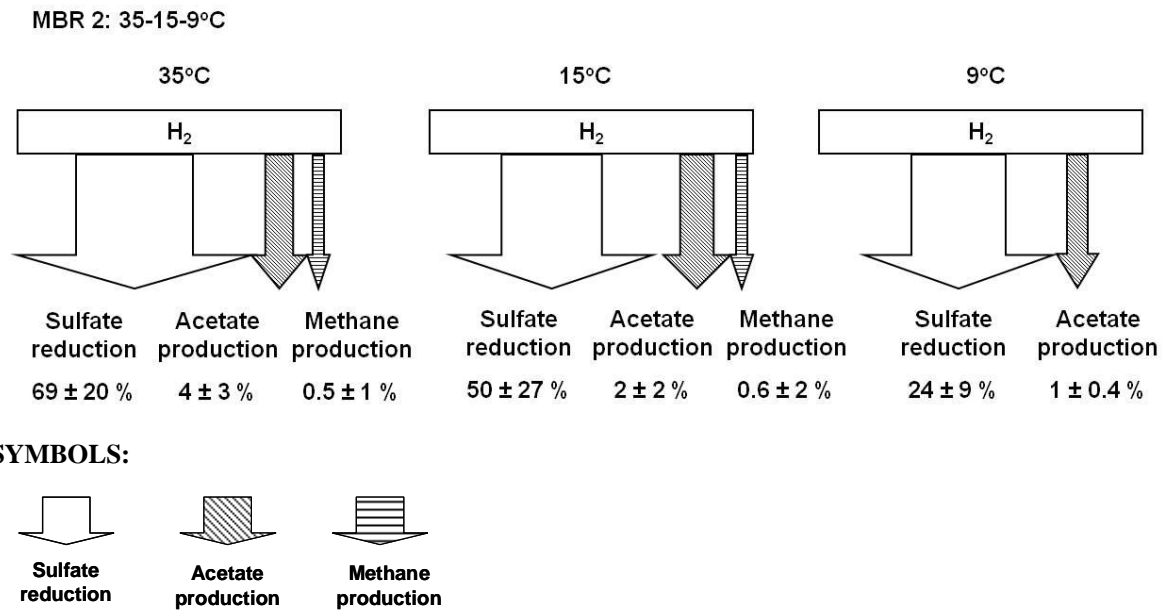


Figure 22. The effect of temperature on electron flow to sulfate reduction at 35, 15 and 9°C in the H₂/CO₂ fed MBR 2 inoculated with the mesophilic enrichment culture.

Electron flow to sulfate reduction in the studies with psychrotolerant mesophilic enrichment culture

The psychrotolerant mesophilic enrichment culture was used in the FBR 2, MBR 1 and GLB experiments. The electron flow to different processes for the formate-fed FBR 2 operated at 9°C was as shown in Figure 23 (Paper I). The inoculum for FBR 2 was the low temperature sulfidogenic enrichment. Sulfate reduction was the major electron sink. Figure 23 shows that acetate production via homoacetogenesis consumed part of the electrons. Acetate production was highest during the unstable period, when the reactor pH dropped due to high acidity and formic acid loading, resulting in simultaneous inhibition of sulfate reduction. The acetate production via homoacetogenesis from H₂ and CO₂ is highly dependent on the CO₂ partial pressure (Bijmans 2008), but presumably the inhibition of SRB was due to the pH drops, and this factor allowed the acetogens to compete more efficiently for H₂ for short periods of time. During stable operation, acetate was a minor electron sink in FBR 2. The microbial community of FBR 2 was dominated by *Desulfomicrobium* sp. (Paper I), which requires acetate as carbon source; therefore some acetate production is necessary to support the growth of the active SRB species. The acetate consumption was not quantified in this study, thus the electron flow to acetate was calculated based on the measured acetate concentration

in the FBR. Methane production was not observed in the measurements during this experiment.

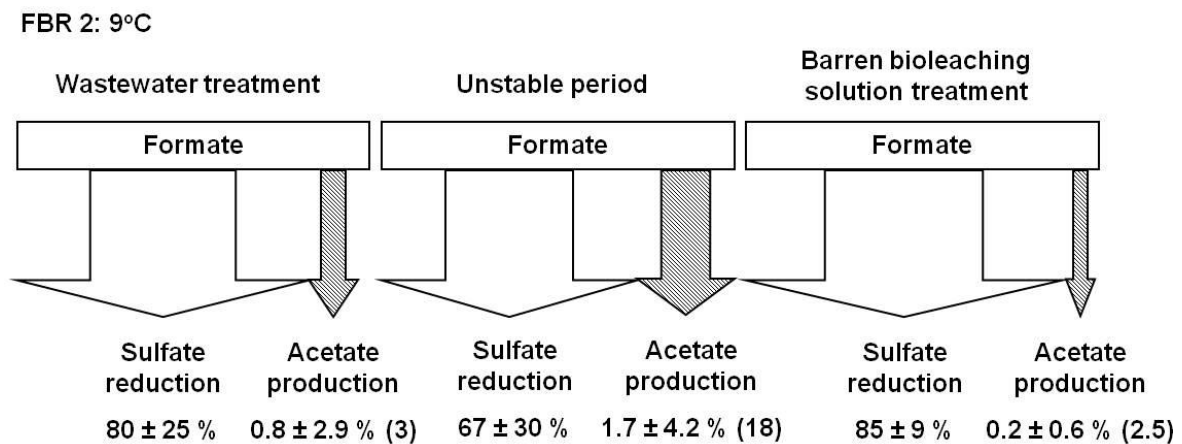


Figure 23. The average electron flow from formate to sulfate reduction and acetate production (via homoacetogenesis) in the formate-fed FBR 2 operated at 9°C during the three operation periods. During the unstable period, there were pH drops in the FBR 2, and this resulted in simultaneous inhibition of sulfate reduction and activation of acetate production. The maximum percent electron flow to acetate production is given inside parenthesis.

The effect of temperature on electron flow with the psychrotolerant mesophilic enrichment culture

The electron flow to sulfate reduction and acetate production (via homoacetogenesis) in the H₂/CO₂-fed MBR 1 operated at 9, 15 and 30°C was as shown in Figure 24 (Paper II). The inoculum for this reactor originated from FBR 2. Sulfate reduction consumed majority of the electrons, but especially at 9°C acetate was a remarkable electron sink (Figure 24). The electron flow to acetate production was controlled by the CO₂ supply. The electron flow to acetate production decreased as temperature was increased, and simultaneously the electron flow to sulfate reduction increased. This indicates that the activity of SRB at 9 and 15°C was limited by the ability to oxidize H₂ and the homoacetogenic bacteria were not able to compete efficiently for H₂ at elevated temperatures. The high electron flow to acetate production was presumably result of the combined effect of almost complete biomass retention, long HRT of the system (6 d) and high CO₂ partial pressure, which allowed the enrichment of the homoacetogenic bacteria. The electron flow to biomass production was 0.2-2.9 (average 0.9 %). The higher electron flow to biomass in MBR 1 than in MBR 2 was presumably due to excessive growth of homoacetogenic bacteria. No methane was produced during this experiment.

MBR 1: 9-15-30°C

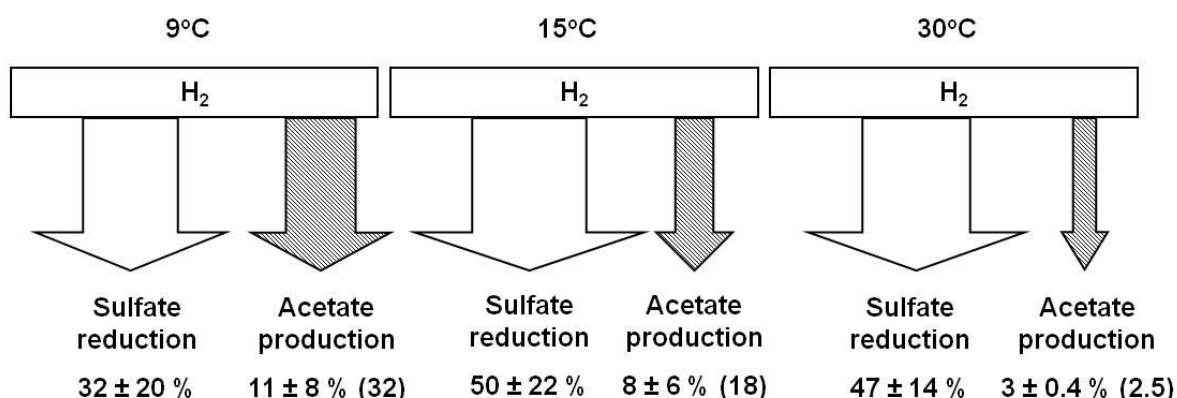


Figure 24. The effect of temperature on electron flow to sulfate reduction at 9, 15 and 30°C in the H₂/CO₂ fed MBR 1 inoculated with the psychrotolerant mesophilic enrichment culture. The maximum percent electron flow to acetate production is given inside parenthesis.

Electron flow to sulfate reduction in the gas-lift bioreactor operated at 9°C

The electron flow to sulfate reduction and acetate production in the H₂/CO₂ fed gas-lift bioreactor operated at 9°C was as shown in Figure 25 (Paper III). Sulfate reduction consumed majority of the electrons. The electron flow to acetate production decreased as the reactor HRT was decreased from 5 days to 1 day, indicating that the acetate production can also be controlled by applying low HRT (Paper III). No methane was produced during the experiment.

GLB: 9°C

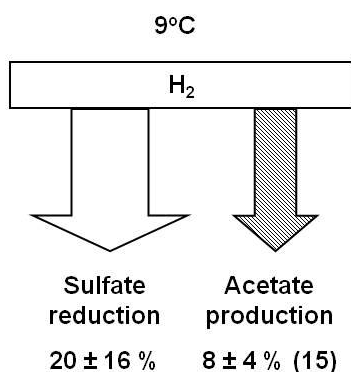


Figure 25. The average electron flow to sulfate reduction and acetate production in the H₂/CO₂ fed gas-lift bioreactor operated at 9°C. The highest electron flow to acetate production is given inside parenthesis.

Table 29 summarizes electron flows to sulfate reduction, acetate production and methanogenesis in the present study and values reported in the literature. The electron flows to different processes have not been reported for low temperature sulfate reduction, therefore the data of Paper II is important, as it also shows the effect of temperature on the electron flow. The results for MBR 1 and MBR 2 show that the electron flow to sulfate reduction decreased with the temperature for mesophilic sulfidogenic enrichments, indicating that the ability of the SRB to utilize hydrogen decreased with the temperature decrease. Majority of

the electron flow to sulfate reduction in the present study, and the values reported in the present study were in the same range to values reported in other studies. The absence of methane production in the reactors operated with the low temperature sulfidogenic enrichment (FBR 2, MBR 1, GLB) indicates that careful enrichment results in culture with no methanogenic activity, although presumably the methanogens have originally been present.

Table 29. Percent electron flow to sulfate reduction, acetate production (via homoacetogenesis) and methanogenesis in the present study and other reactor studies.

Reactor	T (°C)	Electron donor	Sulfate reduction (%)	Acetate production (%)	Methanogenesis (%)	Reference
FBR	35	Ethanol	60-99	nr	nr	Kaksonen et al. (2004a)
FBR	35	Ethanol	77-95	nr	nr	Kaksonen et al. (2003b)
FBR	35	Lactate	60-75	nr	nr	Kaksonen et al. (2003b)
FBR	8	Ethanol	36 ± 6	nr	nr	Sahinkaya et al. (2007)
MBR	30	H ₂	69	9	2-6	Bijmans et al. (2008a)
UASB	30	Acetate	41-65	nr	35-59	Omil et al. (1997)
EGSB	35	Acetate	60-94	nr	nr	Dries et al. (1998)
Gas-lift	30-	Synthesis	53-72*	nr*	28-47	van Houten et al. (2006)
	35	gas				
FBR 1	35	Ethanol-lactate	51 – 87	nr	nr	Paper IV
MBR 2	35	H ₂	69 ± 20	4 ± 3	0.5 ± 1	Paper II
MBR 2	15	H ₂	50 ± 27	2 ± 2	0.6 ± 2	Paper II
MBR 2	9	H ₂	24 ± 9	0.6 ± 0.4	0	Paper II
FBR 2	9	Formate	67 - 85	0.8- 18	0	Paper I
GLB	9	H ₂	20 ± 16	8 ± 4	0	Paper III
MBR 1	9	H ₂	32 ± 20	11 ± 8	0	Paper II
MBR 1	15	H ₂	50 ± 22	8 ± 6	0	Paper II
MBR 1	30	H ₂	47 ± 14	2 ± 0.4	0	Paper II

*Acetate concentration up to 4 g L⁻¹ was observed in the reactor, but specific electron flow to acetate production was not reported. FBR = fluidized-bed bioreactor, MBR = membrane bioreactor, UASB = up-flow anaerobic sludge blanket reactor, EGSB = expanded granular sludge blanket reactor, GLB = gas-lift bioreactor

The electron donor utilization rate shows the capacity of the bioprocess microbial community to oxidize the given substrate. The percent electron flow indicates the efficiency of the overall process. The percent electron flow summarizes the electrons from different substrates, and the electron flow to undesired processes, such as methanogenesis is also shown. With organic electron donors, the acetate oxidation rate should be optimized, and this generally requires lower electron donor loading during the start-up phase of the reactor. High electron flow to sulfate reduction results in high sulfide production rate and alkalinity generation rate, which are the key processes in the precipitation of metals and neutralization of the acidic wastewater.

In the present study, the percent electron flow to sulfate reduction was high in all the reactors except the GLB. The low electron flow to sulfate reduction in the GLB was the result of the carrier material crushing, which caused remarkable biomass loss, and therefore also low sulfidogenic activity. The highest electron flows to sulfate reduction over long operation periods were in the FBRs, showing that this reactor type supports stable sulfate reduction rates with high electron donor utilization rate. This is somewhat contradictory to the low SSA observed in the FBRs. However, the FBR seems to be a preferred option for mine wastewater treatment, when the long time reactor performance is considered.

7.2.4 The electron donor utilization batch assays

The ability of the sulfidogenic enrichment cultures used in the present study to utilize acetate, plant material hydrolyzate and formate was studied using batch assays.

Acetate oxidation kinetics at 35°C

Acetate oxidation kinetics of the FBR 1 biomass was studied by FBR operated in batch mode (Paper IV). In these studies the K_m , the affinity towards acetate and specific sulfidogenic acetate oxidation rate, V_{max} , of the biomass were determined (Figure 26). For acetate oxidation, the K_m was $63 \mu\text{mol L}^{-1}$ and V_{max} was $0.76 \mu\text{mol acetate g VSS}^{-1} \text{min}^{-1}$. The batch assays were performed at neutral pH, so even at the highest total acetate concentration, 52 mmol L^{-1} , the undissociated acetic acid did not have an inhibitory effect. Kaksonen et al. (2003a) reported K_m of $45\text{-}55 \mu\text{mol L}^{-1}$ for acetate for this enrichment culture, thus the K_m reported in the present study was in the same range. The V_{max} measured in the present study was 24 % higher compared to V_{max} of $0.55\text{-}0.58$ reported by Kaksonen et al. (2003a), indicating that the enrichment of acetate oxidizing SRB for the same enrichment culture was a slow process. In conclusion, the SRB in the FBR 1 had high affinity to acetate and the specific acetate oxidation rate was improved steadily as the acetate oxidizing SRB had optimal growth conditions.

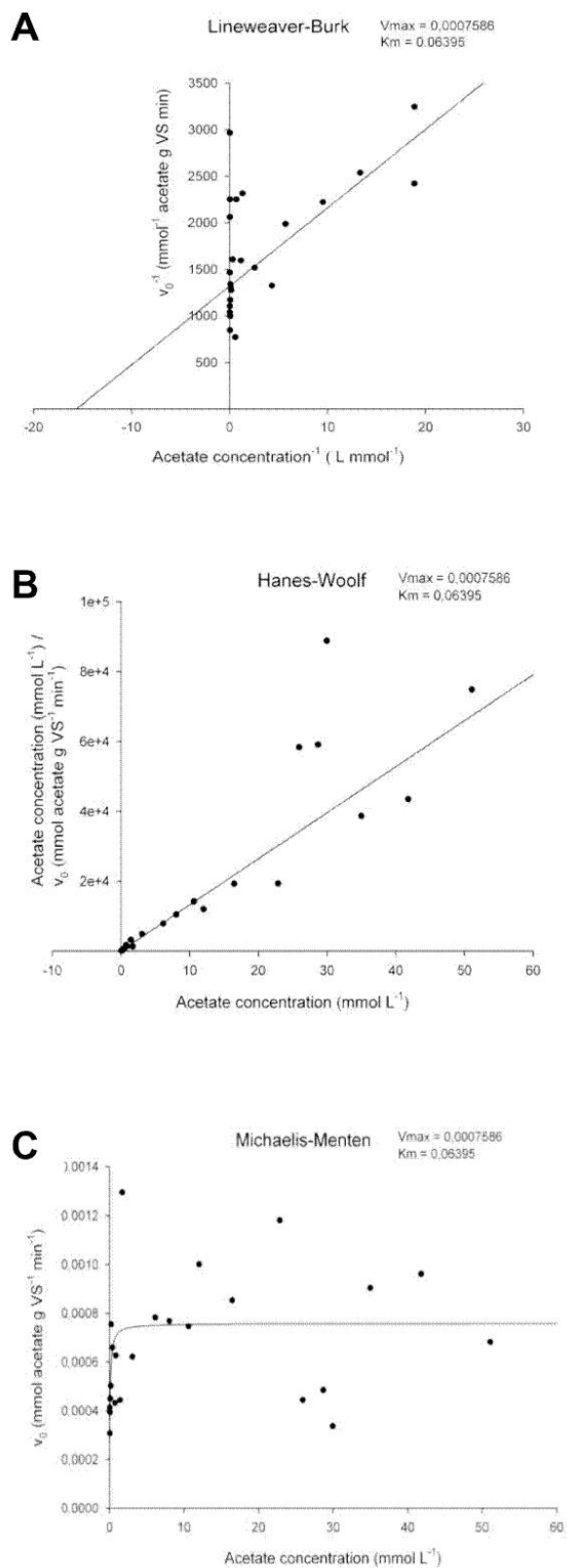
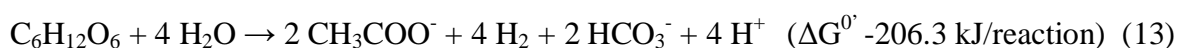


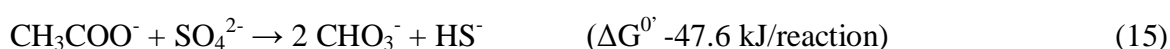
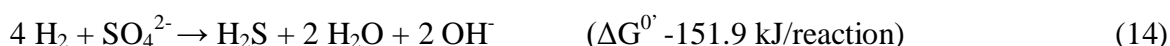
Figure 26. The results of the acetate kinetic batch-assays: A: Lineweaver-Burk plot, B: Hanes-Woolf plot and C: the results fitted into Michaelis-Menten equation. The dots represent measured acetate oxidation rates for various acetate concentrations and the lines the results fitted to equations. This Figure has been published in Paper IV.

Hydrolyzed plant material as substrate for SRB

The suitability of the reed canary grass hydrolyzate (RCGH) and dry plant material as low cost electron donors for SRB was tested with batch bottle assays (Paper V). The RCGH and plant material were degraded to VFAs. The total VFA concentration varied between 300-1300 mg L⁻¹, constituting on average of acetate (84 %), propionate (6%) and butyrate (10%). The microbial community of the inoculum (FBR 1) has been shown to be diverse, and it also contained fermentative bacteria (Kaksonen et al. 2004b; 2004c). Therefore, the soluble sugars of the RCGH were presumably fermented to acetate and hydrogen according to equation (Thauer et al. 1977):



The produced hydrogen and acetate were then oxidized by SRB (Thauer et al. 1977):



Sulfide production started during the first 7 days in all the bottles with RCGH and dry plant material. The highest dissolved sulfide concentration in the batch bottles with RCGH was 530 mg L⁻¹ and with dry plant material 350 mg L⁻¹. The H₂S yields were 6.2 and 0.8 mmol g⁻¹ plant material for RCHG and dry plant material, respectively. The results of the batch bottle assay are discussed in more detail in Paper V.

Formate utilization of the low temperature enrichment culture

A batch bottle assay was performed with the FBR 2 culture to study the degradation of formate at 9°C in the presence and absence of 10 mmol L⁻¹ molybdate to inhibit sulfate reduction (Paper I). In this assay, hydrogen accumulated in all the bottles, and sulfate reduction started only after the hydrogen accumulation. Acetate accumulated both in the presence and absence of molybdate. These results show that formate was first converted to H₂, followed by oxidation by SRB. The CO₂ from dissociated formate was first converted to acetate, which was likely subsequently assimilated by SRB as the carbon source. Similar formate degradation pathway had been observed in a formate fed MBR by Bijmans et al. (2008a), and in this study it was also pointed out that the accumulation of hydrogen made the occurrence of sulfate reduction thermodynamically favorable.

7.3 Biomass growth and retention in the reactor experiments

The biomass growth and retention in the reactor experiments of the present study and in other studies are shown in Table 30. The temperature did not affect the ability of the SRB to retain on carrier material, as the volatile solids (VS) values for the carriers were in the same range independently of the temperature. The volatile suspended solids (VSS) values reported for FBRs were generally lower than in other reactor types, since in the FBRs the majority of the biomass was retained by the carrier material, and unbound biomass was prone to wash-out. The precipitation of metals and retention of these precipitates as TSS in the FBRs improved the retention of VSS in FBR 1 and FBR 2 (Papers I, IV and V). The daily measurement of biomass concentration allowed the determination of biomass doubling times (T_d) in the MBRs. The temperature in both MBRs affected the biomass doubling time. In MBR 1, the high growth rate of homoacetogenic bacteria resulted in the small doubling time at 9°C. Based on the T_d values, the long enrichment time of the mesophilic SRB at low temperature (8-9°C) improved the ability of this culture to grow at decreased temperature. On the other

hand, the T_d was the doubling time of the community, not only the SRB, therefore it is only an indicative parameter in design of anaerobic bioreactors.

Table 30. Biomass growth and retention in sulfate reducing reactors in this study and from the literature. The biomass concentration was measured as volatile solids (VS) and volatile suspended solids (VSS). T_d = biomass doubling time. VSS represents the biomass concentration in the reactor, and the total VSS can be calculated by multiplying the VSS with the total reactor volume.

Reactor	T (°C)	Electron donor	VS (mg g ⁻¹ carrier material)	VSS (mg L ⁻¹)	T_d (d)	Reference
FBR	8	Ethanol	5	nr	nr	Sahinkaya et al. (2006)
FBR	8	Ethanol	9	nr	nr	Sahinkaya et al. (2007)
FBR	35	Lactate	9-17	20-130	nr	Kaksonen et al. (2003b)
FBR	35	Ethanol	3.8-4 ^a	20-150	nr	Kaksonen et al. (2003a)
FBR	35	Ethanol	4.1- 4.3 ^a	100-200	nr	Kaksonen et al. (2004a)
GLB	30	H ₂	nr	1400	nr	Bijmans et al. (2009b)
MBR	30	Formate	nr	4000	nr	Bijmans et al. (2008a)
GLB	30	H ₂	nr	120-260	nr	Bijmans et al. (2009a)
EGSB	35	Acetate	nr	19 000	nr	Dries et al. (1998)
GLB	30-35	Synthesis gas	nr	5.2 ^b	nr	van Houten et al. (2006)
FBR 1	35	Ethanol-lactate	10-40	90-380	nr	Paper IV
FBR 1	35	Plant material hydrolyzate	25-40	100-700	nr	Paper V
MBR 2	35	H ₂	nr	10-200	6	Paper II
MBR 2	15	H ₂	nr	100-210	10	Paper II
MBR 2	9	H ₂	nr	80-190	11	Paper II
FBR 2	9	formate	3-12	500-4000	nr	Paper I
GLB	9	H ₂	50	1-72	nr	Paper III
MBR 1	9	H ₂	nr	20-250	5	Paper II
MBR 1	15	H ₂	nr	110-340	4	Paper II
MBR 1	30	H ₂	nr	91-230	4	Paper II

^a total biomass concentration (g) in the FBR, ^b TSS 129 g L⁻¹, FBR = fluidized-bed bioreactor, MBR = membrane bioreactor, EGSB = expanded granular sludge blanket reactor, GLB = gas-lift bioreactor

7.4 Metal precipitation in the reactor experiments

7.4.1 Metal precipitation rates

The metal precipitation rates measured in FBR 1 and FBR 2 with synthetic mine wastewater (Papers I, IV and V) and values reported in other studies are shown in Table 31. The majority of the metal loading to these reactors consisted of iron, which did not cause toxic effect, and high precipitation rates and percent precipitation were achieved. Another metal precipitated in the FBRs was zinc, which was used only in minor concentrations. The formation of the metal precipitates did not interfere with the reactor performance, actually the precipitates improved reactor performance by retaining suspended biomass (VSS). Paper I described also the treatment of real mine waste water, the diluted barren bioleaching solution, which contained iron and minor concentrations of Ni, Zn, Cu, Cr, Al and As. Stable treatment of this wastewater was obtained in the formate-fed FBR 2 operated at 9°C. The percent metal precipitation was high in all the FBR studies, being 99 % for Fe, Zn, Ni, Zn, Cu, Cr and As, and 60 % for Al.

Table 31. Metal precipitation rates in the FBRs of the present study and values reported in other studies. The percent metal precipitation is given inside parenthesis.

Reactor	T (°C)	pH	Electron donor	Fe precipitation (mmol L ⁻¹ d ⁻¹)	Zn precipitation (mmol L ⁻¹ d ⁻¹)	Precipitation of other metals (mmol L ⁻¹ d ⁻¹)	Reference
FBR	8	7	Ethanol	1.1 (99%)	nr	nr	Sahinkaya et al. 2006
FBR	8	7	Ethanol	1.3 (99%)	nr	nr	Sahinkaya et al. 2007
FBR	35	8	Lactate	1.1 (99 %)	7.7 (99 %)	nr	Kaksonen et al. 2003b
FBR	35	8-9	Ethanol	1.6 (99 %)	7.7 (99 %)	nr	Kaksonen et al. 2003a
FBR	35	9-9	Ethanol	5.4 (99 %)	9.2 (99 %)	nr	Kaksonen et al. 2004a
GBL	30	5.5	H ₂	nr	7.2 (99 %)	nr	Bijmans et al. 2009b
GLB	30	5	H ₂	nr	nr	7.7 Ni	Bijmans et al. 2009a
Packed-bed reactor	30	7.2	Acetate	1.8 (99 %)	1.2 (99 %)	Cu 0.23 (99%) Mn 0.58 (97 %)	Steed et al. (2000)
Anaerobic filter	5	6.5	Ethanol, spent manure	3-7.4 (32-93 %)	nr	nr	Tsukamoto et al. (2004)
FBR 1	35	7	Ethanol-lactate	11 (99 %)	1 (99 %)	nr	Paper IV
FBR 1	35	7	Plant material hydrolyzate	15 (99 %)	0.23 (99 %)	nr	Paper V
FBR 2	9	8	formate	5.4 (99 %)	0.03 (99 %)	0.11 (60-99 %) ^a	Paper I

^a total sum of Al, As, Cu, Cr, Mn and Ni precipitated in the assay with FBR 2 fed with barren bioleaching solution, all the metals except Al were 99 % precipitated, Al was 60 % precipitated. FBR = fluidized-bed bioreactor, MBR = membrane bioreactor, GLB = gas-lift bioreactor, nr = not reported,

7.4.2 Formation of metal sulfides

The formation of the metal sulfides in the FBRs was estimated using a chemical modeling program OLI Stream Analyzer 2.0 (OLI systems Inc., Morris Plains, USA). In FBR 1 (Papers IV and V), iron precipitated as hexagonal iron (II) sulfide (FeS) and zinc precipitated as cubic zinc sulfide (ZnS). The other major mineral components of the influent, Na and Mg, did not precipitate in the FBR conditions. In FBR 2 (Paper I) iron, nickel, copper and zinc precipitated as sulfides, aluminum as hydroxide and manganese mainly as manganese sulfide, but also some manganese carbonates and manganese hydroxides may have formed.

7.5 Alkalinity generation in the sulfate reducing reactors

The neutralization of the acidic influent and alkalinity generation was measured in the FBRs treating synthetic mine wastewaters. In the MBR and GLB experiment the reactor pH was set to 7.5 using a pH controller to provide optimal conditions for SRB. In the FBRs, the acidic influent (3-5) was efficiently neutralized and the SRB were not affected by the low pH of the influent. Also the alkalinity production rate was high. The major form of alkalinity in FBR 1 was bicarbonate alkalinity as a result from sulfidogenic oxidation of organic electron donors. In FBR 2, the majority of the alkalinity was hydroxide alkalinity, as a result from sulfidogenic hydrogen oxidation. The neutralization of acidic influent and alkalinity generation in the present study and other studies are shown in Table 32. Acetate oxidation is desirable, as majority of the sulfidogenic alkalinity is produced in this reaction.

Table 32. Alkalinity generation rates measured in the reactors operated in the present study and values reported in other studies. In the present study, the influent pH was decreased stepwise in each experiment.

Reactor	T (°C)	Electron donor	Influent pH	Effluent pH	Alkalinity generation (mmol L ⁻¹ d ⁻¹)	Reference
FBR	8	Ethanol	5.5	7.5	21	Sahinkaya et al. (2006)
FBR	8	Ethanol	7.2	7	22	Sahinkaya et al. (2007)
FBR	35	Lactate	2.5	8	20	Kaksonen et al. (2003b)
FBR	35	Ethanol	3-4.5	8-9	23	Kaksonen et al. (2003a)
FBR	35	Ethanol	3	8	79	Kaksonen et al. (2004a)
FBR	28-32	Ethanol	2.5-5.5	7	105	Franzmann et al. (2008)
Anaerobic filter	5	Ethanol, spent manure	2.5	6.5	11	Tsukamoto et al. (2004)
FBR 1	35	Ethanol-lactate	4-5	7	225	Paper IV
FBR 1	35	Plant material hydrolyzate	4.5-7.8	7	60	Paper V
FBR 2	9	Formate	3-5.5	8-9	150	Paper I

FBR = fluidized-bed bioreactor, nr = not reported

7.6 The microbial community of the low temperature enrichment culture

The microbial community of the low temperature enrichment culture was analyzed during FBR 2 operation (Paper I). The microbial community of the other enrichment culture used in the present study has been shown to be diverse by Kaksonen et al. (2004b, 2004c), therefore this culture was not analyzed further in this study.

The microbial community of FBR 2 was studied using DGGE. The bacterial community was analyzed using DNA extraction, and the active species were analyzed using RNA extraction. To study the SRB species in FBR 2, SRB specific *dsrB*-primers were used for both DNA and RNA extracts. The results of the DGGE- analyses were as shown in Figure 27 A and B. The species found with the 16S rRNA gene primers were *Desulfomicrobium* sp. (96-99%), *Desulfomicrobium apsheronum* (99 %), *Desulfomicrobium baculatum* (99 %), *Desulfuromonas acetexigens* (96 %), and an uncultured *Clostridiales* bacterium (99 %). The sequences obtained with the *dsrB*-primers aligned all with *Desulfomicrobium baculatum*. The overall quality of the RNA-sequences was poor, and the few good quality sequences belonged to the genus *Desulfomicrobium*. SRB belonging to the genus *Desulfomicrobium* have earlier been reported from this FBR by Sahinkaya et al (2007). The acetate production in FBR 2 was presumably due to activity of *Clostridiales* sp. The *Desulfuromonas acetexigens* is known to be a mesophilic sulfur reducer that only grows on acetate as electron donor (Finster et al. 1994). Mesophilic SRB were also dominant in the FBR operated at 8°C reported by Sahinkaya et al. (2007). Karnachuk et al. (2005) reported *Desulfobulbus* and *Desulfococcus-Desulfosarcina* group mesophilic SRB from MPN incubations of permanently cold sediments, indicating that also these groups contain psychrotolerant species. In summary, the microbial community of a long-term low temperature operated FBR was simple and stable and it consisted of genus *Desulfomicrobium*. It was also the active genus in the reactor based on mRNA-cDNA-DGGE analysis. The genus *Desulfomicrobium* is mesophilic, thus the low temperature FBR was dominated by a psychrotolerant mesophile.

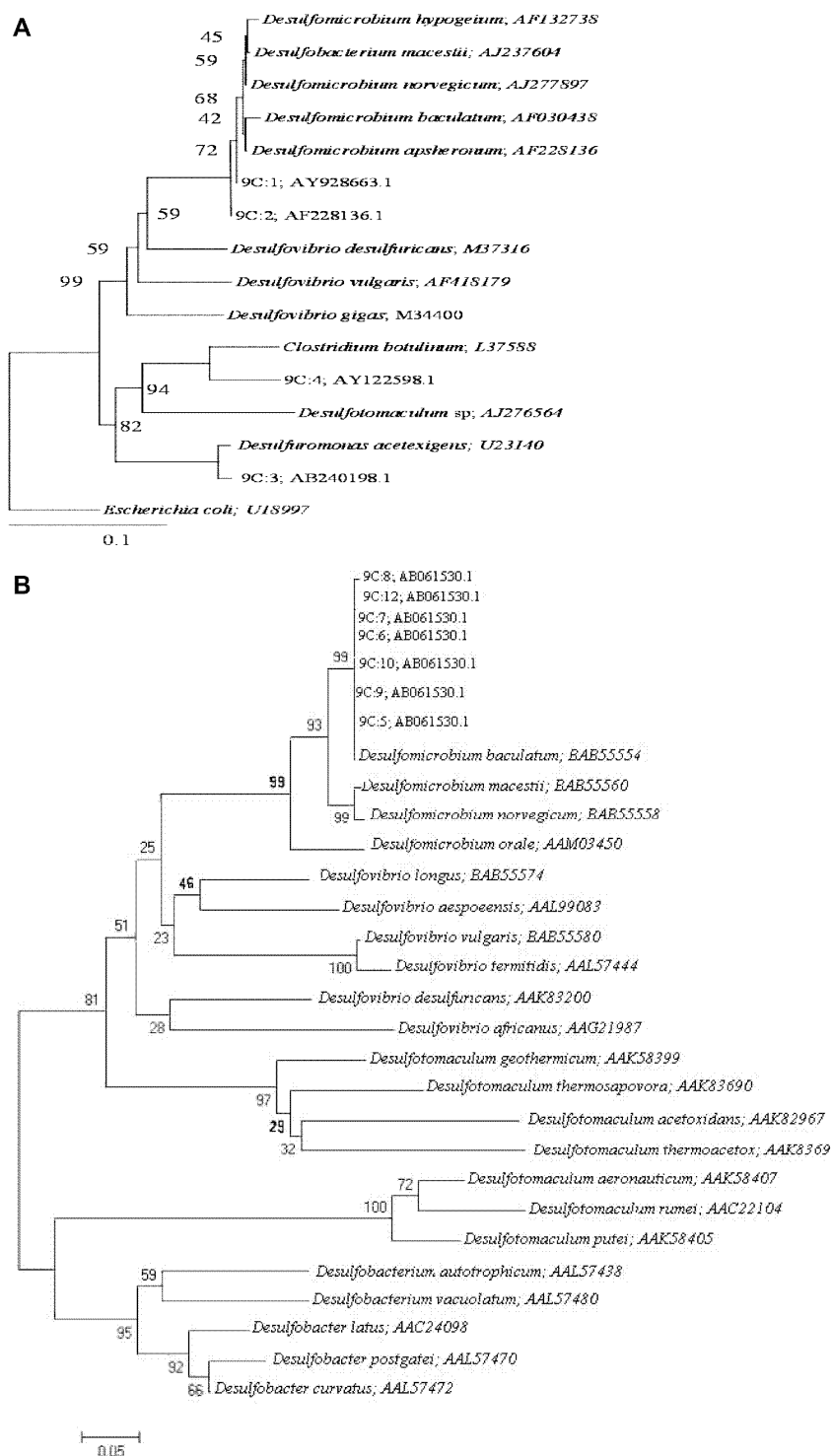


Figure 27. A) The phylogenetic tree of 16S rRNA genes obtained from the FBR and reference sequences from databases (the reference strains include their accession numbers). Numbers at nodes represent bootstrap values in 1000 times resampling analysis. The scale bar indicates 0.1 changes per nucleotide. The length of the aligned sequences was 550 base pairs. *Escherichia coli* represents the out-group of the tree. B) The phylogenetic tree of *dsrB* sequences obtained from the FBR and reference sequences from databases. Numbers at the nodes represent bootstrap values in 1000 times resampling analysis. The length of the aligned sequences was 350 base pairs. The scale bar indicates 0.05 changes per nucleotide. This Figure has been published in Paper I.

8 Conclusions

High sulfate reduction rates were achieved in bioreactors operated at 35 and 9°C. Fluidized-bed bioreactor processes treating synthetic and real mine wastewaters at both temperatures remained stable over the long operation periods. Acidic influent was efficiently neutralized and metals were precipitated at high percentage. Low temperature limited the sulfidogenic acetate oxidation rate. Therefore, the non-acetate yielding electron donors, such as formate and hydrogen are recommended for low temperature mine wastewater treatment, although the described psychrophilic and psychrotolerant SRB species are also able to oxidize acetate. The specific sulfidogenic activities of the sulfidogenic biomass varied depending on the reactor type, and highest sulfidogenic activities were achieved with membrane bioreactors that had complete biomass retention. Membrane bioreactors are not suitable for practical applications of mine wastewater treatment, but rather a tool to study the characteristics of the biomass. Therefore, the high sulfidogenic activities obtained in these reactors are only indicative. The specific sulfidogenic activities obtained with carrier-based bioreactors, gas-lift bioreactor and fluidized-bed bioreactors were lower. However, in the fluidized-bed bioreactors the high electron flow to sulfate reduction and high electron donor utilization rate were maintained over a long period of time. High electron flow to sulfate reduction results in high sulfide production rate and alkalinity generation rate, which are key parameters in the precipitation of metal sulfides and neutralization of the acidic wastewater. This demonstrates that the fluidized-bed bioreactor as reactor type is suitable for mine wastewater treatment and biological hydrogen sulfide production. Fluidized-bed bioreactors are also suitable to enrich sulfate-reducing biomass, especially the slow-growing acetate oxidizing sulfate-reducing bacteria. The acetate oxidation kinetics of the biomass defines the acetate oxidation capacity of the bioreactor, and, therefore, also the efficiency of the treatment process, when acetate yielding organic electron donors are used. The absence of methane production in the reactors operated with the low temperature sulfidogenic enrichment indicated that control of the reactor conditions results in an enrichment culture with insignificant methanogenic activity, although presumably methanogens have been present in the original inoculum.

Choice of an efficient reactor type is related to the chosen electron donor and the design sulfate reduction rate. The gas-lift bioreactor is the choice if H₂ or synthesis gas are used. Fluidized-bed bioreactor and membrane bioreactor are suitable for soluble electron donors, such as ethanol. Both gas-lift bioreactor and fluidized-bed bioreactor are suitable reactor types for low temperature mine wastewater treatment, as high sulfate reduction rate and biomass retention can be achieved in these reactors. The sulfate reduction rates reported for UASB reactors and fluidized-bed bioreactors are slightly lower than for gas-lift bioreactors, but the (design) sulfate reduction rate will depend on the treated wastewater quality and quantity, therefore lower sulfate reduction rates can be sufficient for the treatment process. When considering low-cost electron donors for sulfate-reducing bacteria, organic solid materials are available in high quantities. Solid material is suitable for passive treatment systems and column-type reactors, but not to high rate reactors such as gas-lift bioreactors and fluidized-bed bioreactors. Soluble reed Canary grass (*Phalaris arundinacea*) plant material hydrolyzate is amenable substrate to support sulfate-reducing bacteria in a fluidized-bed bioreactor treating mine wastewater. The dry reed Canary grass (*Phalaris arundinacea*) plant material also supports sulfate reduction and can be considered as electron donor for passive treatment systems.

The analysis of the microbial community, the metabolic and growth parameters of the low temperature sulfidogenic enrichment culture yielded new information on the performance of mesophilic SRB at sub-optimal temperatures. The microbial community analysis with DGGE that was performed for the low temperature sulfidogenic enrichment culture together with the temperature gradient assay demonstrated that the low temperature enrichment culture was a psychrotolerant mesophilic enrichment culture dominated by *Desulfomicrobium* sp. *Desulfomicrobium* sp. was also the active species at the FBR 2 operated at 9°C. The long time FBR 2 operation at 8-9°C resulted in enrichment of mesophilic sulfate-reducing bacteria, which are active at low temperature. The psychrotolerant mesophilic SRB activity at low temperature resulted in stable sulfate reduction rate, although the sulfate reduction rate at 9°C was low, and presumably close to the lower limit of the activity of these sulfate-reducing bacteria. The results of the membrane bioreactor experiments with mesophilic and psychrotolerant mesophilic enrichment cultures indicated that the mesophilic sulfate-reducing bacteria could be more efficiently utilized for mine wastewater treatment at slightly higher temperatures than 9°C. For example, temperature increase to 15°C is sufficient to double the sulfidogenic activity of the biomass. The membrane bioreactor studies showed that the ability of mesophilic sulfate-reducing bacteria to oxidize hydrogen decreases as temperature was decreased. This is contradictory to the fact that the energy gain ($\Delta G^{0'}$) from sulfidogenic H₂ oxidation increases as temperature decreases. Therefore, the limitation is not thermodynamic, but rather a result of lowered affinity and transport rate of the substrate.

9 Recommendations for future research

The present study focused on the sulfate reducing bioreactor technology, the effect of temperature on the activity of mesophilic SRB at sub-optimal temperatures and to the microbiology of the low temperature sulfate reducing enrichment culture. It was shown that stable sulfate reduction rate can be obtained using psychrotolerant mesophilic SRB in bioreactor treatment of AMD and mine wastewaters at 9°C. Although the obtained rates were lower compared to sulfidogenic bioreactors operated at 30-35°C, the low temperature sulfate reduction has potential as practical application. Therefore, more enrichment cultures should be tested and enriched at low temperatures to confirm whether the treatment capacity obtained in the present study could be improved. Also, alternative electron donors should be tested at low temperature, since although sulfidogenic acetate oxidation may proceed at slow rate at decreased temperatures, the enrichment of acetate oxidizing SRB will improve the acetate rate over time. We studied only one psychrotolerant mesophilic enrichment culture, thus more work is required to characterize new psychrophilic and psychrotolerant SRB species and their suitability for biological mine wastewater treatment. The application of a low temperature sulfate reducing bioreactor will be a compromise between the activity, the reactor size and the temperature, therefore adapting the retention times in the treatment could solve the lower activity. In bioreactors operated at low temperature, the biomass retention is crucial to sustain the activity, therefore new applications and bioreactor design for improved biomass retention are required. Apart from bioreactor treatment, low temperature AMD treatment can be done by passive systems, where low-cost electron donors are utilized as substrate. The passive barriers are a less controlled systems, but more economical to operate, and our study showed that the treatment will proceed by mesophilic SRB even at sub-optimal temperatures.

The acetate oxidation capacity of a sulfidogenic bioreactor is dependent on the existence and enrichment of acetate oxidizing species. In mine wastewater treatment applications the optimization of the acetate oxidation is necessary to improve the treatment capacity as was shown in the present study and earlier studies by Kaksonen et al. (2003a; 2003b; 2004a). The acetate oxidation capacity of a sulfidogenic bioreactor can be monitored with chemical parameters, but fast and robust methods to monitor the diversity and activity of the acetate oxidizing SRB would improve the control of the bioreactor conditions to support sulfate reduction. The low-cost electron donors for sulfate reduction are organic waste materials, thus the acetate will be present as intermediate oxidation product. Therefore, the gain of the electron donor costs by using waste products cannot be resolved without optimizing sulfidogenic acetate oxidation.

The analysis of the microbial communities depends mostly of the ability to produce high quality DNA extract, which is always challenging with the environmental samples. Therefore new molecular methods should be tested for environmental samples, although this work is tedious and may not result in desired result. The molecular tool used in the present study, the DGGE, can be used to identify the species, but for relative number of the species a separate analysis is required. DGGE is not a fast method, and the results are also dependent on the sample quality and primer match, therefore this method only gives indication of the members of the microbial community. Molecular analyses that allow simultaneous identification of the species and their relative number, such as quantitative pcr (Q-PCR) and terminal restriction fragment length polymorfism (T-RFLP) would be optimal tools to analyze the microbial communities. These techniques require carefull primer design and build-up of reference libraries, thus the building of these analyses for routine use requires quite some effort, and the

efficiency of DNA (and RNA) extraction and PCR will be also limiting the success of these techniques. The molecular biology methods are improving and new, more efficient enzymes and analyzes are introduced, thus the problems with the molecular analysis of environmental samples are also being resolved.

10 References

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