Microbial aspects of synthesis gas fed bioreactors treating sulfate and metal rich wastewaters

Promotor:	**prof. dr. ir A.J.M. Stams**
	Persoonlijk hoogleraar, Laboratorium voor Microbiologie
	Wageningen Universiteit
Copromotor	**dr. H. Smidt**
	Universitair docent, Laboratorium voor Microbiologie
	Wageningen Universiteit
Leden van de promotiecommissie:	**prof. dr. E. Colleran**
	National University of Ireland, Galway, Ireland
	prof. dr. ir. A.J.H. Janssen
	Wageningen Universiteit
	prof. dr. ir. R.H. Wijffels
	Wageningen Universiteit
	dr. A. Rinzema
	Wageningen Universiteit
	dr. G. Muyzer
	Technische Universiteit Delft

Microbial aspects of synthesis gas fed bioreactors treating sulfate and metal rich wastewaters

Bernd H.G.W. van Houten

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. Dr. M.J. Kropff,
in het openbaar te verdedigen
op vrijdag 16 juni 2006
des namiddags te vier uur in de Aula.

B.H.G.W. van Houten - Microbial aspects of synthesis gas fed bioreactors treating metal and sulfate rich wastewaters -2006

PhH Thesis Wageningen University, Wageningen, The Netherlands - With summary in Dutch

ISBN 90-8504-397-2

Preface

In the past decade, the application of the biological sulfur cycle in anaerobic wastewater treatment systems has increased markedly. The use of sulfate-reducing bioreactors to simultaneously remove both oxidized sulfur compounds and metals show great potential to treat wastewaters generated as a result of flue gas scrubbing, mining activities and galvanic processes. This simultaneous removal is mediated through the process of sulfate reduction. Sulfate reduction is a microbial process during which microorganisms use sulfate as the terminal electron acceptor in their energy metabolism. Using an organic or inorganic electron donor, sulfate is reduced to sulfide and due to their very low solubility, metal sulfides precipitate and can be removed from the wastewater.

The first full-scale hydrogen fed anaerobic gas-lift reactor was constructed at the Budel Zink zinc smelter (Budel-dorplein, The Netherlands) for the simultaneous removal of both sulfate and zinc from the wastewater. Many sulfate-reducing microorganisms are able to use hydrogen as an electron donor for sulfate reduction. However, biological wastewater treatment systems make use of microbial consortia commonly referred to as sludge, and within these consortia sulfate reducing bacteria have to compete with other groups of microorganisms (e.g. methanogens and homacetogens) for the available hydrogen. In order to optimize the use of hydrogen for sulfate reduction, the activity of these competitors should be minimized or prevented.

The research presented in this thesis was part of a larger E.E.T (Economy, Ecology and Technology) project (EETK98028) that aimed to improve the water system of the Zinc Smelter. In co-operation with Paques B.V. (Balk, The Netherlands) and the Sub-Department of Environmental Technology, Wageningen University, The Netherlands, a task group was formed which aimed at optimizing the process of biological sulfate reduction in anaerobic reactors. Within this task group, research conducted at the Laboratory of Microbiology focused on the microbial aspects of sulfate reduction. The research goal of this project was to gain more insight into the competition for hydrogen between sulfate reducing, methanogenic and homoacetogenic microorganisms.

Abstract

The use of synthesis gas fed sulfate-reducing bioreactors to simultaneously remove both oxidized sulfur compounds and metals shows great potential to treat wastewaters generated as a result of flue gas scrubbing, mining activities and galvanic processes. Detailed information about the phylogenetic and functional composition of the microbial communities within these bioreactors however was limited prior of this study. In order to understand and enable the effective control of performance of these bioreactors, an increased understanding of the microbial aspects of sulfate-reducing synthesis gas fed bioreactors is required.

16S rRNA gene analysis demonstrated that the bacterial communities were dominated by the sulfate-reducing genera *Desulfovibrio* and *Desulfomicrobium*. Archaeal communities were comprised of microorganisms belonging to the methanogenic genus *Methanobacterium.* Synthesis gas fed bioreactors however were also able to sustain a diverse bacterial community, not limited to hydrogenotrophic microorganisms. Abundant 16S rRNA clones were also found which showed affiliation to the proteolytic microorganism *Proteiniphilum acetatigenes* and uncultured *Thermotogales*, while other clones clustered within the Chloroflexi subphylum I. A putative role for these organisms as scavengers of dead microbial cells was hypothesized.

Due to the relatively short sludge retention time in these bioreactors hydrogen threshold concentrations are not reached, and instead Monod kinetic parameters controls hydrogen competition. As a result of fluctuations in operating conditions at full-scale a continuous state of hydrogen limitation may not be reached, resulting in suppression of methanogenesis being a slow process. Limiting the carbon dioxide feed rate appeared to be a very effective selective tool to control methanogenesis at full scale, although it did not lead to the complete removal of methanogens from the system.

Heterotrophic SRB and homoacetogens were able to coexist when H_2, CO_2 and sulfate were supplied as the sole substrates as they were not limited by the same substrate; homoacetogens being hydrogen limited, while heterotrophic SRB were acetate limited. This consortium was able to compete effectively with methanogens, as the growth rate of even autotrophic methanogens is negatively affected by the lack of acetate. Even though homoacetogens are reported to have a high hydrogen threshold and a low μ_{max} they posses a high affinity (K_s), which gives them a kinetic advantage over methanogens with intermediate growth rates (0.15 -0 .50 day^{-1}) at 30°C. As growth of methanogens is affected by the availability of acetate, limiting the addition of acetate to the feed of synthesis gas fed bioreactors may provide an additional tool to selectively control methanogenesis.

In conclusion, the increased understanding of the microbial communities of sulfate-reducing synthesis gas fed bioreactors has provided greater insight into the competition for hydrogen and possibilities to control unwanted methanogenesis. Furthermore, the performance and stability of a full-scale reactor over a period of 128 weeks, demonstrate that this technology can be used successfully at full scale to treat sulfate and metal rich wastewaters.

Contents

1

Introduction

Background

The first steps towards biological wastewater treatment were already undertaken at the end of the 19th century, and the first major advance in biological wastewater treatment came when Ardern and Lockett described the aerobic activated sludge process in 1914 (1-3). The aggregation of aerobic microorganisms in activated sludge allowed for their re-use as a biocatalyst in reactors, through separation of the sludge from the effluent by settling. This provided the basis for biological wastewater treatment. Even though the activated sludge process is almost a century old it still is one of the most widespread technologies used for the treatment of sewage and industrial wastewater.

The application of anaerobic wastewater treatment, also over 100 years old, has increased rapidly since the late 1970's (13). This increase was brought about by the discovery of granular sludge, and its application in the upward-flow anaerobic sludge blanket reactor (UASB) (25). Contrary to aerobic wastewater treatment the anaerobic conversion of complex organic pollutants proceeds along a food web (Figure 1), in which interactions between several microorganisms are required to convert organic pollutants to the end product methane (43). Auto-immobilization of these anaerobic communities in dense and highly active sludge granules with good settling properties allowed their usage in high rate upward flow reactors like the UASB rector and the expanded granular sludge bed reactor (EGSB) (21). Anaerobic wastewater treatment has shown some major advantages over aerobic wastewater treatment (24). Anaerobic wastewater treatment does not require a high-energy input for aeration. It even produces a valuable energy source in the form of methane. For aerobic wastewater treatment approximately 50% of the chemical oxygen demand (COD) is used for sludge production. For anaerobic wastewater treatment, up to 90% of the COD is converted to methane and only 10% is used for sludge production, resulting in very low amounts of surplus sludge produced.

Even though UASB and EGSB have proven to be applicable for the treatment of sewage and industrial wastewaters (24), the treatment of wastewaters containing high concentrations of sulfate has shown some problems. The problems caused by sulfate reduction have been reviewed previously (23) and include:

- Sulfate-reducing bacteria can compete very effectively with methanogens for the same substrates resulting in a lowered methane production. The produced sulfide in its gaseous form H_2S contaminates the methane produced, lowering its quality.
- Sulfide can be inhibitory to many anaerobes resulting in a lowered conversion rate of organic pollutants and reactor performance.
- In the effluent, sulfide contributes to the overall oxygen demand, can cause malodor and lead to problems in post-treatment steps.

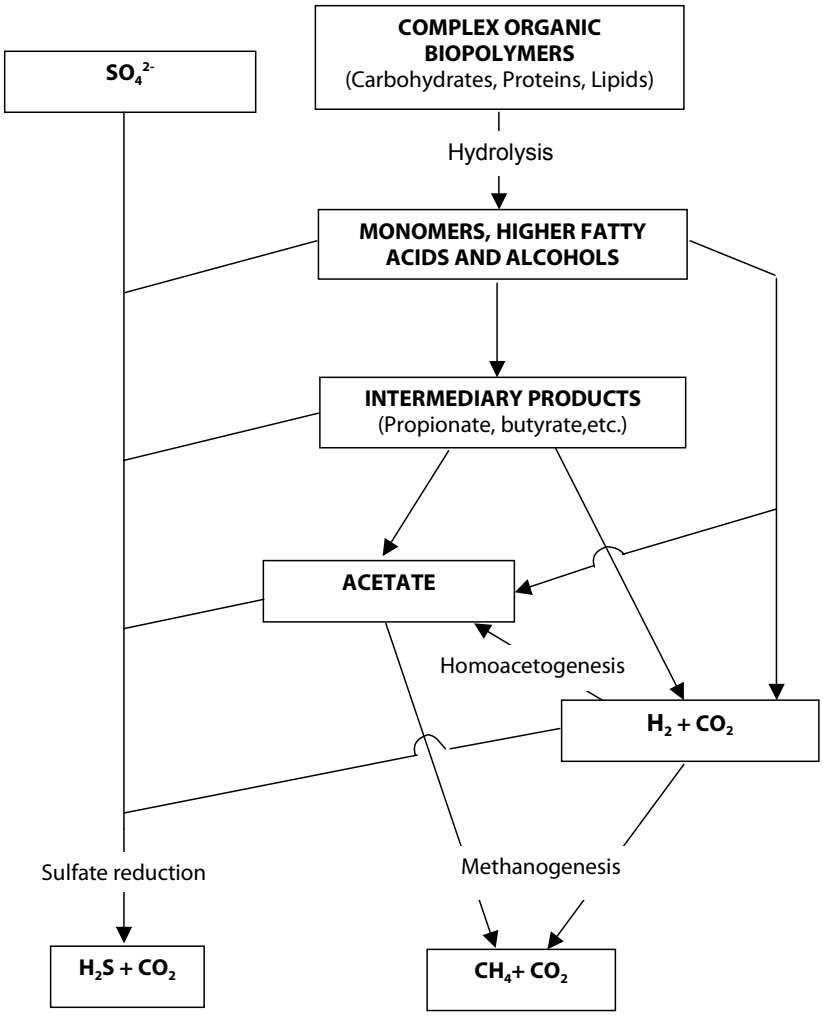

Figure 1 Anaerobic conversion of organic biopolymers in the presence of sulfate.

- Sulfide may also cause corrosion problems, and is toxic. The formation of sulfide requires extra investment during construction and an increase in maintenance costs.

As a result, sulfate reduction in conventional anaerobic bioreactors is considered to be an unwanted process. However, in the last decade microbial sulfate reduction has drawn much attention as an interesting process for the remediation of several types of wastewaters.

Wastewaters containing high amounts of oxidized sulfur compounds are generated as result of several industrial processes including flue gas scrubbing, mining activities and metal smelting (18, 23). Sulfate emissions are not a direct threat to the environment as sulfate is a chemically inert, non-volatile, and non-toxic compound (20, 40). However, sulfate can be considered as a pollutant at the high concentrations, as found in above-mentioned emissions, because it can have a major environmental impact upon discharge. In aquatic environments excess sulfate may affect the natural sulfur cycle, and sulfide formed by sulfate reduction may cause damage to natural ecosystems. After discharge in sewage systems, sulfide production may cause toxicity and malodor problems and damage sewage systems by causing corrosion.

Wastewaters with a high sulfate content can be remediated in anaerobic wastewater treatment systems specifically designed for sulfate reduction. The sulfide produced in these sulfidogenic bioreactors can be removed in a second step. One possibility is the partial oxidation of sulfide to elemental sulfur S^o (6, 7). However, many of the sulfate-containing wastewaters, such as acid mine drainage, galvanic wastewaters and spent liquid from flue gas scrubbing contain high levels of metals. The produced sulfide can be used to (selectively) precipitate and remove metals from wastewater as these metals pose a much larger threat to the environment compared to sulfate.

Sulfidogenic bioreactors

Sulfidogenic bioreactors can roughly be divided into active and passive systems, where passivity refers to the fact that these systems require hardly any maintenance or supervision. Passive systems comprise constructed compost wetlands and compost bioreactors. These systems, especially constructed wetlands, have proven to be popular for the remediation of sulfate and metal containing acid mine drainage (AMD). In both systems compost or another inexpensive bulk organic source is added to stimulate both iron and sulfate reduction for metal and sulfate removal and alkalinity generation. Even though these systems have been shown to be effective for remediation of AMD, a good understanding of the behavior of carbon sources and biogeochemical processes in passive AMD treatment is still lacking (35). Even the contribution of sulfate reduction to the overall remediation of AMD is still debated. The main drawback is that passive systems offer little control over the processes occurring inside, and as a consequence their (long-term) performance is not easy to predict and may be subject to seasonal fluctuations. Furthermore wetlands may require large surface areas.

Although active systems may be more expensive to construct and operate, they offer more control over the process and its performance, generally require less space and offer the potential to produce useful products instead of waste. Numerous sulfidogenic reactor design studies have been reported including trench reactors (53), anaerobic filters (8), completely mixed reactors (31), anaerobic packed bed reactors (32), gas-lift (12, 47), UASB reactors (7) and anaerobic baffled

reactors (14). For wastewater that contains no or insufficient amounts of electron donors and carbon sources for complete sulfate reduction, addition of an appropriate electron donor is required. Several electron donors have been proposed for sulfidogenic bioreactors, including lactate, methanol, ethanol, a mix of volatile fatty acids and hydrogen, and even complex organic substrates such as molasses and primary sewage sludge (19, 30, 37, 45, 50).

The selection of the electron donor depends on the costs of the added electron donor per unit of sulfate reduced sulfate and the possible rest-pollution in the form of COD of the added electron donor in the effluent. Based on the last criterion, simple organic compounds (ethanol, methanol) or hydrogen are preferred to more complex organic substrates (e.g. molasses) as they are easier to dose and can be utilized rapidly and completely. Even though pure H_2-gas is an expensive gas, for high rates of sulfate reduction (>5-10 kmol·hr^{-1}) the use of hydrogen as an electron donor is an attractive option both from the technological and economical point of view (48). Hydrogen can be produced on site from natural gas or other bulk sources like coal, oil or organic waste using a gas reformer. The produced synthesis gas is a mixture containing mainly H_2, CO_2 and CO with minor levels of other components, such as methane, nitrogen and hydrogen sulfide. For small-scale installations (<5-10 kmol·hr^{-1}) methanol or ethanol are more cost effective.

Even though complex substrates may pose problems with rest-pollution, the BioSURE Process, developed at Rhodes University in South Africa, which links AMD treatment and primary sewage sludge disposal has been shown to be a low cost option (37). Sewage sludge serves as the electron donor for the sulfate-reducing bacteria and is simultaneously stabilized. The first reaction in the process comprises the solubilization of the complex organic carbon in the sewage sludge, facilitated in a Falling Sludge Bed Reactor (FSBR) (50). This process was scaled-up to a pilot-installation at Grootvlei Mine (South Africa), and the first plant was officially operational on 18 January 2005, to treat partially remediated AMD from the Grootvlei Mine.

Two other major sulfidogenic bioreactor technologies applied at full-scale have been described to date: the Biosulfide® and the THIOPAQ® processes. The Biosulphide® technology is marketed by Bioteq (Vancouver, B.C., Canada) and THIOPAQ® by Paques B.V (Balk, Netherlands). Both technologies are very similar and both companies share a technology agreement to utilize it. The Biosulfide® system is divided into two stages: a biological stage, and a chemical precipitation stage. Hydrogen is used as an electron donor in the bioreactors. The biological stage functions as a reagent generating system, producing dissolved and gaseous sulfide and alkalinity through sulfate reduction. In the second stage these reagents are used to precipitate the metal pollutants and increase the alkalinity.

The separation of the chemical and biological stages in this manner has several key advantages:

- the entire flow of water for treatment is not passing through the slowest stage of the process (the bioreactors),
- reactions in the two stages can proceed at their optimal (and different) rates,
- the microbial biomass is not exposed to inhibitory or toxic levels of dissolved metals,
- a greater degree of control is possible over the extent of reactions in the two stages.

Several full-scale Biosulphide systems have been built and are currently used for treatment of AMD as well as for metal recovery at 4 different mining sites in North America.

The THIOPAQ® technology comprises two systems: sulfate removal or sulfate and metal removal that can be combined or used separately (Figure 2A). THIOPAQ® for sulfate removal consists of two biological processes, which take place in separate bioreactors. In the sulfidogenic bioreactor, the sulfate is reduced to sulfide under anaerobic conditions, which is subsequently oxidized to sulfur in a second microaerobic reactor. The produced sulfur can be recycled for the production of sulfuric acid, fertilizers or production of sulfide. Due to the increase in alkalinity during the conversion of sulfide into sulfur, influent neutralization can be achieved by recirculation of this stream eliminating the need to add large amounts of alkaline chemicals. Several full-scale sulfate removal plants are currently in operation, for instance at the synthetic fiber production plant of Akzo Nobel (Emmen, The Netherlands) (15).

The THIOPAQ® process for metal and sulfate removal has been successfully used for flue-gas desulfurization, and provides an attractive alternative to the conventional limestone-gypsum process (17). Paques has also installed a THIOPAQ® system, based on combined sulfate reduction and sulfide oxidation and metal precipitation, at the Budel Zink zinc smelter (Budel-dorplein, Netherlands) to remove sulfate, zinc and cadmium (5). This ethanol-fed system, based on an upflow sludge bed reactor, has been operational since 1992, treating metal contaminated groundwater and also the effluent from a conventional lime precipitation reactor that was used to treat the zinc smelters wastewater.

Due to changes in wastewater discharge and treatment legislation prohibiting the formation of solid waste during wastewater treatment, a new wastewater treatment system was necessary to replace the conventional method of lime dosing that produced large amounts of jarosite waste. After tests conducted with a 5-m^3 pilot-scale installation, the first full-scale synthesis gas fed gas lift reactor was constructed to treat wastewater from the zinc smelting plant. The pilot-scale reactor was used to obtain sufficient material to seed the full-scale reactor. This pilot-scale reactor had been seeded with sludge from a pilot scale demonstration reactor that treated effluent from the Kennecott Bingham Canyon copper mine (Utah, USA) (5). The main difference in design between the Kennecott reactor, and the Budel pilot and full-scale reactors was that metal precipitation and

sulfate reduction occurs in the same reactor in the Budel pilot and full-scale reactors (Fig. 2B). The set-up of the Kennecott pilot plant was similar to that presented in figure 2A.

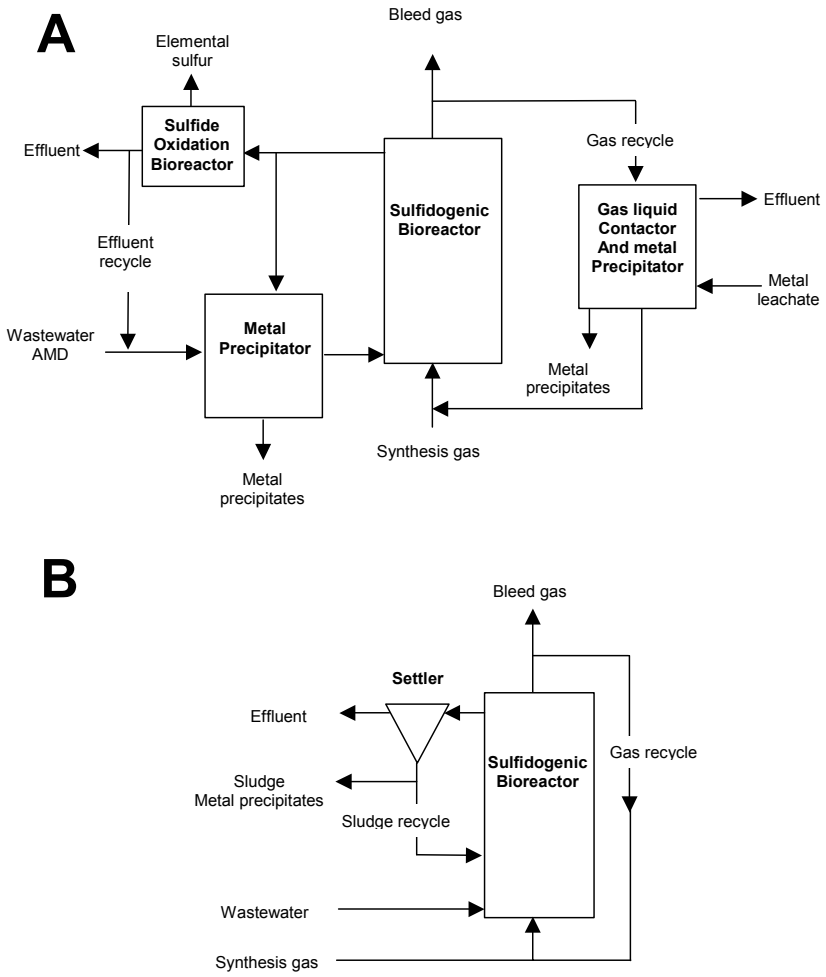

Figure 2 Schematic diagrams of treatment systems for the remediation of sulfate and metal containing wastewaters utilizing sulfidogenic bioreactors. Panel A shows a system in which the synthesis gas fed bioreactor is used solely as a sulfidogenic system. The sulfide produced is used to precipitate the metals in separate precipitation reactors. Excess sulfide is biologically oxidized to elemental sulfur to help neutralize the pH of acidic wastewater e.g. acid mine drainage. For wastewaters that do not contain metal pollutants the precipitation reactors can be omitted. Panel B shows a single reactor set-up in which sulfate reduction and metal precipitation occurs in the sulfidogenic bioreactor.

Competition for hydrogen

Hydrogen is a common electron donor for sulfate-reducing bacteria (52). Although it can also be utilized by many other types of microorganisms like methanogens (51) and homoacetogens (11, 38), previous experiments at lab-scale had revealed that under mesophilic conditions hydrogenotrophic methanogenesis was minimal (46, 47). Hydrogen consumption by these processes would be unwanted as they would lower the overall system efficiency.

The metabolism of hydrogen in natural and man made anaerobic environments e.g. anaerobic wastewater systems and sediments has been studied quite intensively as it is a key intermediate in the anaerobic degradation of complex organic materials. Also the competition between methanogens and sulfate-reducing bacteria in anaerobic bioreactors has received considerable attention because of the detrimental effects sulfate reduction may have in methanogenic bioreactors as discussed in paragraph 1.1, and has been reviewed extensively (9, 16, 34, 43).

Studies with sediments and anaerobic bioreactors have shown that under conditions of excess sulfate, methanogenesis is suppressed by sulfate reduction, as sulfate-reducers outcompete methanogens for their common electron donor hydrogen. Kinetic properties of microorganisms either expressed in the form of Monod or Michaelis-Menten kinetics have been used to explain this outcome. For competition between actively growing microorganisms as is the case in chemostats or other completely stirred tank reactors, growth kinetics based on the Monod equation have been used to predict dominating processes (22, 39). In contrast to this situation, growth rates in granular sludge based bioreactors and sediments are very low and can be considered negligible. Under these 'resting' or non-growth conditions, enzymatic properties of methanogens and sulfate-reducing bacteria using Michaelis-Menten kinetics have been used to explain the outcome of competition for hydrogen (27, 29, 36). Based on the limited Monod and Michaelis-Menten kinetic data that is available for hydrogen (34), sulfate reducers would outcompete methanogens under limiting hydrogen concentrations. However, Michaelis-Menten kinetics failed to accurately describe the competition between methanogens and sulfate-reducing bacteria at the very low hydrogen concentrations encountered in sediments (26).

An alternative model was put forward in which sulfate-reducing bacteria exclude methanogens by lowering the hydrogen concentration below a minimum or threshold concentration necessary for methanogenesis. This threshold concentration can be regarded as the lowest hydrogen concentration at which microorganism can utilize a compound as an electron donor for energy generation. The basis for hydrogen threshold concentrations can be explained by the Gibbs free energy obtained from the oxidation of hydrogen coupled to sulfate reduction, methanogenesis or homoacetogenesis (10) (Table 1).

Under standard conditions the oxidation of hydrogen coupled to sulfate reduction has the most negative Gibbs free energy change ($\Delta G'$). For non-standard conditions the $\Delta G'$ depends on the

temperature and on the concentrations or activities of the reactants. A terminal accepting process should be energy yielding (negative ΔG'), and therefore the hydrogen concentration cannot drop below a certain minimum value. This theoretical minimum concentration is the lowest for sulfate reduction.

Table 1 Standard Gibbs free energy change $\Delta G^{0'}$ for the oxidation of hydrogen coupled to sulfate reduction, methanogenesis and homoacetogenesis. (thermodynamic data derived from Thauer et al. (44)).

Reaction			$\Delta G^{0'}$ (kJ/reaction)
$4\ H_2 + SO_4^{2-} + H^+$	$\rightarrow$	$HS^- + 4\ H_2O$	-151.9
$4\ H_2 + HCO_3^- + H^+$	$\rightarrow$	$CH_4 + 3\ H_2O$	-135.6
$4\ H_2 + 2\ HCO_3^- + H^+$	$\rightarrow$	$Acetate^- + 4\ H_2O$	-104.6

Under conditions of excess electron acceptor concentrations the partial pressure of hydrogen has been shown to correlate with the ΔG' to be obtained (10). Reported hydrogen threshold concentrations observed in pure culture experiments follow the order of sulfate-reduction (0.9-4.5 Pa) < methanogenesis (2.5 -9 Pa) < homoacetogenesis (52 -95) (10, 34, 43). The same order for threshold concentrations for sulfate reduction and methanogenesis has been observed in anaerobic sediments. (28). In general, both kinetic and threshold data indicate that sulfate-reducing bacteria will outcompete methanogens and homoacetogens for hydrogen under hydrogen limited conditions.

Scope and outline of this thesis

Even though earlier studies showed that methanogenesis did not play a dominant role in mesophilic synthesis gas fed lab-scale reactors (46, 47), pilot and full-scale installations have been faced with significant acetate and methane formation. In addition, due to the fact that these hydrogen-fed systems represent a new type of anaerobic wastewater treatment system, no detailed information about the phylogenetic and functional composition of the microbial community is available. In order to understand and control the performance of these bioreactors the research presented in this thesis focused on the microbial aspects of sulfate-reducing synthesis gas fed bioreactors in order to gain more insight into the competition for hydrogen between the different hydrogenotropic microorganisms present in the sludge. The dominant microorganisms in sludge communities were identified and temporal variations in the community structure assessed. In addition the effect of carbon dioxide and acetate addition on the competition for hydrogen was also studied.

Sludge composition

Identifying the dominant microbial populations may provide insight in their metabolic and kinetic properties as well as the interactions that may occur between different functional groups. This information would assist in developing possible means for process control. Previous studies by van Houten showed that community of lab-scale reactors fed with artificial wastewater and synthesis gas consisted of microorganisms tentively identified as *Desulfovibrio* and *Acetobacterium* species [21]. However, more detailed information on sludge communities is lacking.

Temporal community changes were assessed using Denaturing Gradient Gel Electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments, while the relative abundance and identity of dominant bacterial species in the sludge were determined using 16S rRNA gene libraries in combination with Restriction Fragment Length Polymorphism (RFLP) and sequence analysis. These techniques were used to study and monitor the microbial community dynamics in both lab-scale and full-scale reactors.

Carbon dioxide

The availability of carbon dioxide may affect the metabolism of the different microorganisms and therefore their ability to compete for hydrogen. Methanogens and homoacetogens depend on carbon dioxide for their energy metabolism (Table 1), while heterotrophic sulfate-reducing bacteria use bicarbonate only as a carbon source. The latter do not depend on bicarbonate for their energy metabolism and obtain approximately 30% of their cellular carbon from CO_2 (4, 33, 41, 42). Therefore their requirement for carbon dioxide is much lower. If carbon dioxide is not the main carbon source for the sulfate reducing population, lowering the carbon dioxide concentration would favor the growth of this population over methanogens and homoacetogens. Therefore, carbon dioxide could be a possible tool to control the competition for hydrogen.

Acetate

Acetate is likely to play an important role in the competition for hydrogen in the reactor system. The previously identified *Desulfovibrio* species are heterotrophic sulfate reducers, which require an organic carbon source, such as acetate, for growth (52). In both full and pilot scale systems acetate has been added as a carbon source. However, under autotrophic conditions sulfate-reducing bacteria would depend on the production of acetate by homoacetogenic bacteria present in the sludge. In case of acetate-limited sulfate reduction, the growth kinetics of the sulfate reducing bacteria would thus be determined by the growth kinetics of the homoacetogenic bacteria. Alternatively, autotrophic sulfate reducing bacteria might outcompete heterotrophic sulfate reducers. Many methanogens, however, can grow autotrophically with hydrogen and carbon dioxide and do not require an external carbon source. In that case, methanogens may outcompete both heterotrophic sulfate-reducers and homoacetogens.

Outline of this thesis

Chapter 2 describes the identification of the dominant microorganisms in the sludge from the pilot plant used for the treatment of sulfate rich acid mine drainage water from the Kennecott coppermine (Utah, U.S.A.) and the sludge from the full-scale reactor at the Budel zinc smelter. An explanation is given for the occurrence of methanogenesis during the start-up of the full-scale reactor.

In chapter 3 the performance and the microbial community of the full-scale (500 m^3) synthesis gas fed gas-lift reactor at the Budel zinc smelter was investigated over a period of 128 weeks. The effect of lowering the carbon dioxide concentration in the feed gas as a means to control methanogenesis is discussed. In chapter 4 a heterotrophic sulfate-reducing bacterium that was found to be abundant in Budel sludge at a certain point in time is described and its possible ecological role is discussed.

Results obtained by Weijma and coworkers indicated that sulfate-reducers and homoacetogens outcompeted methanogens for hydrogen when an organic carbon source was omitted (49). In order to verify this the bacterial communities of both reactors used in the study by Weijma and coworkers were studied and the dominant microorganisms were identified in chapter 5.

To give an explanation for the observations made by Weijma and coworkers, the role of acetate during the competition for H_2 between methanogens, homoacetogens and sulfate-reducing bacteria was studied in a completely stirred tank reactor using defined cultures. The results are presented in chapter 6. Finally, in chapter 7 a summary and discussion of the results obtained in the previous chapters is given, before concluding remarks are presented.

References

1. **Ardern, E., and W. T. Lockett.** 1914. Experiments on the Oxidation of Sewage without the Aid of Filters. J. Soc. Chem. Ind. **33:**523.

2. **Ardern, E., and W. T. Lockett.** 1914. Experiments on the Oxidation of Sewage without the Aid of Filters, Part II. J. Soc. Chem. Ind. **33:**1122.

3. **Ardern, E., and W. T. Lockett.** 1915. Experiments on the Oxidation of Sewage without the Aid of Filters, Part III. J. Soc. Chem. Ind. **34:**937.

4. **Badziong, W., and R. K. Thauer.** 1978. Growth yields of *Desulfovibrio vulgaris* (Marburg) growing on hydrogen plus sulfate and hydrogen plus thiosulfate as the sole energy sources. Archives of Microbiology **117:**209-214.

5. **Boonstra, J., J. van Lier, G. Janssen, H. Dijkman, and C. J. N. Buisman.** 1999. Biological treatment of acid mine drainage, p. 559-567. *In* R. Amils and A. Ballister (ed.), Biohydrometallurgy and Environment toward the 21st century. Process metallurgy 9B. Elsevier, Amsterdam.

6. **Buisman, C. J. N., B. G. Geraats, P. IJspeert, and G. Lettinga.** 1990. Optimisation of sulphur production in a biotechnological sulphide removing reactor. Biotech.Bioeng. **35:**50–56.

7. **Buisman, C. J. N., R. Post, P. Ijspeert, G. Geraats, and G. Lettinga.** 1989. Biotechnological process for sulphide removal with sulphur reclamation. Acta Biotechnol. **9:**255–267.

8. **Chian, E. S. K., and F. B. DeWalle.** 1983. Removal of heavy metals from a fatty acid wastewater with a completely mixed anaerobic filter. Presented at the 38th Industrial Waste Conference, Purdue University, West Lafayette, IN, USA.

9. **Colleran, E., S. Finnegan, and P. Lens.** 1995. Anaerobic treatment of sulfate-containing waste streams. Antonie Van Leeuwenhoek **67:**29-46.

10. **Cord-Ruwisch, R., H. J. Seitz, and R. Conrad.** 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Archives of Microbiology **149:**350-357.

11. **Diekert, G.** 1991. The Acetogenic Bacteria, p. 517-533. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The Prokaryotes, 2nd edn. ed, vol. Vol. 1. Springer-Verlag, New York.

12. **Du Preez, L. A., and J. P. Maree.** 1994. Pilot-scale biological sulphate and nitrate removal utilizing producer gas as energy source. Water Science & Technology **30:**275–285.

13. **Frankin, R. J.** 2001. Full scale experience with anaerobic treatment of industrial wastewater. Wat. Sci. Technol. **44:**1-6.

14. **Grobicki, A., and D. C. Stuckey.** 1992. Hydrodynamic characteristics of the anaerobic baffled reactor. Water Research **23:**371–378.

15. **Hulshoff Pol, L. W., P. Lens, J. Weijma, and A. J. M. Stams.** 2001. New developments in reactor and process technology for sulfate reduction. Water Science & Technology **44:**67–76.

16. **Hulshoff Pol, L. W., P. N. L. Lens, A. J. M. Stams, and G. Lettinga.** 1998. Anaerobic treatment of sulphate-rich wastewaters. Biodegradation **9:**213 - 224.

17. **Janssen, A., H. Dijkman, and G. Janssen.** 2000. Novel biological processes for the removal of H_2S and SO_2 from gas streams., p. 265–280. *In* P. Lens and L. Hulshoff Pol (ed.), Environmental Technologies to Treat Sulfur Pollution, Principles and Engineering. IWA Pubishing, London.

18. **Johnson, D. B.** 2000. Biological removal of sulfurous compounds from inorganic wastewaters, p. 175-206. *In* P. Lens and L. Hulshoff Pol (ed.), Environmental Technologies. International Association on Water Quality, London.

19. **Kaksonen, A. H., J. J. Plumb, P. D. Franzmann, and J. A. Puhakka.** 2004. Simple organic electron donors support diverse sulfate-reducing communities in fluidized-bed reactors treating acidic metal- and sulfate-containing wastewater. FEMS Microbiology Ecology **47:**279-289.

20. **Karhadkar, P. P., J.-M. Audic, G. M. Faup, and P. Khanna.** 1987. Sulfide and sulfate inhibition of methanogenesis. Water Research **21:**1061–1066.

21. **Kato, M. T., J. A. Field, P. Versteeg, and G. Lettinga.** 1994. Feasibility of expanded granular sludge bed reactors for the anaerobic treatment of low-strength soluble wastewaters. Biotechnology and Bioengineering **44:**469-479.

22. **Kristjansson, J. K., P. Schoenheit, and R. K. Thauer.** 1982. Different Ks values for hydrogen of methanogenic bacteria and sulfate reducing bacteria: an explanation for the apparent inhibition of methanogenesis by sulfate. Archives of Microbiology **131:**278-282.

23. **Lens, P. N. L., A. Visser, A. J. H. Janssen, L. Hulshoff Pol, and G. Lettinga.** 1998. Biotechnological treatment of sulfate-rich wastewaters. Critical Reviews in Environmental Science and Technology **28:**41 - 88.

24. **Lettinga, G.** 1995. Anaerobic digestion and wastewater treatment systems. Antonie van Leeuwenhoek **67:**3-28.

25. **Lettinga, G., A. F. M. van Velsen, S. W. Hobma, W. de Zeeuw, and A. Klapwijk.** 1980. Use of upflow sludge blanket reactor concept for biological waste water treatment, especially for anaerobic treatment. Biotechnology and Bioengineering **22:**699-734.

26. **Lovley, D. R.** 1985. Minimum threshold for hydrogen metabolism in methanogenic bacteria. Applied and Environmental Microbiology **49:**1530-1531.

27. **Lovley, D. R., D. F. Dwyer, and M. J. Klug.** 1982. Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. Applied And Environmental Microbiology **43:**1373-1379.

28. **Lovley, D. R., and S. Goodwin.** 1988. Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. Geochim. Cosmochim. Acta. **52:**2993-3003.

29. **Lupton, F. S., and J. G. Zeikus.** 1984. Physiological basis for sulfate-dependent hydrogen competion between sulfidogens and methanogens. Current microbiology **11:**7-12.

30. **Maree, J. P., and E. Hill.** 1989. Biological removal of sulphate from industrial effluents and concomitant production of sulphur. Wat. Sci. Tech. **21:**265–276.

31. **Maree, J. P., and E. Hill.** 1989. Biological removal of sulphate from industrial effluents and concomitant production of sulphur. Water Science & Technology **21:**265–276.

32. **Maree, J. P., and W. F. Strydom.** 1987. Biological sulfate reduction in an upflow packed bed reactor. Water Research **21:**141-146.

33. **Noguera, D. R., G. A. Brusseau, B. E. Rittmann, and D. A. Stahl.** 1998. A unified model describing the role of hydrogen in the growth of *Desulfovibrio vulgaris* under different environmental conditions. Biotechnology and Bioengineering **59:**732-746.

34. **Oude Elferink, S. J. W. H., A. Visser, L. W. Hulshoff Pol, and A. J. M. Stams.** 1994. Sulfate reduction in methanogenic bioreactors. FEMS Microbiology Reviews **15:**119-136.

35. **Pulles, W.** 2000. Passive mine water treatment systems developed. Water Sewage & Effluent**:**12–14.

36. **Robinson, J. A., and J. M. Tiedje.** 1984. Competition for hydrogen between sulfate-reducing and methanogenic bacteria for hydrogen under resting and growing conditions. Archives of Microbiology **137:**26-32.

37. **Rose, P. D., B. Pletschke, and C. Whiteley.** 2000. Complex organic carbon compounds as electron donors for sulphate reduction – the Rhodes "Biosure" process in the treatment of mine drainage wastewaters. Presented at the Technology Transfer Workshop on Biological Sulphate Removal of the Anaerobic Processes Division of the Water Institute of South Africa, Pretoria, South Africa, 22 & 23 August.

38. **Schink, B., and M. Bomar.** 1991. The genera *Acetobacterium*, *Acetogenium*, *Acetoanaerobium* and *Acetitomaculum*, p. 1925-1936. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The Prokaryotes. Springer-Verlag, New York.

39. **Schönheit, P., J. K. Kristjansson, and R. K. Thauer.** 1982. kinetic mechanism for the ability of sulfate reducers to out-compete methanogens for acetate. Archives of Microbiology **132:**285-288.

40. **Shin, H. S., J. Y. Jung, B. U. Bae, and B. C. Paik.** 1995. Phase-separated anaerobic toxicity assaysfor sulfate and sulfide. Wat. Environ. Res. **67:**802–806.

41. **Sorokin, Y. I.** 1966. Investigation of the structural metabolism of sulfate-reducin bacteria with C^{14}. Microbiologiya **35:**967-977.

42. **Sorokin, Y. I.** 1966. Sources of energy and carbon for biosynthesis in sulfate-reducing bacteria. Microbiologiya **35:**761-766.

43. **Stams, A. J. M.** 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie van Leeuwenhoek **66:**271-294.

44. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriological Reviews **41:**100–180.

45. **Tsukamoto, T. K., and G. C. Miller.** 1999. Methanol as a carbon source for microbiological treatment of acid mine drainage. Water Research **33:**1365-1370.

46. **van Houten, R. T.** 1996. Biological sulfate reduction with synthesis gas. Ph.D. thesis. Wageningen Agricultural University, Wageningen, Netherlands.

47. **van Houten, R. T., L. Hulshoff Pol, and G. Lettinga.** 1994. Biological sulfate reduction using gas-lift reactors fed with hydrogen and carbon dioxide as energy and carbon source. Biotechnology and Bioengineering **44:**586-594.

48. **van Houten, R. T., and G. Lettinga.** 1994. Biological sulphate reduction with synthesis gas: microbiology and technology, p. 793-799. *In* R. H. Wijffels, R. M. Buitelaar, C. Bucke, and J. Tramper (ed.), Progres in Biotechnology, vol. Vol. 11. Elsevier, Amsterdam.

49. **Weijma, J., F. Gubbels, L. Hulshoff Pol, A. J. M. Stams, P. Lens, and G. Lettinga.** 2002. Competition for H_2 between sulfate reducers, methanogens and homoacetogens in a gas-lift reactor. Wat. Sci. Tech. **45:**75-80.

50. **Whiteley, C. G., G. Enongene, B. Pletschke, P. D. Rose, and K. Whittington-Jones.** 2003. Co-digestion of primary sewage sludge and industrial wastewater under anaerobic sulphate reducing conditions: enzymatic profiles in a recycling sludge bed reactor. Water Science & Technology **48:**129–138.

51. **Whitman, W. B., T. L. Bowen, and D. R. Boone.** 1991. The Methanogenic Bacteria, p. 719-767. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The Prokaryotes, 2nd edn. ed, vol. Vol. 1. Springer-Verlag, New York.

52. **Widdel, F., and T. A. Hansen.** 1991. The dissimilatory sulfate- and sulfur-reducing bacteria, p. 583-624. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The Prokaryotes, 2nd edn. ed, vol. Vol. 1. Springer-Verlag, New York.

53. **Younger, P. L., T. P. Curtis, A. Jarvis, and R. Pennell.** 1997. Effective passive treatment of aluminium-rich acidic colliery spoil drainage using a compost wetland at Quaking Houses, County Durham. J. Chartered Inst. Water and Environment Management **11:**200-208.

2

Occurrence of methanogenesis during start-up of a full-scale synthesis gas fed reactor treating sulfate and metal rich wastewater

Bernd H.G.W. van Houten [a], Kees Roest [a], Vesela A. Tzeneva[a], Henk Dijkman[b], Hauke Smidt [a] and Alfons J.M. Stams [a]

a *Laboratory of Microbiology, Hesselink van Suchtelenweg 4, Wageningen University, 6703 CT, Wageningen, The Netherlands.*

b *Paques B.V., T. de Boerstraat 24, P.O Box 52, 8560 AB Balk, The Netherlands*

Water Research **40:**553-560

Abstract

The start-up of a full-scale synthesis gas fed gas-lift reactor treating metal and sulfate rich wastewater was investigated. Sludge from a pilot-scale reactor was used to seed the full-scale reactor. The main difference in design between the pilot and full-scale reactor was that metal precipitation and sulfate reduction occurred in the same reactor. After 7 weeks the full-scale reactor achieved the sulfate conversion design rate of 15 $kg \cdot m^{-3} \cdot day^{-1}$. Zinc sulfide precipitation inside the reactor did not interfere with obtaining a high rate of sulfate reduction. 16S rRNA gene analysis demonstrated that the bacterial communities in both reactors were dominated by the sulfate-reducing genus *Desulfomicrobium*. Archaeal communities of both reactors were dominated by the methanogenic genus *Methanobacterium*. MPN counts confirmed that heterotrophic sulfate-reducing bacteria were dominant (10^{11}-10^{12} cells/g VSS) compared to homoacetogens (10^{5}-10^{6} cells/g VSS) and methanogens (10^{8}-10^{9}cells/g VSS). Methanogenesis was not suppressed during start-up of the full scale-reactor, despite the predominance of sulfate-reducing bacteria, which have a lower hydrogen threshold. Due to the short sludge retention time (4 to 7 days) competition for hydrogen is determined by Monod kinetics, not hydrogen thresholds. As the kinetic parameters for SRB and methanogens are similar, methanogenesis may persist which results in a loss of hydrogen.

Keywords

Gas-lift reactor; Wastewater; Sulfate reduction; Methanogenesis; Hydrogen; Monod Kinetics

Introduction

Sulfate and metal rich wastewaters that are low in organic carbon are produced by several industrial processes, such as metal smelting, flue gas scrubbing and mining. Sulfate reducing bioreactors have been previously shown to be suitable systems to treat these types of wastewater (Boonstra et al., 1999). These bioreactors utilize the sulfidogenic activity of sulfate-reducing bacteria (SRB) to simultaneously remove sulfate and metals, in the form of metal sulfides. A full-scale ethanol fed sulfate-reducing bioreactor had been operational at the Budel Zink zinc smelter site since 1992. The reactor influent consisted of metal-contaminated groundwater and effluent from a conventional lime precipitation reactor that was used to treat the zinc smelters wastewater. However, new wastewater discharge and treatment legislation prohibited the formation of solid waste during wastewater treatment. As a consequence an alternative to the conventional lime precipitation reactor was needed.

Paques B.V. (Balk, Netherlands) has developed a sulfidogenic THIOPAQ® system, which is based on a gas-lift reactor fed with hydrogen gas as the electron donor for sulfate reduction. For large-scale applications synthesis gas is an attractive source of hydrogen (van Houten and Lettinga, 1994). Synthesis gas is a mix containing mainly H_2, CO_2 and CO with minor levels of other components, such as methane and nitrogen. The main advantage of this system is that the metal sulfides produced can be re-used in the metal smelting process, and no solid waste products are produced. After tests conducted with a pilot-scale reactor, the first full-scale synthesis gas fed reactor was constructed to treat the wastewater from the zinc smelter. The original pilot-scale reactor was used to obtain sufficient material to seed the full-scale reactor. This pilot-scale reactor had been seeded with sludge from a pilot scale demonstration reactor that treated effluent from the Kennecott Bingham Canyon copper mine (Boonstra et al., 1999). The design of the Kennecott reactor differed from the Budel reactors with regards to metal precipitation. Sulfate reduction and metal precipitation took place in two separate reactors in the Kennecott set-up (Fig. 1).

Reports on the application of synthesis gas fed systems have so far have been limited to lab-scale (van Houten and Lettinga, 1994) and pilot-scale test studies (Boonstra et al., 1999). The Budel reactor is the first synthesis gas fed reactor to be used on full-scale to treat industrial wastewater. Ecological studies of engineering applications utilizing SRB have, so far, focused on conventional systems treating organic waste materials such as UASB and EGSB systems. Only Kaksonen et al. (2004) have studied the bacterial diversity of lactate and ethanol-utilizing sulfate-reducing lab-scale reactors that were fed with synthetic mineral processing. Currently, no detailed information on the microbial composition of this type of synthesis gas fed reactors was available.

The main objective of this study was to monitor the start-up process of the full-scale Budel reactor. To do this the performance of the full-scale reactor was monitored for a period of 20 weeks after start-up. Secondly, because the operation of the Budel reactor differed from the Kennecott reactor

with regards to metal precipitation, and the lack of knowledge on the microbial composition in these systems, we wanted to identify the predominant microorganisms in both sludges. To identify the predominant microorganisms we have used both cultivation dependent and independent approaches. Most Probable Number (MPN) estimates were used to determine the abundance of different hydrogenotrophic groups of microorganisms. The abundant species in the Kennecott seed sludge and Budel sludge were assessed using Restriction Fragment Length Polymorphism (RFLP), and identified by 16S rRNA sequence analysis.

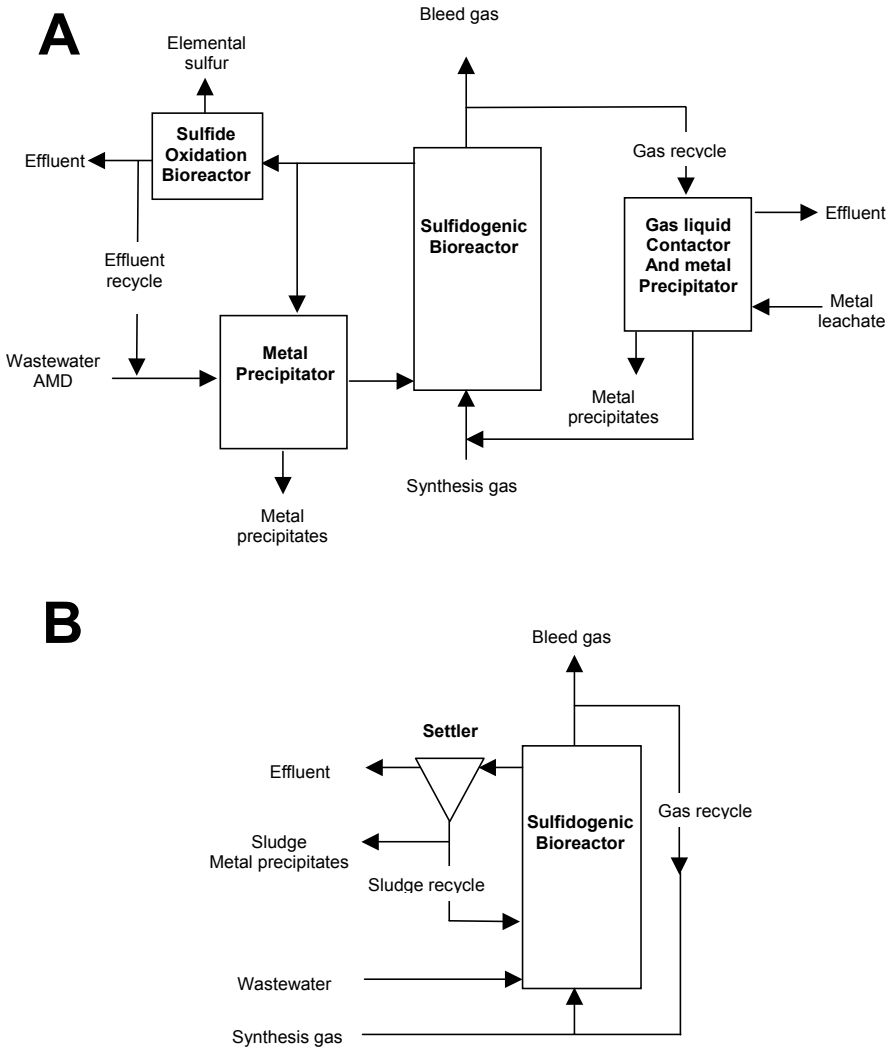

Figure 1 Simplified flow diagrams of the Kennecott (A) and Budel (B) reactors.

Materials & methods

Sludge origin

Anaerobic sludge was obtained from the gas lift reactors of two THIOPAQ ® systems used for metal recovery and sulfate removal. The first reactor was part of a pilot scale system used for the treatment of Acid Mine Drainage (AMD) and the recovery of copper from waste rock leachate (Kennecott Bingham Canyon copper mine, Utah, USA).

The second reactor was a full-scale reactor treating sulfate and heavy metal rich wastewater from a zinc smelter in Budel, the Netherlands. This reactor was seeded with sludge originating from the aforementioned Kennecott pilot scale reactor. Before seeding, the Kennecott sludge was pre-grown in a 5-m^3 pilot scale reactor for 9 months at the Budel site in order to obtain sufficient sludge for start-up. Both reactors were fed with synthesis gas. The feed gas of both reactors had an average composition of H_2 (76.2 % v/v), CO_2 (20.4 % v/v) N_2 (2.9 % v/v). The trace gasses methane and carbon monoxide did not exceed 1 % (v/v).

The main pollutants in the Budel reactor feed were sulfate (5-10 g/L), zinc (3-5 g/L) and magnesium (10 – 300 mg/L). The main pollutants in the Kennecott reactor feed were sulfate (30 g/L), magnesium (4.5 g/L), aluminium (2.2 g/L), iron (675 mg/L) and manganese (350 mg/L). Ethanol was added as an organic carbon source to the Kennecott reactor, and Acetate was added as an organic carbon source to the Budel reactor at 5 % of the COD required to reduce all the sulfate in feed. Schematic flow diagrams and operating conditions of both systems are presented in figure 1 and table 1.

Table 1 Overview of the operating conditions of the Kennecott and Budel reactors.

Parameter	Kennecott	Budel
Volume Reactor	5 m^3	500 m^3
Sludge Retention Time (SRT)	4 - 7 days	4 - 7 days
Hydraulic Retention Time (HRT)	1 day.	1 - 3 days
Temperature	30 - 35 °C	30 - 35 °C
pH	7.0 - 7.5	7.0 - 7.5
Carbon source	Ethanol	Acetate

Most probable number (MPN) estimates

The different groups of hydrogenotrophic bacteria were enumerated in selective media using the MPN technique (n=3). Sludge from the Kennecott reactor had been stored at 4 °C for a period of 9 months. No fresh samples could be obtained because the reactor was no longer operational. Sludge from the Budel reactor was sampled 19 weeks after start-up of the reactor. Samples were

diluted tenfold with anaerobic bicarbonate-buffered medium and crushed as previously described Oude Elferink (1995). The crushed sludge was serially diluted in 120-mL serum vials containing 50 ml of bicarbonate buffered medium (Stams et al., 1993). The different groups of hydrogenotrophic bacteria were grown using a H_2/CO_2 (4:1) gas phase of 1.7 bar. For selective enrichment of heterotrophic sulfate reducers the medium was supplemented with 20 mM sulfate and 2 mM acetate. Homoacetogens were enriched in basal medium supplemented with 10 mM 2-bromoethanesulfonate (BES). Methanogens were enriched using unsupplemented basal bicarbonate medium. Growth was monitored over a period of 7 months. Cultures were scored positive for growth if substrate consumption, product formation, and an increase in turbidity were observed.

Chemical analyses

For the MPN estimates acetate and sulfate were analyzed by HPLC, and hydrogen and methane by gas chromatography (GC) as described previously (Stams et al., 1993; Scholten and Stams, 1995). Sulfide was measured colorimetrically (Trüper and Schlegel, 1964). The total and volatile suspended solids (TSS and VSS) content of the sludge was determined according to Dutch standard methods (NEN, 1992).

For the Budel reactor acetate and sulfate concentrations were measured using a Dionex DX 100 ion chromatograph equipped with an AG 14 guard column and an AS 14 separator column. The eluent contained 3.5 mM sodium carbonate and 1.0 mM sodium bicarbonate. Injection volume was 25 µl and the eluent flow rate was 1.2 ml min^{-1}. Zinc was measured by inductively coupled plasma atomic emission spectrometry on a Thermo Electron Corporation, IRIS Advantage Dual view, according to Dutch standard methods (NEN, 1995). Organic N content of the sludge was measured on an Interscience Elemental Analyzer EA1110 equipped with a Thermal Conductivity Detector (TCD). Carrier gas was helium, flow rate 120 ml min^{-1}. Column temperature was 60 °C, reactor temperature was 1000 °C. Hydrogen, methane, and carbon dioxide were measured by GC on a Hewlett Packard 5890 series II equipped with a TCD. Carrier gas was helium, flow rate 17.5 ml min^{-1}. Column temperature was 60 °C, and detector temperature was 200 °C.

Nucleic acids isolation, PCR, cloning, RFLP and sequence analysis

Sludge for nucleic acid isolation was taken from the Budel reactor 14 weeks after start-up. Samples (6 ml) were fixed with 9.5 ml ethanol and 0.5 ml 0.8 M sodium chloride prior to storage at -25 °C. A pre-treatment was necessary in order to isolate nucleic acids from the fixed Budel sample. The method was based on a low speed centrifugation step in a sodium pyrophosphate buffer in order to separate cells from the zinc sulfide precipitate. The fixed sample was centrifuged for 10 minutes at 15,000 rpm. Approximately 10 g of wet pellet was resuspended in 100 ml 0.1 % sodium pyrophosphate solution, and centrifuged for 3 minutes at 1,000 rpm. This procedure was repeated

twice and supernatants were pooled. The pooled supernatants were centrifuged at 10,000 rpm for 20 minutes. The pellet was resuspended in 0.8 ml 120 mM sodium phosphate buffer. For Kennecott sludge no pretreatment was required and 1 ml of sludge was used.

Total DNA was extracted as previously described by Oude Elferink (1997). 16S rRNA genes were PCR amplified from the sludge DNA extract using a Taq DNA polymerase kit (Life Technologies, Gaithersburg, Md.). All primers were purchased from MWG-Biotech (Ebersberg, Germany). Bacterial 16S rRNA genes were selectively amplified for sequence analysis using 7-f and 1510-r primers (modified from Lane, 1991) with the following thermocycler program: 94ºC for 5 min; 25 cycles of 94ºC for 30 s, 52ºC for 20 s, and 68ºC for 40 s; and 68ºC for 7 min. For Archaea, 16S rRNA genes were selectively amplified for sequence analysis using primers A109-f (Großkopf et al., 1998), and 1510-r (modified from Lane, 1991). PCR reactions were performed with the following thermocycler program: 94 ºC for 5 min; 35 cycles of 94 ºC for 30 s; 52 ºC for 40 s, 68 ºC for 1 min , and 68 ºC for 7 min. Amplification and size of the PCR products were verified using a 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania) by 1 % (w/v) agarose gel electrophoresis and ethidium bromide staining. according to the manufacturer's manual. Amplified total 16S rRNA genes were purified using a QIAquick Kit (Qiagen GmbH, Hilden, Germany) and cloned in *E. coli* JM109 using the pGEM®-T vector system (Promega, Leiden, The Netherlands). Clones (36 bacterial clones and 36 archaeal clones per sample) were screened by Restriction Fragment Length Polymorphism (RFLP) analysis, using the enzyme MspI (Invitrogen, Breda, The Netherlands). Fragments were separated by 2% (w/v) agarose (Boehringer Mannheim GmbH, Mannheim, Germany) gel electrophoresis and visualized by ethidium bromide staining. Clones were grouped based on similar RFLP patterns and considered as an operational taxonomic unit. The plasmid of one selected transformant from each clone group was purified using the QIAprep spin miniprep kit (Qiagen GmbH, Hilden, Germany). Sequence analysis was carried out with the Sequenase sequencing kit (Amersham, Slough, United Kingdom) using IRD8000 labeled sequencing primers Sp6 and T7, complementary to the adjacent sequences of the pGEM®-T cloning site. The sequences were automatically analyzed on a LI-COR (Lincoln, NE, USA) DNA sequencer 4000L and checked manually. Partial 16S rRNA gene sequences from the sludge clones were searched against the NCBI database using BLAST (http://www.ncbi.nlm.nih.gov/blast/). Sequences generated from this work are deposited at GenBank under accession numbers AY831636 - AY831649, and DQ098652 and DQ098653.

Results and discussion

The sulfate conversion rate of the Budel full-scale reactor was monitored for 20 weeks (Fig. 2A). After 7 weeks the sulfate conversion rate reached the design rate of 15 $kg \cdot m^{-3} \cdot day^{-1}$. This coincided with an increased biomass concentration in the reactor (Fig. 2B). During the 20-week period the reactor experienced a number of disturbances. Between weeks 8 and 10 the phosphate dosage pump did not work properly, which lowered the sulfate conversion rate. After restoring the dosage of this nutrient, the sulfate conversion rate recovered within 1 week. In week 15 the reactor was fed with too much zinc containing wastewater, which resulted in an unknown free zinc concentration inside the reactor. This incident lowered the sulfate reduction rate to below 1 $kg \cdot m^{-3} \cdot day^{-1}$. In week 17 the reactor was taken off line to modify the synthesis gas production system. Between weeks 13 to 16 less wastewater was produced which resulted in a lower sulfate feed to the reactor.

Overall the performance of the full-scale reactor was good. The average sulfate removal efficiency was 88% between weeks 6 and 20, and the reactor was able to overcome disturbances within 1 week. Zinc sulfide precipitation inside the Budel reactor did not interfere with obtaining high rates of sulfate reduction. This is contrary to the findings of Utgikar et al. (2002) who have reported a strong inhibitory effect of metal sulfide formation on a sulfate-reducing community in a stirred tank reactor fed with acetate. They identified precipitation of metal sulfides on bacterial cells as the probable reason for this. Precipitation inside the reactor did change the appearance and solid composition of the sludge. Sludge from the original Kennecott reactor was black, and had a TSS content of 11 $g \cdot L^{-1}$ and a VSS content of 8 $g \cdot L^{-1}$. Budel sludge was grey and had a TSS content of 129 $g \cdot L^{-1}$. The VSS content of Budel sludge could not be determined accurately because of the high metal sulfide concentration. Based on organic N content, 4 % of the total solids of the Budel sludge was organic, as opposed to 27 % of the Kennecott sludge based on VSS content. The TSS of the Budel sludge consisted of 95 % zinc sulfide and could be re-used in the zinc smelting process.

During the first 6 weeks after start-up of the Budel reactor acetate formation of up to 4 $g \cdot L^{-1}$ was observed (Fig. 2C). With the increase of the biomass concentration (Fig. 2B) and an increased rate of sulfate reduction (Fig. 2A), the acetate concentration decreased to undetectable amounts. Homoacetogens were probably not able to compete effectively for hydrogen because of their relatively poor growth kinetic parameters for growth on hydrogen compared to SRB and methanogens (Cord-Ruwisch et al., 1988; Peters et al., 1998; Stams, 1994). They were however not completely washed out of the reactor. Twelve weeks after start-up acetate formation was again observed. This coincided with a period with a lower sulfate reduction rate (Figure 2A).

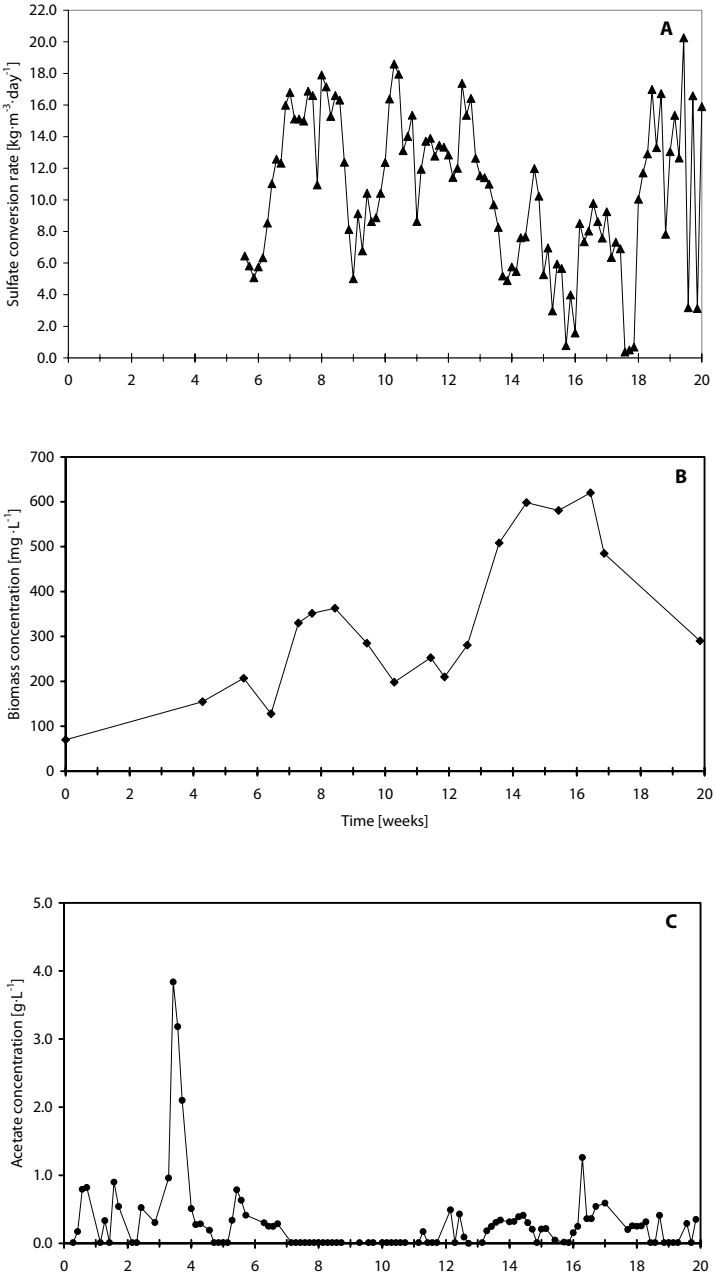

Figure 2 Sulfate conversion rate (A), biomass concentration (B), and acetate concentration (C) in the Budel reactor. The biomass concentration is based on the organic N.

Previous studies by van Houten (1996) showed that methanogenesis did not occur in synthesis gas fed lab-scale reactors. Studies with sediments and sludge from other types of anaerobic bioreactors have shown that at an excess of sulfate, hydrogen is mainly consumed by sulfate reducers (Oude Elferink et al., 1994). In anaerobic granular sludge based reactors the activity of hydrogenotrophic methanogens was completely suppressed within a few weeks by the addition of sulfate (Visser et al., 1993). This suppression of methanogenesis by sulfate reduction has been attributed to the fact that SRB have a lower hydrogen threshold concentration (Oude Elferink et al., 1994).

However, during 20 weeks of operation of the Budel reactor, methanogenesis was not suppressed. High methane concentrations ranging from 26 to 67 % v/v could be detected in the gas recycle, indicating considerable methane production. Based on estimates of the feed, bleed gas flow and composition we estimate that approximately 28 to 47 % of the hydrogen fed to the reactor was used for methanogenesis.

The MPN estimates showed that heterotrophic SRB dominated Budel sludge 19 weeks after start-up (Table 2). The original Kennecott sludge also showed predominance of heterotrophic SRB. Heterotrophic SRB outnumbered methanogens, approximately 200-fold for Kennecott sludge, and 1,000-fold for Budel sludge.

Table 2 Most probable numbers (n=3) of sulfate reducers, homoacetogens and methogens present in Kennecott and Budel sludge. *VSS content of the Budel sludge was calculated based on organic N content.

Trophic group	Kennecott	Budel Zink
	cells/ g VSS	cells/ g VSS*
Heterotrophic sulfate reducers	1.5×10^{11}	4.4×10^{12}
Methanogens	6.8×10^{8}	4.5×10^{9}
Homoacetogens	3.6×10^{5}	6.2×10^{6}

However, based on the estimates of the hydrogen consumption for methanogenesis the numbers of methanogens and SRB should have been in the same order of magnitude for the Budel reactor. Even though the MPN estimates appeared to underestimate the number of methanogens, they confirmed the presence of high numbers of methanogens. Overall, Kennecott sludge showed a lower cell number per g VSS, but this could be due to possible cell death in the Kennecott sludge during the 9 month storage period.

Partial 16S rRNA gene libraries for Bacteria and Archaea were constructed from both Budel (week 14) and Kennecott sludge in order to identify the predominant microorganisms. The sequence analysis results are summarized in table 3. The majority of predominant bacterial clones obtained from Budel and Kennecott sludge showed the highest levels of sequence similarity to the genus *Desulfomicrobium*.

Table 3 Relative abundance and closest relatives of the clones obtained from Kennecott and Budel sludge based on partial sequence analysis of the 16S rRNA gene. Closest relative were found by comparison of the sequences with sequences in the GenBank data base using BLAST. Closest cultured relatives are given separately when the closest relative was uncultured. Relative abundance was calculated based on the number of similar clones found by RFLP analysis for each individual sludge sample.

Clone	Relative Abundance %	Accession number	Source	Closest relative	Similarity %	Closest cultured Relative	Similarity %
Bacteria							
BKV01	52.2	AY831636	Budel	uncultured bacterium mle1-31, AF280859.1	95	*Desulfomicrobium norvegicum*, AJ277897	95
BKV02	30.4	AY831637	Budel	*Desulfomicrobium sp*. 'Delta +', AF443593.1	94	*Desulfomicrobium norvegicum*, AJ277897	94
BKV08	17.4	DQ098652	Budel	Uncultured Clone Gitt-GS-109, AJ582209	92	*Proteophilum aceticum* TB107, AY742226.1	
BKV09	82.1	AY831638	Kennecott	*Desulfomicrobium sp*. 'Delta +', AF443593.1	90	*Desulfomicrobium norvegicum*, AJ277897	90
BKV10	3.6	AY831639	Kennecott	*Acetobacterium wieringa*, X96955	87		
BKV13	3.6	AY831640	Kennecott	*Desulfovibrio* sp. SB1, AY726757	92		
BKV14	3.6	AY831641	Kennecott	*Desulfovibrio* sp. SB1, AY726757	95		
BKV17	3.6	DQ098653	Kennecott	Uncultured bacterium mle1-42, AF280863	95	*Synergistes jonesii*, L08066.1	88
Archaea							
BKV18	26.2	AY831642	Budel	*Methanobacterium* sp. OM15, AJ550160	94		
BKV19	21.4	AY831643	Budel	*Methanobacterium bryantii* AF028688	92		
BKV21	7.1	AY831644	Budel	*Methanobacterium formicicum* AF02868	96		
BKV22	7.1	AY831645	Budel	clone:BA03, AB092917	97	*Methanospirillum hungatei*, AY196683	95
BKV23	2.4	AY831646	Budel	clone OuI-24, AJ556504	87	*Methanobacterium curvum*, AF276958	86
BKV25	14.3	AY831647	Budel	*Methanobacterium subterraneum*, X99045	96		
BKV28	94.1	AY831648	Kennecott	*Methanobacterium formicicum* AF028689	96		
BKV29	5.9	AY831649	Kennecott	*Methanobacterium formicicum* AF028689	94		

Growth on hydrogen coupled to sulfate reduction is common for this genus, but requires an additional carbon source such as acetate (Widdel and Bak, 1991). This is in agreement with the results obtained from the MPN estimates (week 19), which showed that both sludges were dominated by heterotrophic SRB. The archaeal sequences retrieved from both sludges were related to the genera *Methanobacterium* and *Methanospirillum* (table 4). *Methanobacterium* and *Methanospirillum* species are known to grow on hydrogen and carbon dioxide (Whitman et al., 1991). As only a limited number of clones from of each reactor were screened at only one point in time, the clone library will not reflect total microbial diversity of these reactors. However, the relative abundance of a small number of sulfate-reducing and methanogenic species in these two gas-lift reactors is likely to be characteristic of these systems. The two gas-lift systems are characterized by a low SRT and hydrogen as the main electron donor. These very restrictive conditions are likely to select for the abundance of a limited number of hydrogenotrophic sulfate-reducing and methanogenic species. These conditions are in contrast to more conventional anaerobic granular sludge based wastewaters treatment systems.

Granular sludge based systems normally display a high microbial diversity (Godon et al., 1997; Grotenhuis et al., 1991; Oude Elferink et al., 1998; Roest et al., 2005), as they are characterized by the presence of a complex anaerobic food web needed for the degradation of a mix of organic and inorganic substrates. The hydrogen thresholds of the methanogens detected within the sludge, *Methanobacterium* and *Methanospirillum* spp., are reported to be in the range of 2.5 to 16 Pa at temperatures between 28 and 37 °C (Cord-Ruwisch et al., 1988; Stams, 1994). Thresholds for *Desulfovibrio* spp. are reported to be lower, in the range of 0.9 to 4.5 Pa. Based on these threshold values methanogenesis should have been suppressed rapidly. However, because of the relatively short SRT of 4 to 7 days, the specific growth rate of the microorganisms has to be in the range of 0.14 to 0.25 day^{-1}. To sustain this growth rate, hydrogen threshold concentrations will not be reached (Fig. 3). Limited data is available on Monod saturation constants (K_s), however maximum growth rates (μ_{max}) and Michaelis-Menten constants (K_m) are in the same range for *Desulfovibrio, Methanobacterium* and *Methanospirillium* species (Oude Elferink et al., 1994). The persistence of methanogenesis could therefore be explained by the relatively similar Monod kinetics of SRB and methanogens. Although homoacetogenesis was suppressed (Fig. 2C), the MPN estimates showed that homoacetogens were still present in the sludge after 19 weeks (Table 2).

As hydrogenotrophic methanogens require carbon dioxide as their terminal electron acceptor, limiting the carbon dioxide feed to the reactor might be a suitable method to control methanogenesis. Heterotrophic SRB also utilize carbon dioxide as a carbon source but to a much lower extent as they obtain most of their carbon from the organic carbon source provided (Sorokin, 1966; Sorokin, 1966). Limiting the carbon dioxide feed to the reactor had already been planned as a means to minimize the addition of sodium hydroxide to the reactor for pH correction, and indeed proved to be a suitable and simple method to control methanogenesis (Chapter 3).

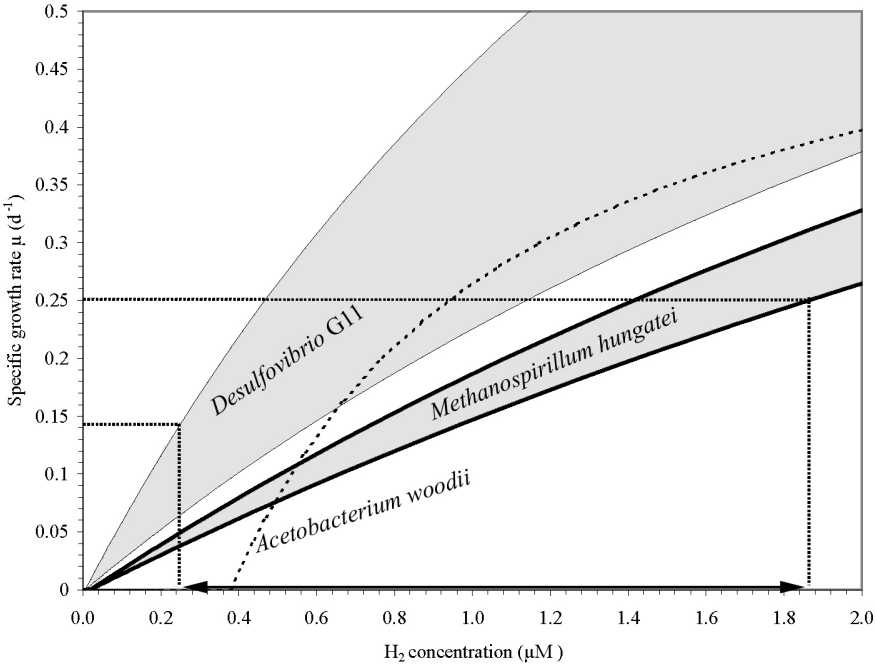

Figure 3 Hydrogen concentration range to sustain growth rates of 0.14 to 0.25 d^{-1} based on Monod kinetics* for *Desulfovibrio* G11, *Methanospirillum hungatei* JF1 and *Acetobacterium woodii*.

*Calculations are based on a modified Monod equation (Pavlostathis and Giraldo-Gomez, 1991) to account for threshold concentrations;

$$\mu = \mu_{\max} \cdot \frac{S - S_t}{(S - S_t) + K_s}$$

Monod kinetic parameters for *Desulfovibrio* G11, *Methanospirillum hungatei* JF1 are based on the ranges given by Robinson and Tiedje (1984) for growth at 37 °C. Monod parameters for *Acetobacterium woodii* were obtained from Peters et al. (1998) for growth at 30 °C. Hydrogen thresholds measured at 28 – 34 °C ,were obtained from Cord-Ruwisch et al. (1988).

Conclusions

After 7 weeks the sulfate conversion rate of the full-scale reactor reached the design rate of 15 $kg \cdot m^{-3} \cdot day^{-1}$. Zinc sulfide precipitation inside the reactor did not interfere with obtaining this high rate of sulfate reduction and the averaged sulfate removal efficiency was 88%. Homoacetogenesis was suppressed after an increase in the sludge concentration and an increased rate of sulfate reduction. However, methanogenesis was not suppressed.

16S rRNA gene sequence analysis showed that the bacterial community 14 weeks after start-up of the full-scale reactors was mainly related to the sulfate-reducing genus *Desulfomicrobium*. The original seed reactor showed the same predominance. This is in agreement with the MPN estimates, which showed that heterotrophic sulfate-reducing bacteria were dominant in both sludges. The archaeal communities were closely related to the methanogenic genera *Methanobacterium* and *Methanospirillum*. RFLP analysis also showed the relative abundance of a limited number of sulfate-reducing and methanogenic species. These findings suggest that a specialized community has developed which can be attributed to the fact that hydrogen was the only readily available electron donor, and to the relatively short SRT of 4 to 7 days. Due to this relatively short SRT, competition for hydrogen will be a result of Monod kinetics, and not hydrogen thresholds concentrations. As kinetics of parameters of SRB and methanogens are relatively similar, methanogenesis was probably able to persist. Although methanogenesis did not interfere with obtaining a high rate of sulfate reduction, it is an unwanted process as it results in a loss of hydrogen.

Acknowledgements

This research was supported by a grant from the E.E.T. program of the Dutch ministries of Economic Affairs, Education, Culture and Sciences and Housing, Spatial Planning and the Environment (EETK98028). We would like to thank C. Copini and W. Vermeulen from Zinifex Budel Zink for valuable discussion and J.E. Edwards for critically reviewing the manuscript.

References

Boonstra, J., van Lier, J., Janssen, G., Dijkman, H. and Buisman, C.J.N. (1999) Biological treatment of acid mine drainage. in Biohydrometallurgy and environment toward the 21st century. Process metallurgy 9b. Amsterdam: Elsevier.

Cord-Ruwisch, R., Seitz, H.J. and Conrad, R. (1988) The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor, Archives of Microbiology. p. 350-357.

Godon, J., Zumstein, E., Dabert, P., Habouzit, F. and Moletta, R. (1997) Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis, Applied and Environmental Microbiology. p. 2802-2813.

Großkopf, R., Janssen, P.H. and Liesack, W. (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16s rrna gene sequence retrieval, Applied and Environmental Microbiology. p. 960-969.

Grotenhuis, J.T.C., Smit, M., Plugge, C.M., Yuansheng, X., Van Lammeren, A.A.M., Stams, A.J.M. and Zehnder, A.J.B. (1991) Bacteriological composition and structure of granular sludge adapted to different substrates, Applied And Environmental Microbiology. p. 1942-1949.

Kaksonen, A.H., Plumb, J.J., Franzmann, P.D. and Puhakka, J.A. (2004) Simple organic electron donors support diverse sulfate-reducing communities in fluidized-bed reactors treating acidic metal- and sulfate-containing wastewater, FEMS Microbiology Ecology. p. 279-289.

Lane, D.J. (1991) 16s/23s rRNA sequencing. in Nucleic acid techniques in bacterial systematics. New York: John Wiley and Sons.

NEN (1992) Waste water and sludge - determination of the content of suspended solids after drying and after ignition - gravimetric method, NEN 6621, C1. Dutch Standardization Institute (NEN): Delft.

NEN (1995) Water - determination of 40 elements by inductively coupled plasma atomic emission spectrometry, NEN 6426:1995 nl. Dutch Standardization Institute (NEN): Delft.

Oude Elferink, S.J.W.H., Maas, R.N., Harmsen, H.J.M. and Stams, A.J.M. (1995) *Desulforhabdus amnigenus* gen. Nov. Sp. Nov. A sulfate reducer isolated from anaerobic granular sludge, Archives of Microbiology. p. 119-124.

Oude Elferink, S.J.W.H., Rinia, H.A., Bruins, M.E., de Vos, W.M. and Stams, A.J.M. (1997) Detection and quantification of *desulforhabdus amnigenus* in anaerobic granular sludge by dot blot hybridization and pcr amplification, Journal of Applied Microbiology. p. 102-110.

Oude Elferink, S.J.W.H., Visser, A., Hulshoff Pol, L.W. and Stams, A.J.M. (1994) Sulfate reduction in methanogenic bioreactors, FEMS Microbiology Reviews. p. 119-136.

Oude Elferink, S.J.W.H., Vorstman, W.J.C., Sopjes, A. and Stams, A.J.M. (1998) Characterization of the sulfate-reducing and syntrophic population in granular sludge from a full-scale anaerobic reactor treating papermill wastewater, FEMS Microbiology Ecology. p. 185-194.

Pavlostathis, S.G. and Giraldo-Gomez, E. (1991) Kinetics of anaerobic treatment: A critical review, Critical Reviews in Environmental Control. p. 411-490.

Peters, V., Janssen, P.H. and Conrad, R. (1998) Efficiency of hydrogen utilization during unitrophic and mixotrophic growth of *acetobacterium woodii* on hydrogen and lactate in the chemostat, FEMS Microbiology Ecology. p. 317-324.

Robinson, J.A. and Tiedje, J.M. (1984) Competition for hydrogen between sulfate-reducing and methanogenic bacteria for hydrogen under resting and growing conditions, Archives of Microbiology. p. 26-32.

Roest, K., Heilig, H.G.H.J., Smidt, H., de Vos, W.M., Stams, A.J.M. and Akkermans, A.D.L. (2005) Community analysis of a full-scale anaerobic bioreactor treating paper mill wastewater, Systematic and Applied Microbiology. p. 175-185.

Scholten, J.C.M. and Stams, A.J.M. (1995) The effect of sulfate and nitrate on methane formation in a freshwater sediment, Antonie van Leeuwenhoek. p. 309-315.

Sorokin, Y.I. (1966) Investigation of the structural metabolism of sulfate-reducin bacteria with C^{14}, Microbiologiya. p. 967-977.

Sorokin, Y.I. (1966) Sources of energy and carbon for biosynthesis in sulfate-reducing bacteria, Microbiologiya. p. 761-766.

Stams, A.J.M. (1994) Metabolic interactions between anaerobic bacteria in methanogenic environments, Antonie van Leeuwenhoek. p. 271-294.

Stams, A.J.M., Van Dijk, J.B., Dijkema, C. and Plugge, C.M. (1993) Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria, Applied and Environmental Microbiology. p. 1114-1119.

Trüper, H.G. and Schlegel, H.G. (1964) Sulphur metabolism in thiorhodaceae 1. Quantitative measurements on growing cells of *chromatium okenii*, Antonie van Leeuwenhoek. p. 225-238.

Utgikar, V.P., Harmon, S.M., Chaudhary, N., Tabak, H.H., Govind, R. and Haines, J.R. (2002) Inhibition of sulfate-reducing bacteria by metal sulfide formation in bioremediation of acid mine drainage, Environmental Toxicology. p. 40-48.

van Houten, R.T. (1996) Biological sulfate reduction with synthesis gas. Wageningen Agricultural University: Wageningen, Netherlands. p. 125.

van Houten, R.T. and Lettinga, G. (1994) Biological sulphate reduction with synthesis gas: Microbiology and technology. in Progres in biotechnology. Amsterdam: Elsevier.

Visser, A., Beeksma, I., van der Zee, F., Stams, A.J.M. and Lettinga, G. (1993) Anaerobic degradation of volatile fatty acids at different sulphate concentrations, Applied Microbiology and Biotechnology. p. 549-556.

Whitman, W.B., Bowen, T.L. and Boone, D.R. (1991) The methanogenic bacteria. in The prokaryotes. New York: Springer-Verlag.

Widdel, F. and Bak, F. (1991) Gram-negative mesophilic sulfate-reducing bacteria. in The prokaryotes. New York: Springer-Verlag.

3

Microbial community analysis and long-term performance of a full-scale synthesis gas fed reactor treating sulfate and zinc rich wastewater

Bernd H.G.W. van Houten [a], Wim van Doesburg [a], Henk Dijkman [b], Cris Copini [c], Hauke Smidt [a] and Alfons J.M. Stams [a]

a Laboratory of Microbiology, Hesselink van Suchtelenweg 4, Wageningen University, 6703 CT, Wageningen, The Netherlands.

b Paques B.V., T. de Boerstraat 24, P.O Box 52, 8560 AB Balk, The Netherlands

c Zinifex Budel Zink B.V. P.O Box 2001, 6020 AA Budel, The Netherlands

To be submitted

Abstract

The performance of a full-scale (500 m^3) synthesis gas fed gas-lift reactor treating metal and sulfate rich wastewater was investigated over a period of 128 weeks. The average sulfate conversion rate was 181 $kg \cdot hr^{-1}$ for the first 92 weeks. After 92 weeks the sulfate input rate was increased and the average sulfate conversion rate was 289 $kg \cdot hr^{-1}$. The carbon dioxide feed rate was gradually lowered during the first 6 weeks. Consequently, methane production rate decreased and, between week 8 and 93, less than 1% of the hydrogen supplied was used for methanogenesis. Denaturing Gradient Gel Electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene fragments showed that the archaeal community decreased in diversity, but did not disappear completely. After the carbon dioxide feed rate increased in week 88, the methane production rate increased from week 93 onwards; but no obvious change in archaeal diversity was detected. Clone libraries of PCR-amplified 16S rRNA gene fragments demonstrated that diversity within the bacterial community was not limited to hydrogenotropic sulfate-reducing bacteria (SRB). Members of Chloroflexi subphylum I were identified as potential scavengers of lysed cells. Fluctuations in feed rates and sludge retention time, which frequently occurred during operation at full scale might sustain multiple populations of hydrogenotropic SRB. This functional redundancy within the SRB present in the reactor, could be an important factor in maintaining a stable reactor performance under process fluctuations. The long-term performance and stability together with the ability to control methanogenesis by lowering the carbon dioxide feed rate demonstrate that this technology can be used successfully at full scale to treat metal and sulfate rich wastewater.

Introduction

Sulfate and metal rich wastewaters that are low in organic carbon are produced as a result of several industrial processes, such as metal smelting, flue gas scrubbing and mining. Sulfate-reducing bioreactors have been previously shown to be suitable systems to remediate these types of wastewater (3). These bioreactors utilize the sulfidogenic activity of sulfate-reducing bacteria (SRB) to simultaneously remove sulfate and metals in the form of metal sulfides. Paques B.V. (Balk, Netherlands) has developed a sulfidogenic THIOPAQ® system, which is based on a gas-lift reactor fed with hydrogen gas as the electron donor for sulfate reduction. After tests conducted with a pilot-scale reactor, the first full-scale synthesis gas fed reactor was constructed to treat the wastewater from the Budel Zink zinc smelting plant. The main advantage of this system over a conventional lime stone neutralization process is that the zinc sulfide produced can be re-used in the metal smelting process, and no solid waste is produced. Furthermore, since the solubilities of most metal sulfides are much lower than that of their respective hydroxides, considerably lower effluent metal concentrations can be achieved. For large-scale applications synthesis gas is an attractive source of hydrogen (22). Synthesis gas is a mix containing mainly H_2, CO_2 and CO with minor levels of other components, such as methane and nitrogen.

While the start-up of this synthesis gas fed reactor was successful and the desired rate of sulfate reduction was achieved, methanogenesis was not suppressed despite the predominance of sulfate-reducing bacteria (20). This persistence of methanogenesis was unexpected, as prior research had shown suppression of methanogenesis by sulfate reduction in similar lab scale systems (21, 23). A possible explanation would be that the fluctuations that frequently occur during operation at full scale with regards to feed rates and sludge retention time lead to fluctuations in the hydrogen level. As a result a continuous state of hydrogen limitation may not be reached. Since Monod kinetic parameters for methanogens are relatively similar to those of SRB (14), they are not readily outcompeted.

An alternative strategy to hydrogen limitation would be to limit the carbon dioxide feed to the reactor. As hydrogenotrophic methanogens require carbon dioxide as their terminal electron acceptor, limiting the carbon dioxide feed to the reactor might be a suitable method to control methanogenesis. Heterotrophic SRB also utilize carbon dioxide as a carbon source but to a much lower extent as they obtain most of their carbon from the organic carbon source provided (2, 18, 19) Limiting the carbon dioxide feed to the reactor had already been planned as a means to minimize the addition of sodium hydroxide to the reactor for pH correction.

The main objectives of this study were to monitor the reactor performance over a prolonged period of 128 weeks, and to investigate the effect of lowering the effect of the carbon dioxide feed on the rate of methanogenesis. Furthermore, we studied the composition and dynamics of the bacterial and archaeal community over this period. Information on the composition of the

microbial community will provide information that can be used to understand and control the performance of these bioreactors in the future. Temporal community changes were assessed using denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA gene, while the relative abundance and identity of dominant bacterial species in the sludge were determined using restriction fragment length polymorphism (RFLP) and sequence analysis.

Materials and methods

Reactor operating conditions

The reactor was a 500 m^3 full-scale gas-lift reactor treating sulfate and heavy metal rich wastewater from a zinc smelter in Budel-Dorplein, The Netherlands. A schematic flow diagram of the Budel reactor has been presented previously(20). The input of the reactor consisted of two waste streams from the zinc production process. The reactor was operated between 30 to 35 °C and pH 7.0 to 7.5. The sludge retention time (SRT) was 4 to 7 days. The hydraulic retention time (HRT) was between 1 and 3 days. Acetate was added as an organic carbon source at a rate of 31 mg per gram of sulfate fed to the reactor.

Chemical analyses

Sulfate concentrations of the influent and effluent were measured using ion chromatography and hydrogen, methane and carbon dioxide concentrations of the reactor feed, bleed and recycle gas were measured by gas chromatography as previously described (20).

Nucleic acids isolation and PCR

Sludge samples for nucleic acid isolation were taken from the sludge recycle. Samples (6 ml) were fixed with 9.5 ml ethanol and 0.5 ml 0.8 M sodium chloride prior to storage at –20 °C.

Total DNA was extracted as previously described (13). 16S rRNA genes were PCR amplified from the sludge DNA extract using a *Taq* DNA polymerase kit (Life Technologies, Gaithersburg, Md.). All primers were purchased from MWG-Biotech (Ebersberg, Germany). Bacterial 16S rRNA genes were selectively amplified for clone library construction and sequence analysis using 7-f and 1510-r primers (8) with the following thermocycler program: 94 °C for 5 min; 25 cycles of 94 °C for 30 s, 52 °C for 20 s, and 68 °C for 40 s; and 68 °C for 7 min. For DGGE, partial bacterial 16S rRNA gene fragments were amplified using 968-GC-f and 1401-r primers (11) with the same thermocycler program, but an increased number of 35 cycles and an annealing temperature of 56 °C was used. For archaeal DGGE analyses, A109(T)-f (original (4)), 3rd bp changed into T) and 515-GC-r (8) were used for amplification. Reactions were performed with the following thermocycler program: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 52 °C for 40 s, 68 °C for 1 min , and 68 °C for 7 min. Amplification and size of PCR products were verified by 1 % (w/v) agarose gel electrophoresis with

ethidium bromide staining, and comparison of amplification products to a 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania).

DGGE and RFLP analysis

DGGE analysis was performed on 8% (w/v) polyacrylamide gels containing a denaturing gradient of 35 to 60% for bacterial amplicons, and 30 to 45% for archaeal amplicons. 100% denaturant corresponded to 7 M urea and 40 % (v/v) formamide. Electrophoresis was performed in 0.5 x TAE buffer (20 mM Tris, 10 mM acetic acid and 0.5 mM EDTA pH 8) at 85 V and 60ºC for 16 hours (after 10 minutes at 200 V) using a DCode System (BioRad, Hercules, CA). Gels were silver stained as described previously (16).

For the generation of bacterial clone libraries, amplified full-length 16S rRNA genes were purified using a QIAquick Kit (Qiagen GmbH, Hilden, Germany) and cloned into *E. coli* JM109 by using the pGEM®-T Easy vector system (Promega, Leiden, The Netherlands). Clone libraries (96 clones for weeks 10 and 50, and 48 for week 128) were screened for number and relative abundance of unique clone types by Restriction Fragment Length Polymorphism (RFLP) analysis (digestion with *Msp*I, *Cfo*I and *Alu*I, Invitrogen) and DGGE. Plasmids of selected transformants were then purified using the QIAprep spin miniprep kit (Qiagen GmbH, Hilden, Germany).

Sequence determination and analysis

Only abundant operational taxonomic units (OTUs) with unique RFLP and DGGE migration patterns, and represented by 2 or more clones, were sequenced. Sequence determination was carried out with the Sequenase sequencing kit (Amersham, Slough, United Kingdom). 16S rRNA gene sequences were searched against the NCBI database using BLAST (http://www.ncbi.nlm.nih.gov/blast/) (10). Sequences were checked for chimera using PINTAIL software (1).

Alignment and phylogenetic analysis of 16S rRNA gene sequences retrieved from the full scale reactor at weeks 10, 50 and 128, and reference sequences, were performed using the ARB software package (9). Phylogenetic analysis was performed using the neighbour joining method with Felsenstein correction as implemented in the ARB software package (15). The tree was constructed using *E. coli* positions 37 to 1491, using a general bacterial 50% conservation filter provided with the latest release of the ARB small subunit rRNA database. Sequences generated from this work are deposited at GenBank under accession numbers DQ447163-DQ447176.

Results and discussion

The sulfate input and output rates of the 500 m^3 Budel full-scale reactor were monitored for 128 weeks (Fig. 1). For the first 92 weeks, the sulfate conversion rate was on average 181 kg hr^{-1}. After 92 weeks the sulfate input rate increased due to changes in the composition of the wastewater. The reactor was able to accommodate this increased sulfate load, and from week 94 to 128 the average sulfate conversion rate was 289 kg hr^{-1}. High sulfate conversion rates of up to 400 kg hr^{-1} could be sustained for a period of 8 weeks (Fig. 1). Fluctuations in the sulfate input rates during the 128 weeks were mainly caused by the rates at which the influents were produced. Other causes for shorter periods with lower or no sulfate reduction include: maintenance and inspection, problems with nutrient addition to the reactor or with feed gas production, and free zinc toxicity (week 4). In general, the reactor performed well and demonstrated good stability over the 128 week period.

The methane production rate and carbon dioxide feed rate were monitored for 128 weeks (Fig. 2). Before the carbon dioxide feed rate was lowered, the reactor had a high methanogenic activity of 46 $Nm^3 \cdot hr^{-1}$ (Normal m^3: T = 293 K, p = 101.325 Pa). The carbon dioxide concentration in the feed gas was gradually lowered from 20-22 % to 6-3 % (v/v) during the first 4 weeks, resulting in a lowered carbon dioxide feed rate of 20-15 $Nm^3 \cdot hr^{-1}$. From week 6 onwards the carbon dioxide feed rate was kept below 15 $Nm^3 \cdot hr^{-1}$ and by week 8 the methanogenic activity had decreased to 3 Nm^3 methane hr^{-1}. Between weeks 8 and 93, at a calculated hydrogen consumption rate of 2 $Nm^3 \cdot hr^{-1}$, less than 1% of the hydrogen supply was used for methanogenesis. From week 88 the feed gas carbon dioxide concentration varied between 3.4 and 10.2 %, due to a problem with the gas absorber used for carbon dioxide removal from the feed gas. This caused an increased feed of carbon dioxide to the reactor (Fig. 2). After a period of little methane production, an increase in methane production was detected from week 93 onwards. At a calculated hydrogen consumption rate of 132 Nm^3 hr^{-1} in week 103 approximately 25% of the supplied hydrogen was used for methanogenesis.

The abundant archaea in this system had been identified previously as members of the hydrogenotrophic genus *Methanobacterium (20)*. To assess the influence of these changes in operating conditions on the archaeal community structure, DGGE profiles were generated (Fig. 3). After the carbon dioxide feed rate had been lowered initially, the archaeal profiles decreased in complexity and maintained a consistent profile up to week 128. These results indicate that the lowered carbon dioxide feed decreased the archaeal diversity. Nevertheless, the archaea were not washed out completely. After the rate of methanogenesis had increased again in week 93, no obvious change in diversity was detected. The increase in the rate of methanogenesis after the carbon dioxide feed had increased, suggests that the archaeal community was carbon dioxide limited between week 13 and week 95.

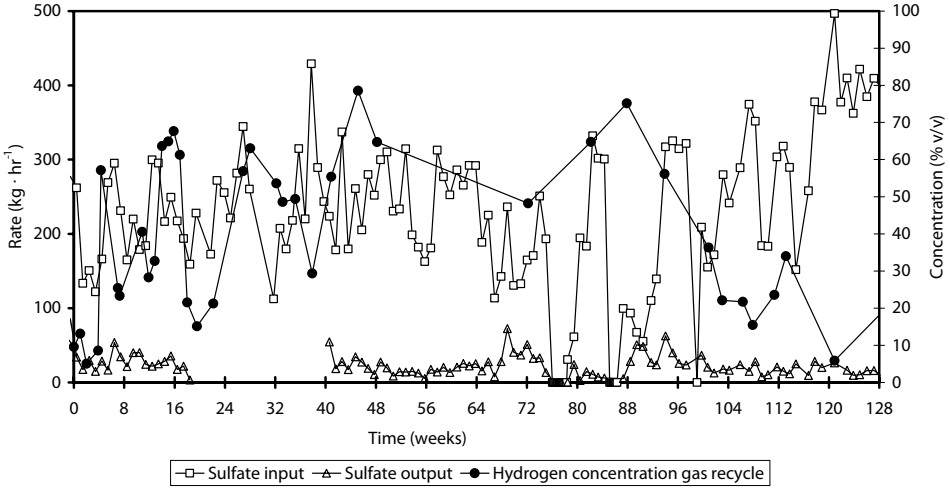

Figure 1 Averaged weekly sulfate input and output rate, and the hydrogen concentration in gas recycle of the Budel full-scale reactor.

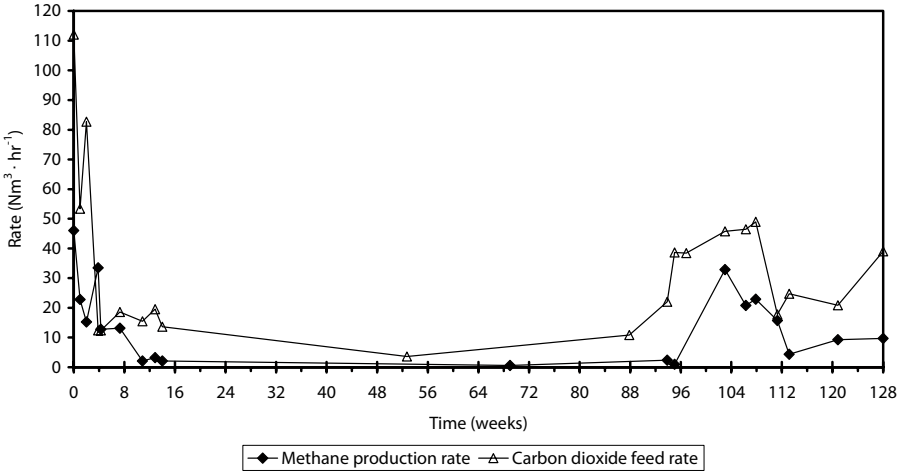

Figure 2 Methane production rate and carbon dioxide feed rate of the Budel full-scale reactor, volume is given in Normal m^3 (T = 293 K, p = 101.325 Pa)

This is also reflected by the decrease in diversity observed by DGGE, as the competition for a single growth limiting substrate could be expected to decrease diversity. Limiting the carbon dioxide

feed proved to be a good tool to control the methanogenic activity, although it did not result in a complete washout of methanogens from the reactor.

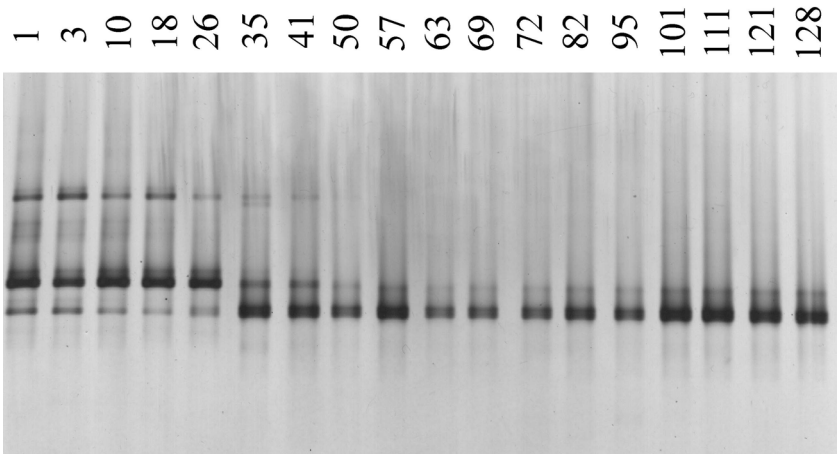

Figure 3 Analysis of archaeal community dynamics by 16S rRNA gene-targeted PCR-DGGE. Numbers indicate week of sampling.

The temporal dynamics of the bacterial community in the reactor were also monitored for the complete period of 128 weeks (Fig 4) using DGGE. After successive changes until week 41, the community structure became relatively stable between week 41 and 101 with regard to the dominant bands. This period was characterized by a stable operation of the reactor with only minor fluctuations in the sulfate input rate, the hydrogen concentration in the gas recycle (Fig 1), and the feed gas rate and composition (data not shown).

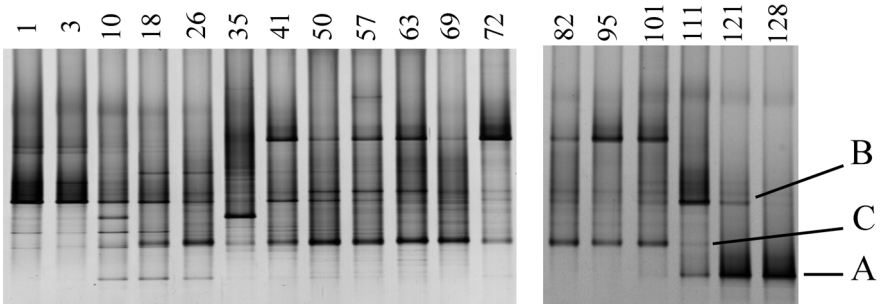

Figure 4 Analysis of bacterial community dynamics by 16S rRNA gene-targeted PCR-DGGE. Numbers indicate week of sampling. Band position A, B and C correspond with library clones BUD16, BUD03 and BUD11 respectively.

After week 101 a shift in the bacterial community to only one predominant band (A) was observed. This shift coincided with a period of a low hydrogen concentration in the gas recycle (Fig. 1). This lowered hydrogen concentration is likely to have resulted in a stronger competition for hydrogen,

and consequently would have lowered the diversity of hydrogenotrophic microorganisms. 16S rRNA gene clone libraries were constructed for the bacterial community from samples from weeks 10, 50 and 128 (Table1). The low bacterial diversity found in the week 128 clone library is in agreement with reduction in diversity observed by DGGE after week 101. In week 128, the microorganisms represented by clone BUD16 appear to be predominant as indicated by RFLP abundance (27.7%). The migration pattern of clone BUD16 on DGGE was the same as predominant band A observed in the DGGE profiles from weeks 111, 121 and 128. It is likely that the microorganisms represented by clone BUD16, which showed the highest level of sequence identity to *Desulfovibrio giganteus*, possess a high affinity for hydrogen, giving them a selective advantage at low hydrogen concentrations. Two predominant bands B and C were observed over prolonged periods during the reactor operation. The DGGE migration patterns of the abundant clones BUD03 and BUD11 coincided with bands B and C, respectively. Band C became predominant during the stable period with a high hydrogen concentration in the gas-recycle (week 18 - 101). Band B was detectable over the longest period of time (week 1 - 121) and remained prominent for a longer time during the period with a lower hydrogen concentration in the gas-recycle. These two taxa, belonging to the sulfate reducing genera of *Desulfomicrobium* and *Desulfovibrio* (Fig. 3), appear to have been important microorganisms for the performance of the Budel reactor.

The diversity found within the clone libraries (Table 1) appeared to contrast with the results obtained by DGGE (Fig. 2). The lower microbial diversity observed by DGGE may have been caused by several factors. The high relative abundance of a few taxa will have interfered with the amplification of less abundant species during PCR. A high number of faint bands, hardly distinguishable from the background, were visible on DGGE. Furthermore, the universal primer pair 968-GC-f and 1401-r for DGGE showed 2 mismatches with clones BUD04, BUD07, BUD09, BUD13, BUD14 and 3 mismatches with clones BUD05, BUD10 and BUD12, resulting in a poor amplification of the individual clones (data not shown). A large number of clones represented by a single RFLP pattern were found: 59.7 %, 27% and 47% for weeks 10, 50 and 128 respectively. However, an unknown number of these unique clones are likely to be sulfate reducers considering the high rates of sulfate reduction of the sludge. The fluctuations that frequently occur during operation at full scale with regards to feed rates, SRT and in particular hydrogen levels is likely to enable multiple hydrogenotrophic SRB to be sustained. Based on conditions prevailing in the reactor, only a limited number will become abundant. This functional redundancy of a part of the SRB community may be an important factor in enabling a stable reactor performanceto be maintained under process fluctuations.

The predominance of the genus *Desulfomicrobium* found previously (20) was not found in week 10. The RFLP data for week 10 showed a relative abundance of only 6% for the only sequenced clone (BUD03) that belonged to this genus. A large number of abundant clones found in week 10

did not belong to known genera of SRB. Clone BUD01 showed affiliation to the proteolytic microorganism *Proteiniphilum acetatigenes* strain TB107. Clone BUD04 from week 10, and clones BUD13, BUD09 and BUD14 from later time points, cluster within the Chloroflexi subphylum I (Fig. 5) as defined by Hugenholtz and co-workers (5).

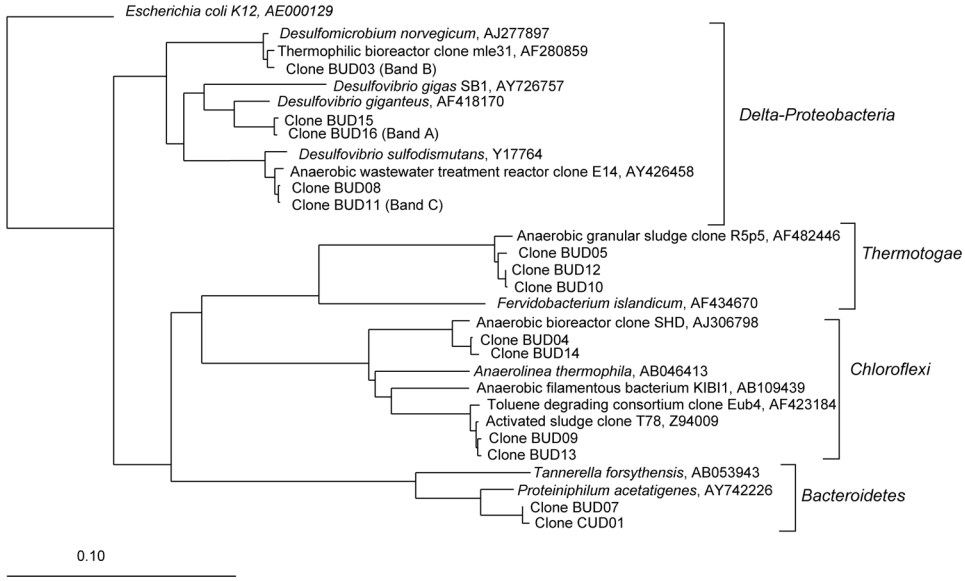

Figure 5 Phylogenetic tree of 16S rRNA gene sequences retrieved from the full-scale reactor at weeks 10, 50 and 128, and reference sequences. Accession numbers of reference sequences are indicated. The scale bar indicates the branch length that represents 10 % sequence dissimilarity.

Even though very little is known about the physiology of these organisms, a cultured representative strain UNI-1T has been shown to grow on complex substrates such as sugars and amino acids (17). This strain did not show growth on hydrogen. As the ingoing wastewater did not contain complex organic compounds, these organisms could be involved in the degradation of dead microbial cells in sludge. Okabe and co-workers (12) presented data that strongly suggested that members of the Chloroflexi preferentially utilize microbial products derived from biomass decay. Consequently, the relative abundance of these organisms in week 10 may be explained by the occurrence of the calamity in week 4. Too much zinc containing wastewater was fed to the reactor, which resulted in an unknown free zinc concentration inside the reactor. The drop in performance (Fig. 1) suggests that toxic concentrations were reached, which would have resulted in substantial cell death and lysis. Under normal conditions, due to the relatively high growth rate in the reactors (0.14 to 0.25 day^{-1}), there will be a continuous production of dead cells allowing these putative scavengers to persist in the system. Koizumi and co-workers have observed coexistence of SRB and Chloroflexi in the top sediment of a saline meromictic lake (7).

Table 1 Relative abundance and closest relatives of cloned bacterial 16S rRNA gene sequences obtained from Budel sludge (weeks 10, 50 and 128) based on sequence analysis of the 16S rRNA gene. Closest relatives were found by comparison of the sequences against those deposited in the GenBank database using BLAST. Relative abundance was calculated based on the number of similar clones found by RFLP analysis for each individual sludge sample.

Week	Clone	Genbank Accession nr	Relative Abundance	Closest relative	Genbank Accession nr	Identities
10	BUD05	DQ447166	13.4 %	Uncultured Thermotogales bacterium clone R5p5	AF482446	97 %
	BUD10	DQ447170	9.0 %	Uncultured Thermotogales bacterium clone R5p5	AF482446	96 %
	BUD03	DQ447164	6.0 %	Uncultured Desulfomicrobium bacterium mle1-31	AF280859	99 %
	BUD04	DQ447165	4.5 %	Uncultured Chloroflexi bacterium SHD-231	AJ306798	96 %
	BUD01	DQ447163	4.5 %	*Proteiniphilum acetatigenes* strain TB107	AY742226	94 %
	Unidentified clones		62.7 %			
50	BUD13	DQ447173	14.7 %	Unidentified Chloroflexi bacterium T78	Z94009	99 %
	BUD11	DQ447171	11.6 %	Uncultured Desulfovibrio bacterium clone E14	AY426458	98 %
	BUD10	DQ44717	11.6 %	Uncultured Thermotogales bacterium clone R5p5	AF482446	96 %
	BUD07	DQ447167	10.5 %	*Proteiniphilum acetatigenes* strain TB107	AY742226	95 %
	BUD12	DQ447174	9.5 %	Uncultured Thermotogales bacterium clone R5p5	AF482446	97 %
	BUD09	DQ447169	7.4 %	Unidentified Chloroflexi bacterium T78	Z94009	99 %
	BUD08	DQ447168	4.2 %	Uncultured Desulfovibrio bacterium clone E14	AY426458	98 %
	Unidentified clones		30.6 %			
128	BUD16	DQ447176	27.7 %	*Desulfovibrio giganteus*	AF418170	95%
	BUD15	DQ447175	21.3 %	*Desulfovibrio giganteus*	AF418170	95%
	BUD14	DQ447174	4.3 %	Uncultured Chloroflexi bacterium SHD-231	AJ306798	97%
	Unidentified clones		46.8 %			

They proposed a syntrophic relationship between SRB and Chloroflexi, where members of subphylum I might be initial degraders of macromolecules, providing fatty acids and hydrogen as electron donors for SRB. If this interaction also takes place in the Budel reactor, these intermediates might enable a more diverse microbial community than just hydrogenotrophs to be sustained. Kaksonen and co-workers (6) demonstrated that sulfate-reducing fluidized bed reactor communities, enriched and maintained on either ethanol or lactate treating acidic metal-containing wastewater, were composed of a diverse mixture of bacteria. Diversity and flexibility of microbial communities were suggested to enhance the robustness of the reactor under varying operational conditions, such as metal and acidity shocks. Our current study demonstrated that even a hydrogen fed sulfate-reducing bioreactor can sustain a diverse bacterial community, not limited to hydrogenotrophic SRB. This diverse microbial community and the functional redundancy within the SRB may have contributed to the reactor stability, during its prolonged period of operation**.** In general, the ability to control methanogenesis by limiting the carbon dioxide feed together with the good performance and stability of the reactor over a period of 128 weeks, demonstrate that this new technology can be used successfully at full scale to treat sulfate and metal rich wastewaters.

Acknowledgements

This research was supported by a grant from the E.E.T. program of the Dutch ministries of Economic Affairs, Education, Culture and Sciences and Housing, Spatial Planning and the Environment (EETK98028). We would like to thank W. Vermeulen from Zinifex Budel Zink for help and valuable discussion and J.E. Edwards for critically reviewing the manuscript.

References

1. **Ashelford, K. E., N. A. Chuzhanova, J. C. Fry, A. J. Jones, and A. J. Weightman.** 2005. At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. Appl. Environ. Microb. **71:**7724-7736.

2. **Badziong, W., and R. K. Thauer.** 1978. Growth yields of *Desulfovibrio vulgaris* (Marburg) growing on hydrogen plus sulfate and hydrogen plus thiosulfate as the sole energy sources. Arch. of Microbiol. **117:**209-214.

3. **Boonstra, J., J. van Lier, G. Janssen, H. Dijkman, and C. J. N. Buisman.** 1999. Biological treatment of acid mine drainage, p. 559-567. *In* R. Amils and A. Ballister (ed.), Biohydrometallurgy and Environment toward the 21st century. Process metallurgy 9B. Elsevier, Amsterdam.

4. **Großkopf, R., P. H. Janssen, and W. Liesack.** 1998. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl. Environ. Microbiol. **64:**960-969.

5. **Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace.** 1998. Novel division level of bacterial diversity in a Yellowstone hot spring. J. Bacteriol **180:**366-376.

6. **Kaksonen, A. H., J. J. Plumb, P. D. Franzmann, and J. A. Puhakka.** 2004. Simple organic electron donors support diverse sulfate-reducing communities in fluidized-bed reactors treating acidic metal- and sulfate-containing wastewater. FEMS Microbiol. Ecol. **47:**279-289.

7. **Koizumi, Y., H. Kojima, and M. Fukui.** 2004. Dominant Microbial Composition and Its Vertical Distribution in Saline Meromictic Lake Kaiike (Japan) as Revealed by Quantitative Oligonucleotide Probe Membrane Hybridization. Appl. Environ. Microbiol. **70:**4930-4940.

8. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York.

9. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucl. Acids Res. **32:**1363-1371.

10. **McGinnis, S., and T. L. Madden.** 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucl. Acids Res. **32:**20-25.

11. **Nübel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig, and H. Backhaus.** 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. **178:**5636-5643.

12. **Okabe, S., T. Kindaichi, and T. Ito.** 2005. Fate of 14C-Labeled Microbial Products Derived from Nitrifying Bacteria in Autotrophic Nitrifying Biofilms. Appl. Environ. Microbiol. **71:**3987-3994.

13. **Oude Elferink, S. J. W. H., H. A. Rinia, M. E. Bruins, W. M. de Vos, and A. J. M. Stams.** 1997. Detection and quantification of *Desulforhabdus amnigenus* in anaerobic granular sludge by dot blot hybridization and PCR amplification. J. Appl. Microbiol. **83:**102-110.

14. **Oude Elferink, S. J. W. H., A. Visser, L. W. Hulshoff Pol, and A. J. M. Stams.** 1994. Sulfate reduction in methanogenic bioreactors. FEMS Microbiology Reviews **15:**119-136.

15. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4:**406-425.

16. **Sanguinetti, C. J., E. Dias Neto, and A. J. G. Simpson.** 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques **17:**914-921.

17. **Sekiguchi, Y., T. Yamada, S. Hanada, A. Ohashi, H. Harada, and Y. Kamagata.** 2003. Anaerolinea thermophila gen. nov., sp. nov. and Caldilinea aerophila gen. nov., sp. nov., novel filamentous thermophiles that represent a previously uncultured lineage of the domain Bacteria at the subphylum level. Int J Syst Evol Microbiol **53:**1843-1851.

18. **Sorokin, Y. I.** 1966. Investigation of the structural metabolism of sulfate-reducin bacteria with C^{14}. Microbiologiya **35:**967-977.

19. **Sorokin, Y. I.** 1966. Sources of energy and carbon for biosynthesis in sulfate-reducing bacteria. Microbiologiya **35:**761-766.

20. **van Houten, B. H. G. W., K. Roest, V. A. Tzeneva, H. Dijkman, H. Smidt, and A. J. M. Stams.** 2006. Occurence of methanogenesis during start-up of a full-scale synthesis gas fed reactor treating sulfate and metal rich wastewater. Wat. Res. **40:**553-560.

21. **van Houten, R. T., L. Hulshoff Pol, and G. Lettinga.** 1994. Biological sulfate reduction using gas-lift reactors fed with hydrogen and carbon dioxide as energy and carbon source. Biotechnol. Bioeng. **44:**586-594.

22. **van Houten, R. T., and G. Lettinga.** 1994. Biological sulphate reduction with synthesis gas: microbiology and technology, p. 793-799. *In* R. H. Wijffels, R. M. Buitelaar, C. Bucke, and J. Tramper (ed.), Progres in Biotechnology, vol. Vol. 11. Elsevier, Amsterdam.

23. **Weijma, J., F. Gubbels, L. Hulshoff Pol, A. J. M. Stams, P. Lens, and G. Lettinga.** 2002. Competition for H_2 between sulfate reducers, methanogens and homoacetogens in a gas-lift reactor. Wat. Sci. Tech. **45:**75-80.

4

Isolation and description of a dominant hydrogenotrophic sulfate reducer, Desulfovibrio strain SB1, from a gas-lift reactor treating zinc and sulfate rich wastewater

Bernd H.G.W. van Houten, Roel Meulepas, Wim van Doesburg, Hauke Smidt and Alfons J.M. Stams

Summary

In this paper we describe the isolation and characterization of a dominant sulfate reducer from the sulfidogenic sludge of a full-scale gas lift reactor used for treating wastewater from a zinc smelter (Budel-Dorplein, The Netherlands). Strain SB1, a hydrogenotrophic sulfate-reducing bacterium, was found to be one of the most abundant hydrogenotrophic sulfate reducers in the Budel full-scale reactor at the time of isolation. Considering its relatively high growth rate of 1.9 - 2.8 d^{-1}, strain SB1 may have become abundant during a period of hydrogen excess after a disturbance in the reactor. Phylogenetic analysis of the 16S rRNA gene showed *Desulfovibrio gigas* DSM 1382^T to be the closest relative of strain SB1, with a sequence similarity of 98.3%. Strain SB1 has very similar physiological properties to *Desulfovibrio gigas* DSM 1382^T with regards to substrate utilization, morphology and low NaCl requirement. However, strain SB1 did not show the ability to utilize malate fermentatively unlike *Desulfovibrio gigas*. Moreover, strain SB1 can utilize glycerol as an electron donor for sulfate reduction, while *Desulfovibrio gigas* DSM 1382^T lacks this property. Based on the current data it is likely that strain SB1 is a novel strain of *Desulfovibrio gigas,* Strain SB1 has been added to the culture collection of from the Deutsche Samlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany as DSM16681.

Introduction

Sulfate and metal rich wastewaters that are low in organic carbon are produced as a result of several industrial processes, such as metal smelting, flue gas scrubbing and mining (9)(5). Sulfate-reducing bioreactors have been previously shown to be suitable systems to remediate these types of wastewater (1, 26). These bioreactors utilize the sulfidogenic activity of sulfate-reducing bacteria (SRB) to simultaneously remove sulfate and metals in the form of metal sulfides. Due to the very low solubility, metal sulfides precipitate, making them easy to remove from the wastewater. We have shown that the sulfidogenic communities of these bioreactors are dominated by heterotrophic sulfate-reducing bacteria *(26)(Chapter 3)*. In this paper we describe the isolation and characterization of a dominant sulfate reducer from the sulfidogenic sludge of a full-scale gas lift reactor used for treating wastewater from a zinc smelter (Budel-Dorplein, The Netherlands).

Methods

Origin and isolation of strain SB1

Strain SB1 was isolated from anaerobic sludge of a full-scale 500 m^3 gas lift reactor treating sulfate and zinc rich wastewater from a zinc smelter located in Budel-Dorplein, The Netherlands. This reactor was fed with hydrogen rich purified synthesis gas and operated at a temperature of 30 - 35^oC and has been previously described in more detail (26), (Chapter 3). A sludge sample (10 ml) was taken from the reactor in week 7 as previously defined (Chapter 3). The sample was diluted 10-fold and crushed as previously described (14). This crushed sludge was used to make 10-fold serial dilution series in liquid medium containing 20 mM of sulfate and 4 mM of acetate, and a H_2/CO_2 gas-phase. Unless stated otherwise, all bacteria were cultivated at 37^oC in 120-ml serum bottles with a 1.7 kPa H_2/CO_2 or N_2/CO_2 gas phase (80/20 v/v) as described previously (23). The serial dilutions were incubated at 30^oC, and the highest dilution that showed growth was selected for further isolation. A pure culture was obtained by application of the agar roll tube dilution method as described by Hungate (4). Purity was confirmed by microscopic observation, uniform colony formation and by testing for anaerobic contaminants on Wilkins-Chalgren anaerobe broth (Oxoid).

Growth experiments

Tests for utilization of carbon and energy sources were performed using the same bicarbonate buffered medium as used for isolation. Growth was established by detection of substrate consumption and product formation and increase in culture turbidity. For determination of the

optimum temperature, cultures were incubated at different temperatures ranging from 10°C to 45°C.

Analytical methods

Volatile fatty acids and sulfate were analyzed by HPLC and Ion Chromatography, and hydrogen and methane were determined by gas chromatography as previously described (23)(20). Sulfide was measured colorimetrically (25). Desulfoviridin was determined according to Postgate (17).

The G+C content of the genomic DNA was determined by HPLC at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Cells were disrupted in a French pressure cell and DNA was purified on hydroxyapatite according to Cashion et al. (2) DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase (12). The resulting deoxyribonucleotides were analyzed by HPLC (Shimadzu) using a method adapted from Tamaoka, J., and K. Komagata (24). Calibration was performed with non-methylated lambda DNA (Sigma), G+C content 49.858 mol % (12). The DNA G+C content was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) according to Mesbah et al. (12).

Nucleic acids isolation, PCR, DGGE

Total DNA of strain SB1 and *Desulfovibrio gigas* DSM 1382^T and the individual serial dilutions was extracted as described previously (15). *Desulfovibrio gigas* DSM 1382^T was obtained from the DSMZ (Braunschweig, Germany). For DGGE analysis, the partial 16S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) using primers 968-GC-f and 1401-r (13). The following thermocycling program was used: 94^{o}C for 5 min; 35 cycles of 94^{o}C for 30 s, 56^{o}C for 20 s, and 68^{o}C for 40 s; and 68^{o}C for 7 min. Amplification and size of PCR products were verified by 1 % (w/v) agarose gel electrophoresis with ethidium bromide staining, and comparing amplification products to a 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania). Denaturing gradient gel electrophoresis (DGGE) analysis was performed on 8% (w/v) polyacrylamide gels containing a denaturing gradient of 35 to 60%. 100% denaturant corresponded to 7 M urea and 40 % (v/v) formamide. Electrophoresis was performed in 0.5 x TAE buffer (20 mM Tris, 10 mM acetic acid and 0.5 mM EDTA pH 8) at 85 V and 60^{o}C for 16 hours (after 10 minutes at 200 V) using a DCode System (BioRad, Hercules, CA). Gels were silver stained as previously described (19).

Sequence determination

For sequencing of the 16S rRNA gene of strain SB1 and *Desulfovibrio gigas* DSM 1382^T DNA was PCR amplified using a *Taq* DNA polymerase kit (Life Technologies, Gaithersburg, Md.), and 7-f and 1510-r primers (8). The following thermocycling program was used: 94^{o}C for 5 min; 25 cycles of 94^{o}C for 30 s, 52^{o}C for 20 s, and 68^{o}C for 40 s; and 68^{o}C for 7 min. The size and amount of PCR

products were estimated by 1% agarose gel (wt/vol) electrophoresis and ethidium bromide staining. Amplified almost full-length 16S rRNA gene fragments were purified using a QIAquick Kit (Qiagen GmbH, Hilden, Germany) and cloned in *E. coli* JM109 by using the pGEM®-T Easy vector system (Promega, Leiden, The Netherlands). The inserts of the selected transformants were purified using the QIAprep spin miniprep kit (Qiagen GmbH, Hilden, Germany). Sequencing analysis was carried out with the Sequenase sequencing kit (Amersham, Slough, United Kingdom) using the IRD8000 labelled (MWG-Biotech, Ebersberg, Germany) sequencing primers Sp6 (5'-GATTTAGGTGACACTATAG-3'), and T7 (5'-AATACGACTCACTATAGG-3'). The sequences were automatically analysed on a LI-COR (Lincoln, NE, USA) DNA sequencer 4000L and checked manually.

Sequence analysis

A similarity search of the 16S rRNA gene sequence derived from strain SB1 against sequences deposited in publicly accessible databases were performed using the NCBI BLAST search tool http://www.ncbi.nlm.nih.gov/BLAST (11). Using the ARB software package, sequences were aligned with the FastAligner tool, followed by manual control according to secondary structures (10). A phylogenetic tree was constructed with Felsenstein correction and a filter for δ-Proteobacteria in ARB, using the neighbor joining method (*E. coli* positions 52 to 1365). The sequence of strain SB1 has been deposited in GenBank under accession number AY726757, the new sequence for *Desulfovibrio gigas* DSM 1382^T was deposited under accession number DQ447183.

Results & Discussion

Isolation

Strain SB1 was enriched from a sludge sample obtained from the Budel full-scale reactor by serial dilution in liquid medium. This sample was taken from the reactor in week 7 as defined previously (Chapter 3). Strain SB1 was obtained from the highest dilution that showed growth (10^8) indicating that it was an abundant hydrogenotrophic SRB at the time of isolation. To illustrate this further the bacterial diversity of the individual dilutions was assessed using DGGE (Fig. 1). The DGGE fingerprint of the strain SB1 is characterized by a distinct pattern of two strong and two weak bands. The 2 strong bands could be observed throughout the highest dilutions. Only one other band could be observed in the highest dilution. Microscopic observation of the highest dilution showed a predominance of strain SB1. Strain SB1 was isolated by a second application of serial dilution in liquid media, followed by a serial dilution in solid media using the agar roll-tube dilution method (4). A single colony was picked and serially diluted in liquid medium.

Morphology

Cells were vibroid to slightly spiral shaped (1 µm wide and 5-8 µm long) and appeared singly or in chains of up to 8 cells. Cells of the strain were motile in a corkscrew like motion. Strain SB1 was desulfoviridin positive and stained Gram-negative. Phase contrast microscopy showed refractive structures, and spores were never observed. On solid medium the strain produced lens-shaped colonies.

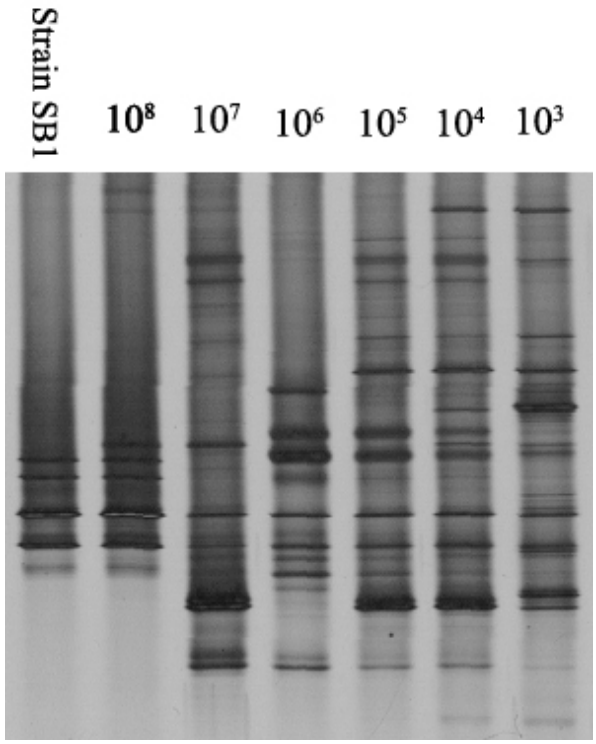

Figure 1 DGGE analysis of the bacterial diversity of the serial dilutions used to enrich strain SB1.

Physiology

The optimum temperature for growth on hydrogen and sulfate was between 37 and 40°C. No growth was observed at 45°C. Optimum pH for growth was between 7.1 and 7.5, no growth was observed below pH 6.5. Strain SB1 did not show a requirement for NaCl and growth was inhibited at NaCl concentrations higher than 12 $g \cdot l^{-1}$.

Strain SB1 used hydrogen (with 4 mM acetate as an organic carbon source), pyruvate (20 mM), lactate (40 mM), malate (20 mM), fumarate (20 mM), succinate (20 mM), ethanol (40 mM) and glycerol (20 mM) as electron donors for sulfate reduction. The organic substrates were incompletely oxidized to acetate. Acetate, propionate, butyrate, methanol, cysteine and choline (all 20 mM) did not serve as electron donors. Formate in the presence of 4 mM acetate was

converted but did not support growth. Pyruvate and fumarate (both 40 mM) could be used fermentatively. Malate and choline were not used fermentitavely. Sulfate, sulfite and thiosulfate (10 mM) were used as electron acceptors for growth on hydrogen. Nitrate (40 mM) did not serve as an electron acceptor for growth on hydrogen. The maximum growth rate for batch growth at 37°C on lactate and sulfate was 1.8 day^{-1} (T_d = 9.8 h). The maximum growth rate on hydrogen and sulfate was estimated at to be between 1.9 - 2.8 d^{-1} (T_d = 8.7 - 5.9 h).

Phylogenetic analysis

The DNA base G+C composition of strain SB1 was 62.2%. A similarity search of 16S rRNA gene sequence of strain SB1 using the NCBI Blast search tool indicated that *Desulfovibrio giganteus* DSM 4370 (GenBank accession number AF418170) was most closely related with a sequence identity of 92.0 %. However, further phylogenetic analysis showed that strain SB1 was more closely related to *Desulfovibrio gigas* DSM 1382^T. This apparent discrepancy was caused by 112 unidentified nucleotides in the sequence of *Desulfovibrio gigas* DSM 1382^T (GenBank accession number M34400.1), making accurate phylogenetic comparison impossible (21). We therefore resequenced the 16S rRNA gene of *Desulfovibrio gigas* DSM 1382^T and have submitted it to GenBank under accession number DQ447183. Alignment of the full sequences showed a sequence homology of 98.3 %. Phylogenetic analysis confirmed *Desulfovibrio gigas* DSM 1382^T to be the closest relative of strain SB1 (Fig. 2).

Sequence comparison between two separate clones, which yielded the dominant bands observed during DGGE analysis showed a base pair difference at E.coli position 1326. The lower band corresponded to a clone with a C at this position, and the higher band a clone with T.

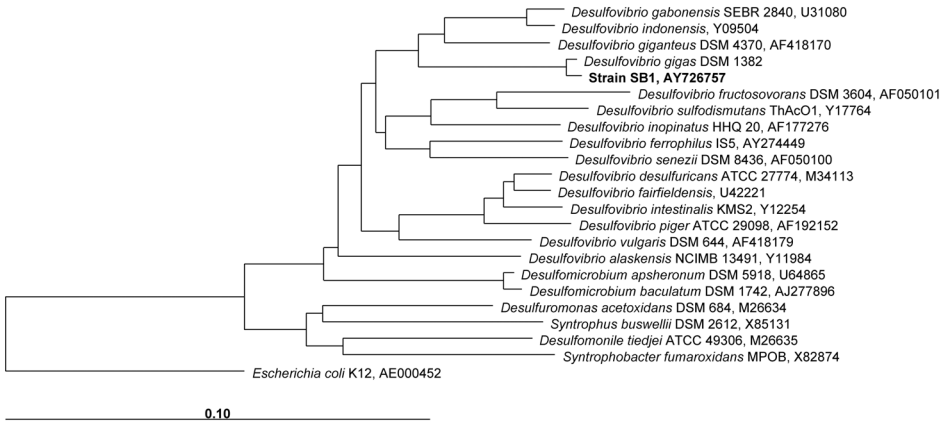

Figure 2 Phylogenetic tree based on 16S rRNA gene sequences of selected δ-Proteobacteria. GenBank accession numbers of reference sequences are indicated. *Escherichia coli* was used as an outgroup. The reference bar indicates 10% of nucleotide sequence divergence.

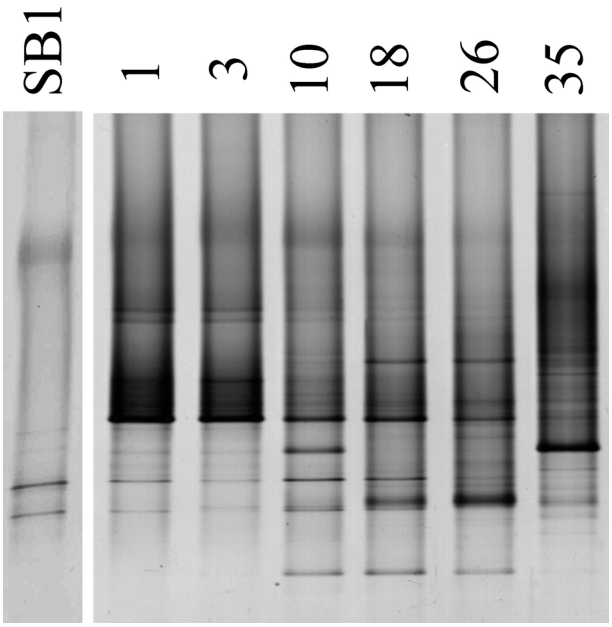

Figure 3 Analysis of bacterial community dynamics by 16S rRNA gene-targeted PCR-DGGE. Numbers indicate week of sampling.

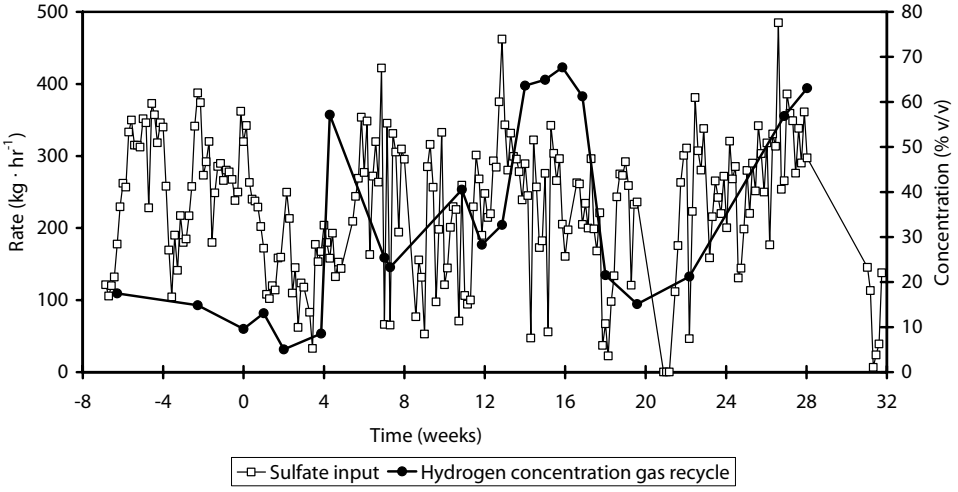

Figure 4 Averaged weekly sulfate input rate, and the hydrogen concentration in gas recycle of the Budel full-scale reactor.

Since the double band pattern was observed consistently an amplification error seems unlikely, and therefore it seems likely that strain SB1 has two different 16S rRNA operons within its genome

Ecological importance of strain SB1

It can only be speculated which conditions contributed most to the dominance of strain SB1 at the time of its isolation. The temporal changes occurring in the sludge community from which strain SB1 was isolated were followed using DGGE analysis of 16S rRNA gene fragments (Fig. 3). The distinct 2 band pattern characteristic for SB1 could be observed over a long time period (week 1–18). Its abundance in week 7 may have been linked to the rapid increase in the hydrogen concentration in the gas recycle in week 4 combined with a low sulfate conversion rate (Fig. 4). This lowered sulfate conversion rate had been caused by short exposure of the sludge to free Zn^{2+}. As a result the sludge was exposed to a period of hydrogen excess. Considering its relatively high growth rate of 1.9 - 2.8 d^{-1}on hydrogen compared to other sulfate-reducing bacteria (16) strain SB1 may have been able to become abundant during this period of hydrogen excess. The presence of this fast growing SRB in the sludge community may explain the fast recovery of the reactor after the calamity.

Summary

Strain SB1, a hydrogenotrophic sulfate-reducing bacterium was found to be one of the most abundant hydrogenotrophic sulfate reducers in the Budel full-scale reactor at the time of its isolation. Considering its relatively high growth rate of 1.9 - 2.8 d^{-1}, strain SB1 may have become abundant during a period of hydrogen excess after a disturbance in the reactor.

Strain SB1 showed typical physiological characteristics of the genus *Desulfovibrio*. It oxidizes organic substrates incompletely to acetate, stains gram-negative and is desulfoviridin positive. In addition, it has very similar physiological properties as *Desulfovibrio gigas* DSM 1382^T with regards to substrate utilization, morphology and low NaCl requirement. However, strain SB1 did not show the ability to utilize malate fermentatively unlike *Desulfovibrio gigas* (7). Moreover, strain SB1 can utilize glycerol as electron donor for sulfate reduction, while *Desulfovibrio gigas* DSM 1382^T lacks this property (3, 6).

Phylogenetic analysis of the 16S rRNA gene showed *Desulfovibrio gigas* DSM 1382^T to be the closest relative of strain SB1 (Fig. 2) with a sequence similarity of 98.3%. The DNA base G+C composition of strain SB1 was 62.2% is comparable to the 60.2% found for *Desulfovibrio gigas* DSM 1382^T, and falls within the 5% that is normally observed within the same species (18). Based on the current data it is likely that strain SB1 is a novel strain of *Desulfovibrio gigas,* However, other analysis analysis such as DNA-DNA reassociation analysis should be performed to resolve this (22).

Strain SB1 has been added to the culture collection of the Deutsche Samlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany as DSM16681.

References

1. **Boonstra, J., J. van Lier, G. Janssen, H. Dijkman, and C. J. N. Buisman.** 1999. Biological treatment of acid mine drainage, p. 559-567. *In* R. Amils and A. Ballister (ed.), Biohydrometallurgy and Environment toward the 21st century. Process metallurgy 9B. Elsevier, Amsterdam.
2. **Cashion, P., M. A. Holder-Franklin, J. McCully, and M. Franklin.** 1977. A rapid method for the base ratio determination of bacterial DNA. Analytical Biochemistry. **81:**461-466.
3. **Esnault, G., P. Caumette, and J.-L. Garcia.** 1988. Characterization of *Desulfovibrio giganteus* sp. nov., a sulfate reducing bacterium isolated from a brackish coastal lagoon. Systematic and Applied Microbiology **10:**147-151.
4. **Hungate, R. E.** 1969. A roll-tube method for the cultivation of strict anaerobes, p. 117 - 132. *In* J. Norris and D. W. Ribbons (ed.), Methods in Microbiology, vol. 3B. Academic Press, London.
5. **Johnson, D. B.** 2000. Biological removal of sulfurous compounds from inorganic wastewaters, p. 175-206. *In* P. Lens and L. Hulshoff Pol (ed.), Environmental Technologies. International Association on Water Quality, London.
6. **Kremer, D. R., and T. A. Hansen.** 1987. Glycerol and dihydroxyacetone dissimilation in *Desulfovibrio* strains. Archives of Microbiology **147:**249-256.
7. **Kremer, D. R., H. E. Nienhuis-Kuiper, C. J. Timmer, and T. A. Hansen.** 1988. Catabolism of malate and related dicarboxylic acids in various *Desulfovibrio* strains and the involvement of an oxygen-labile NADPH dehydrogenase. Archives of Microbiology **151:**34-39.
8. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York.
9. **Lens, P. N. L., A. Visser, A. J. H. Janssen, L. Hulshoff Pol, and G. Lettinga.** 1998. Biotechnological treatment of sulfate-rich wastewaters. Critical Reviews in Environmental Science and Technology **28:**41 - 88.
10. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Research **32:**1363-1371.
11. **McGinnis, S., and T. L. Madden.** 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Research **32:**20-25.
12. **Mesbah, M., U. Premachandran, and W. B. Whitman.** 1989. Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. International Journal of Systematic Bacteriology **39:**159–167.
13. **Nübel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig, and H. Backhaus.** 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. **178:**5636-5643.

14. **Oude Elferink, S. J. W. H., R. N. Maas, H. J. M. Harmsen, and A. J. M. Stams.** 1995. *Desulforhabdus amnigenus* gen. nov. sp. nov. a sulfate reducer isolated from anaerobic granular sludge. Archives of Microbiology **164:**119-124.
15. **Oude Elferink, S. J. W. H., H. A. Rinia, M. E. Bruins, W. M. de Vos, and A. J. M. Stams.** 1997. Detection and quantification of *Desulforhabdus amnigenus* in anaerobic granular sludge by dot blot hybridization and PCR amplification. Journal of Applied Microbiology. **83:**102-110.
16. **Oude Elferink, S. J. W. H., A. Visser, L. W. Hulshoff Pol, and A. J. M. Stams.** 1994. Sulfate reduction in methanogenic bioreactors. FEMS Microbiology Reviews **15:**119-136.
17. **Postgate, J. R.** 1984. The Sulfate-reducing Bacteria, 2nd edn. ed. Cambridge University Press, Cambridge, U.K.
18. **Rossello-Mora, R., and R. Amann.** 2001. The species concept for prokaryotes. FEMS Microbiology Reviews **25:**39-67.
19. **Sanguinetti, C. J., E. Dias Neto, and A. J. G. Simpson.** 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques **17:**914-921.
20. **Scholten, J. C. M., and A. J. M. Stams.** 1995. The effect of sulfate and nitrate on methane formation in a freshwater sediment. Antonie van Leeuwenhoek **68:**309-315.
21. **Stackebrandt, E., W. Frederiksen, G. M. Garrity, P. Grimont, P. Kampfer, M. Maiden, X. Nesme, R. Rossello-Mora, J. Swings, H. G. Truper, L. Vauterin, A. C. Ward, and W. B. Whitman.** 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. International Journal of Systematic and Evolutionary Microbiology **52:**1043-1047.
22. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. International Journal of Systematic Bacteriology **44:**846-849.
23. **Stams, A. J. M., J. B. Van Dijk, C. Dijkema, and C. M. Plugge.** 1993. Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Applied and Environmental Microbiology **59:**1114-1119.
24. **Tamaoka, J., and K. Komagata.** 1984. Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol Lett **25:**125–128.
25. **Trüper, H. G., and H. G. Schlegel.** 1964. Sulphur metabolism in Thiorhodaceae 1. Quantitative measurements on growing cells of *Chromatium okenii*. Antonie van Leeuwenhoek **30:**225-238.
26. **van Houten, B. H. G. W., K. Roest, V. A. Tzeneva, H. Dijkman, H. Smidt, and A. J. M. Stams.** 2006. Occurence of methanogenesis during start-up of a full-scale synthesis gas fed reactor treating sulfate and metal rich wastewater. Water Research **40:**553-560.

5

Coexistence of homoacetogens and heterotrophic sulfate-reducing bacteria in a synthesis gas fed lab-scale reactor

Bernd H.G.W. van Houten , Wim van Doesburg, Hauke Smidt and Alfons J.M. Stams

To be submitted

Abstract

Heterotrophic sulphate-reducing bacteria (SRB), methanogenic archaea and homoacetogens were previously detected in synthesis gas-fed gas lift reactors treating sulfate and metal rich wastewater (16). Addition of hydrogen and carbon dioxide as the sole substrates without addition of an organic carbon source would result in dependence of heterotrophic SRB on homoacetogens. Based on the order of competitiveness autotrophic methanogens should be able to outcompete heterotrophic SRB for hydrogen if organic carbon sources are not available (3, 10). Weijma et al. (17) have studied this situation using lab scale gas-lift reactors. Their results showed that methanogenesis was suppressed, while small amounts of acetate were produced. Hydrogen was more readily consumed by homoacetogens than by methanogens. A cooperative interaction between SRB and homoacetogens was proposed.

We have studied the microbial community of both reactors used by Weijma et al. (17), using restriction fragment length polymorphism (RFLP) and sequence analysis of the 16S rRNA gene. Temporal community changes were assessed using 16S rRNA gene based denaturing gradient gel electrophoresis (DGGE). The bacterial communities of both reactors were found to dominated by a limited number of microorganisms belonging to the genera *Desulfovibrio*, *Acetobacterium* and *Desulfomicrobium*. This confirms the earlier conclusions by Weijma et al. (17). We speculate both trophic groups were able to coexist because they were not limited by the same substrate. Homoacetogens were hydrogen limited, while heterotrophic SRB were acetate limited**.** The archaeal diversity appears to have been limited to a single species related to *Methanobacterium formicicum* strain Fcam (99.9%). We hypothesize that the growth of the methanogens may have been acetate limited, resulting in a reduced ability to compete for hydrogen.

Keywords

Sulfate reduction; Methanogenesis; Homoacetogenesis; Hydrogen; Acetate; Competition;

Introduction

Hydrogen threshold concentrations have been used to explain the competition for hydrogen by microorganisms in sediments, and also anaerobic bioreactors (3, 10). Reported values of hydrogen threshold concentrations indicate a competitive order of heterotrophic sulfate-reducing bacteria (SRB) > methanogenic archaea > homoacetogenic bacteria. However, hydrogen threshold concentration cannot always predict the outcome of the competition for hydrogen. We have reported previously on the occurrence of methanogenesis during start-up of a full-scale THIOPAQ® reactor at the Budel Zink zinc Smelter (Budel-Dorplein, The Netherlands) (16). Because of the relatively short sludge retention time (SRT) of 4 to 7 days, hydrogen threshold concentrations were not reached. The persistence of methanogenesis was explained by the similar Monod kinetics of SRB and methanogens. Homoacetogenic avtivity was also observed initially, but was suppressed after rates of sulfate reduction and methanogenesis had increased. Homoacetogens were probably unable to compete effectively for hydrogen, as they show relatively poor growth kinetics on hydrogen compared to SRB and methanogens (3, 11, 15). However, despite this, they were not completely washed out of the reactor within a period of 20 weeks.

Omission of an organic carbon source to the reactor would cause that heterotrophic SRB to become dependent on homoacetogens for the production of an organic carbon source. Under these conditions autotrophic methanogens should be able to compete effectively with heterotrophic SRB for hydrogen as their growth is dependent on the growth of homoacetogens. Weijma et al. (17) have studied this situation using gas-lift reactors inoculated with sludge obtained from the full-scale THIOPAQ® reactor at the Budel Zink zinc smelter (Budel-Dorplein, The Netherlands). Their results showed that methanogenesis was suppressed, while small amounts of acetate were produced. In contrast with the order of competitiveness, hydrogen was more readily consumed by homoacetogens than by methanogens. A cooperative interaction between SRB and homoacetogens was proposed. Furthermore it was noted that the competition between methanogens and SRB was not determined by growth and substrate-conversion kinetics. It was speculated that the better retention of sulfidogenic biomass in the reactor, compared to methanogenic biomass, played a role in the competition. However, a follow up study showed that methanogens were outcompeted at both short and long sludge retention times (2.6 and 19.8 days) indicating biomass retention may not play a role (5).

To verify if heterotrophic SRB and homoacetogens were both present we have studied the microbial community of both reactors used in the earlier study by Weijma et al. (17). The predominant microorganisms were identified using culture-independent approaches. Relative abundance and the identity of dominant microbial populations in the sludge were determined using restriction fragment length polymorphism (RFLP) and sequence analysis of the 16S rRNA

gene. Temporal community changes were assessed using 16S rRNA gene based denaturing gradient gel electrophoresis (DGGE).

Materials and methods

Nucleic acids isolation and PCR

Sludge for nucleic acid extraction was taken from both reactors (R+ and R-) used by Weijma et al. (17) at days -30, 10, -4, +5, and +35 as defined before. The operation and medium composition of reactor R+ and R- were identical, except the medium of R+ contained 200 mg/L sodium acetate. Samples (6 ml) were fixed with 9.5 ml ethanol and 0.5 ml 0.8 M sodium chloride prior to storage at -25 °C. Total DNA was extracted as previously described (Chapter 3). 16S rRNA genes were PCR amplified from the sludge DNA extract using a Taq DNA polymerase kit (Life Technologies, Gaithersburg, Md.). All primers were purchased from MWG-Biotech (Ebersberg, Germany). Bacterial 16S rRNA genes were selectively amplified for clone library construction and sequence analysis using 7-f and 1510-r primers (7) with the following thermocycler program: 94 °C for 5 min; 25 cycles of 94 °C for 30 s, 52 °C for 20 s, and 68 °C for 40 s; and 68 °C for 7 min. For DGGE, partial bacterial 16S rRNA gene fragments were amplified using 968-GC-f and 1401-r primers (9) with the same thermocycler program, but an increased number of 35 cycles and an annealing temperature of 56 °C was used. For Archaea, 16S rRNA genes were selectively amplified for sequence analysis using primers A109-f (16), and 1510-r (7). For archaeal DGGE analyses, A109(T)-f (original (6), 3rd bp changed into T) and 515-GC-r (7) were used for amplification. Both reactions were performed with the following thermocycler program: 94 °C for 5 min; 35 cycles of 94 °C for 30 s; 52 °C for 40 s, 68 °C for 1 min , and 68 °C for 7 min. Amplification and size of PCR products were verified by 1 % (w/v) agarose gel electrophoresis with ethidium bromide staining, and comparison amplification products to a 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania).

DGGE, cloning and RFLP analysis

DGGE analysis was performed on 8 % (w/v) polyacrylamide gels containing a denaturing gradient of 35 to 60 % for bacterial amplicons, and 30 to 45% for archaeal amplicons. 100% denaturant corresponded to 7 M urea and 40 % (v/v) formamide. Electrophoresis was performed in 0.5 x TAE buffer (20 mM Tris, 10 mM acetic acid and 0.5 mM EDTA pH 8) at 85 V and 60°C for 16 hours (after 10 minutes at 200 V) using a DCode System (BioRad, Hercules, CA). Gels were silver stained as described previously (13).

For the generation of bacterial clone libraries, amplified full-length 16S rRNA genes were purified using a QIAquick Kit (Qiagen GmbH, Hilden, Germany) and cloned in *E. coli* JM109 by using the pGEM®-T Easy vector system (Promega, Leiden, The Netherlands). Clone libraries (48 clones for weeks -30 and week 35) of reactor R+ were screened for number and relative abundance of unique

clone types by Restriction Fragment Length Polymorphism (RFLP) analysis (digestion with *Msp*I, *Cfo*I and *Alu*I, Invitrogen) and DGGE. Plasmids of selected transformants were then purified using the QIAprep spin miniprep kit (Qiagen GmbH, Hilden, Germany). Only abundant operational taxonomic units OTUs with unique RFLP and DGGE migration patterns, and represented by 2 or more clones, were sequenced.

Sequence analysis

Sequence analysis was carried out with the Sequenase sequencing kit (Amersham, Slough, United Kingdom). 16S rRNA gene sequences from the sludge clones were searched against the NCBI database using BLAST (http://www.ncbi.nlm.nih.gov/blast/) (8). Sequences were checked for chimera using the PINTAIL software (2).Sequences generated from this work are deposited at GenBank under accession numbers DQ447177-DQ447182.

Results and discussion

Weijma and coworkers (17) studied the effect of acetate addition on the competition between SRB, methanogens and homoacetogens using two hydrogen fed lab scale reactors (R+ and R-). To reactor R+ 200 mg/l acetate was added as an additional organic carbon source, while reactor R- did not receive any additional substrate. The reactors were initially fed with a low amount of sulfate to obtain both sulfidogenic and methanogenic activity. After approximately 30 days, the sulfate load for both reactors was increased, yielding a molar ratio of H_2 to SO_4^{2-} of 2.5 to 1. This ratio of hydrogen to sulfate is not sufficient for a complete sulfate reduction. The day of sulfate load increase was defined as day 0. Weijma et al. (17) showed that methanogens were outcompeted by SRB under non sulfate-limited conditions, irrespective of whether acetate was added as an additional organic carbon source. Reactor R- showed a net production of acetate during the whole experiment, indicating homoacetogenic activity.

The bacterial communities of both reactors were compared by DGGE profiling (Fig. 1), and 16S rRNA gene libraries. Libraries were constructed for the bacterial community with the start-up sludge, and the sludge of reactor R+ for day +35 (Table1). DGGE analyses showed a very low microbial diversity for both reactors with the predominance of a very limited number of amplicons. Bacterial amplicons observed on DGGE for both reactors varied from four to seven at different time points. This predominance of a limited number of bandss was previously observed in both pilot and full-scale synthesis gas fed reactors and appears to be typical for these types of reactors (16) (Chapter 3). One predominant band could be observed throughout the whole experiment in reactor R-. The migration pattern of this band was identical to clones 1B8 and A25B2, and a high relative clone library abundance (Table 1) confirmed the predominance as observed by DGGE analysis (Fig. 1). Sequence analysis of clone A25B2 showed that this

microorganisms belonged to the genus *Acetobacterium*, a genus well known for homoacetogenic growth (4). This species was also enriched from reactor R- and displayed homoacetogenic growth (Chapter 6). Based on the fact that this species was observed throughout the experiment and that it had a high relative abundance in the clone libraries, it is likely that this bacterium was responsible for the acetate production observed.

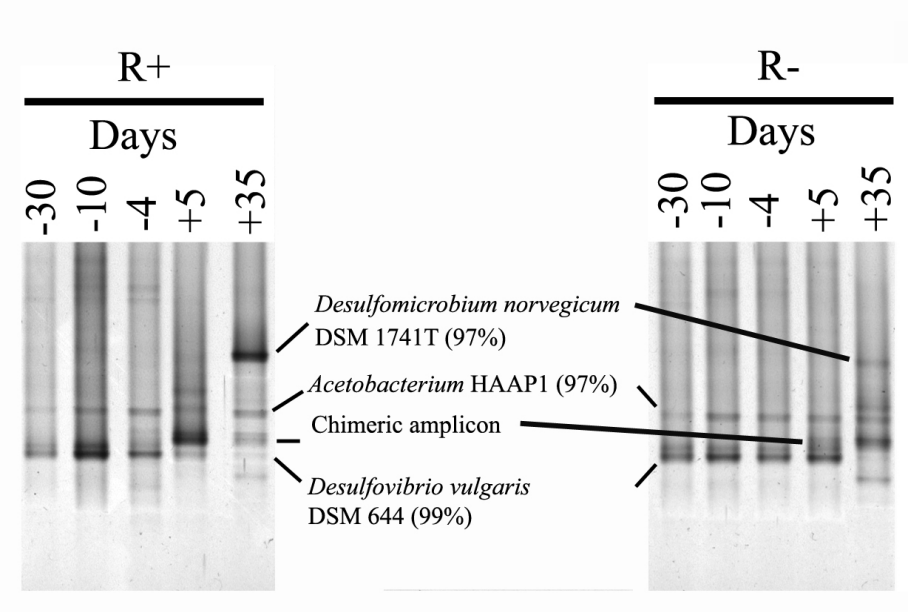

Figure 1 Analysis of bacterial community dynamics by 16S rRNA gene-targeted PCR-DGGE. Numbers indicate week of sampling as defined by Weijma et al. (17). Values in parentheses are sequence similarities of clones with an identical migration pattern.

The most abundant clone from the start-up sludge, clone 1A2, displayed a very high sequence similarity to the heterotrophic sulfate-reducing bacterium *Desulfovibrio vulgaris*. Based on DGGE this bacterium remained abundant in reactor R- and R+ until at least day +5. On day +35 the band associated with this clone was hardly visible in either reactor.This low abundance is also reflected by the fact that it was not among the abundant micro-organisms in the clone library of day +35 for reactor R+. A clone, which migrated to the same position as a predominant band in the DGGE fingerprints of both reactors after day 0, was identified as a chimera using the Pintail sequence analysis software (2).This chimeric amplicon consisted of 2 fragments, one similar to 1A2 and one similar to A25B2. Clone A25B1 which belonged to the genus *Desulfomicrobium* was found to be predominant based on DGGE and clone library abundance in R+. The genus *Desulfomicrobium* consists of heterotrophic SRB. A band with an identical migration pattern was also observed in reactor R-.

Table 1 Relative abundance and closest relatives of the cloned bacterial 16S rRNA sequences obtained from the start-up sludge and sludge from reactor R+ on day +35. Closest relatives were found by comparison of the sequences against those deposited in the GenBank database using BLAST. Relative abundance was calculated based on the number of similar clones found by RFLP analysis within each individual sludge sample clone library. For each sample, a total number of 48 clones was analyzed. Unidentified clones had a relative abundance of <2% (i.e. RFLP type with only 1 clone), and were not further subjected to sequence analysis.

			BLAST Results			
Clone Name	Accession number	Relative Abundance	Closest Hit	Identities	Closest Cultured Hit	Identities
		%		(%)		(%)
Day -30						
1A2	DQ447177	35.4	*Desulfovibrio vulgaris* DSM 644	99		
1B8	DQ447180	8.3	*Acetobacterium* sp. HAAP-1	98		
1B1	-	4.2	Uncultured bacterium mle1-2	99	*Bacteroides merdae*	93
1C5	DQ447181	4.2	Uncultured bacterium IIIB-28	99	*Cytophagales* str. QSSC5-1	88
1A12	DQ447178	2.1	Uncultured bacterium MTCE-T2	97	*Soehngenia saccharolytica*	94
Unidentified		41.7				
Day +35 R+						
A25B1	DQ447179	58.3	*Desulfomicrobium norvegicum* DSM 1741T	97		
A25B2	DQ447180	18.8	*Acetobacterium* sp. HAAP-1	98		
A25B8	DQ447181	4.2	Uncultured bacterium IIIB-28	99	*Cytophagales* str. QSSC5-1	88
Unidentified		18.8				

The relative abundance of heterotrophic SRB and homoacetogenic species agrees with the earlier conclusions of Weijma et al. (17) that a community of heterotrophic SRB and homoacetogenic microorganisms was responsible for the observed sulfate reduction and acetate production.
In addition to the analysis of bacterial populations, we also compared archaeal communities by DGGE fingerprinting (Fig. 3). Only two archaeal bands were observed for the two reactors, and both showed very similar migration patterns. The archaeal diversity remained limited to these two amplicons in both reactors during the experiment.

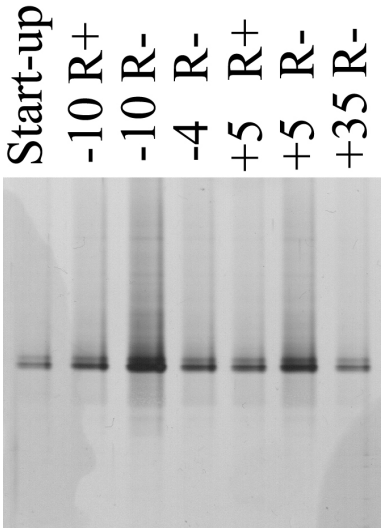

Figure 3 Analysis of archaeal community dynamics by 16S rRNA gene-targeted PCR-DGGE. Numbers indicate week of sampling as defined by Weijma et al. (17).

Archaeal16S rRNA gene libraries were constructed for reactor R- and only two different clones were found. These clones showed nearly identical sequences. Clone 4F11 (Genbank accession number DQ447182) displayed a sequence homology of 99 % to *Methanobacterium formicicum* strain Fcam, and its amplicon showed an identical DGGE migration pattern to the dominant band in the community profile. These results indicate that the archaeal diversity may have been limited to a single species and that methanogens were not completely washed out of either reactor by day +35. The very low diversity observed, or predominance of a single species, will have affected the ability of the methanogenic community to compete with other trophic groups under the changing conditions applied to the bioreactors. The seed sludge had been pre-cultivated under sulfate-limiting conditions. These conditions of hydrogen excess may have selected for the observed species to become predominant. As a consequence, the fast growing methanogens selected at these hydrogen excess conditions are probably poor competitors at low hydrogen

concentrations. This may explain the fact that methanogenesis was suppressed under sulfate excess conditions after day 0.

The results of Weijma et al. (17) showed that both the sulfate and the methanogenic activity did not appear to be hydrogen limited towards the end of the experiment; hydrogen therefore did not appear to be the growth limiting substrate. Towards the end of their experiments the hydrogen feed rate was increased to investigate whether H_2 was the growth limiting substrate (reactor R+ at day 28 and reactor R- at day 23). The SRB and methanogens did not appear to be limited by hydrogen. However, following the hydrogen feed rate increase on day 23 the acetate production rate increased in reactor R-. The homoacetogenic activity in R- thus seems to be hydrogen limited. This also illustrates that hydrogen mass transfer did not limit the growth of SRB and methanogens. For reactor R+ however, it is very speculative to attribute the increase of the acetate production rate solely to the increase in the hydrogen feed rate, as on day 32 also acetate addition to this reactor was stopped. As neither heterotrophic SRB nor methanogens were hydrogen limited, another substrate must have been limiting their growth.

The fact that homoacetogenic activity was hydrogen limited may have been central to the competition between SRB and methanogens. For the heterotrophic SRB the growth limiting substrate may have been acetate as expected, because they require an organic carbon source for growth. However, this may have also been the case for methanogens. Though methanogens are known for autotrophic growth on H_2 and CO_2, they display very poor growth under these conditions, and are normally cultured on relatively organic rich media that contain acetate, yeast extract and other complex organic carbon sources (18). Growth kinetic parameters of methanogens on H_2 and CO_2 have so far only been determined using media relatively rich in organic carbon (1, 12, 14). Under autotrophic conditions these growth kinetic parameters may very well be different, resulting in a reduced ability to compete for hydrogen with homoacetogens. Methanogens may have been faced with a dual limitation. If both heterotrophic SRB and methanogens were acetate limited they might have competed for this limiting substrate.

Conclusions

The bacterial communities of both reactors used in the study by Weijma et al (17) were dominated by a limited number of species, belonging to the genera *Desulfovibrio*, *Acetobacterium* and *Desulfomicrobium*. The relative abundance of heterotrophic SRB and homoacetogenic species agrees with the earlier conclusions by Weijma et al. (17) that a community of heterotrophic SRB and homoacetogenic bacteria were responsible for the observed sulfate reduction and acetate production. Both trophic groups were able to coexist because they were not limited by the same substrate. Homoacetogens were hydrogen limited, while heterotrophic SRB were acetate limited.

The archaeal diversity appears have been limited to a single species (99% sequence identity to *Methanobacterium formicicum* strain Fcam). DGGE analyses showed that methanogens were not washed out of either reactors by day +35. As an alternative to the selective wash-out of methanogenic sludge as suggested by Weijma et al. (17) we propose a different explanation for the suppression of methanogenesis. We hypothesize that the growth of the methanogens may have been acetate limited, resulting in a reduced ability to compete for hydrogen.

Acknowledgements

This research was supported by a grant from the E.E.T. program of the Dutch ministries of Economic Affairs, Education, Culture and Sciences and Housing, Spatial Planning and the Environment (EETK98028). We would like to thank Jan Weijma and Look Hulshof Pol for valuable discussion during the project and for providing access to their unpublished data.

References

1. **Archer, D. B., and G. E. Powell.** 1985. Dependence of the specific growth rate of methanogenic mutualistic cocultures on the methanogen. Archives of Microbiology **141:**133-137.

2. **Ashelford, K. E., N. A. Chuzhanova, J. C. Fry, A. J. Jones, and A. J. Weightman.** 2005. At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. Applied and Environmental Microbiology **71:**7724-7736.

3. **Cord-Ruwisch, R., H. J. Seitz, and R. Conrad.** 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Archives of Microbiology **149:**350-357.

4. **Diekert, G.** 1991. The Acetogenic Bacteria, p. 517-533. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The Prokaryotes, 2nd edn. ed, vol. Vol. 1. Springer-Verlag, New York.

5. **Esposito, G., J. Weijma, F. Pirozzi, and P. N. L. Lens.** 2003. Effect of the sludge retention time on H_2 utilization in sulphate reducing gas-lift reactors. Process Biochemistry **39:**491-498.

6. **Großkopf, R., P. H. Janssen, and W. Liesack.** 1998. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Applied and Environmental Microbiology **64:**960-969.

7. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York.

8. **McGinnis, S., and T. L. Madden.** 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Research **32:**20-25.

9. **Nübel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig, and H. Backhaus.** 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. Journal of. Bacteriology **178:**5636-5643.

10. **Oude Elferink, S. J. W. H., A. Visser, L. W. Hulshoff Pol, and A. J. M. Stams.** 1994. Sulfate reduction in methanogenic bioreactors. FEMS Microbiology Reviews **15:**119-136.

11. **Peters, V., P. H. Janssen, and R. Conrad.** 1998. Efficiency of hydrogen utilization during unitrophic and mixotrophic growth of *Acetobacterium woodii* on hydrogen and lactate in the chemostat. FEMS Microbiology Ecology **26:**317-324.

12. **Robinson, J. A., and J. M. Tiedje.** 1984. Competition for hydrogen between sulfate-reducing and methanogenic bacteria for hydrogen under resting and growing conditions. Archives of Microbiology **137:**26-32.

13. **Sanguinetti, C. J., E. Dias Neto, and A. J. G. Simpson.** 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques **17:**914-921.

14. **Shauer, N. L., and J. G. Ferry.** 1980. Metabolism of formate in *Methanobacterium formicicum*. Journal of Bacteriology **142:**800-807.

15. **Stams, A. J. M.** 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie van Leeuwenhoek **66:**271-294.

16. **van Houten, B. H. G. W., K. Roest, V. A. Tzeneva, H. Dijkman, H. Smidt, and A. J. M. Stams.** 2006. Occurence of methanogenesis during start-up of a full-scale synthesis gas fed reactor treating sulfate and metal rich wastewater. Water Research **40:**553-560.

17. **Weijma, J., F. Gubbels, L. Hulshoff Pol, A. J. M. Stams, P. Lens, and G. Lettinga.** 2002. Competition for H_2 between sulfate reducers, methanogens and homoacetogens in a gas-lift reactor. Wat. Sci. Tech. **45:**75-80.

18. **Whitman, W. B., T. L. Bowen, and D. R. Boone.** 1991. The Methanogenic Bacteria, p. 719-767. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The Prokaryotes, 2nd edn. ed, vol. Vol. 1. Springer-Verlag, New York.

6

The effect of acetate on the competition for H_2 between methanogens, homoacetogens and sulfate-reducing bacteria

Bernd H.G.W. van Houten, Hauke Smidt and Alfons J.M. Stams

Abstract

Heterotrophic sulfate-reducing bacteria SRB and homoacetogens were able to coexist in a lab-scale gas-lift reactor fed with hydrogen and carbon dioxide as the sole substrates. This consortium was able to compete effectively with active methanogens for hydrogen. It was hypothisized both heterotrophic SRB and homoacetogens were able to coexist because homoacetogens were hydrogen limited, while heterotrophic SRB were acetate limited. Furthermore, also methanogens may have been acetate limited.

To test this, three model organisms were co-cultured in a completely stirred tank reactor (CSTR) fed with H_2/CO_2 and SO_4^{2-} as the sole substrates. A stable co-culture of the homoacetogen *Acetobacterium* strain HM1 and the heterotrophic sulfate reducer *Desulfovibrio gigas* strain SB1 was established. This consortium was able to outcompete *Methanobacterium formicicum* JF-1. Addition of an organic carbon source resulted in the washout of the homoacetogen and an increased number of the sulfate reducer, illustrating that the co-culture between the homoacetogen and the heterotrophic sulfate reducer was possible because growth of the heterotrophic SRB was carbon limited.

Batch experiments conducted with *M. formicicum* strain JF-1 showed that the maximum specific growth rate on hydrogen was lower without acetate (0.655 day^{-1}, SD = 0.167 day^{-1}) than with acetate (0.973 day^{-1}, SD = 0.273 day^{-1}). A simple model based on Monod kinetics showed that *Acetobacterium woodii* would be able to outcompete *M. formicicum* strain JF-1 under the conditions applied in the CSTR experiment and the earlier experiments of Weijma et al. (15). Even though homoacetogens are reported to have a high hydrogen threshold and a low μ_{max} they posses a high affinity (K_s), which gives them a kinetic advantage over methanogens at intermediate growth rates (0.15 - 0.50 day^{-1}) at 30°C in environments with low or no organic carbon sources.

Keywords

Sulfate reduction; homoacetogenesis; methanogenesis Hydrogen; acetate; Competition;

Introduction

Heterotrophic sulfate-reducing bacteria (SRB), methanogenic archaea and homoacetogens were detected in synthesis gas-fed gas lift reactors treating sulphate and metal rich wastewater (14)(Chapter 3). Addition of hydrogen and carbon dioxide as the sole substrates without addition of an organic carbon source would result in dependence of heterotrophic SRB on homoacetogens for an organic carbon source. Based on the order of competitiveness autotrophic methanogens should be able to outcompete heterotrophic SRB for hydrogen if organic carbon sources are not available (4, 7). Weijma et al. (15) have studied this situation using lab-scale gas-lift reactors inoculated with sludge obtained from the full-scale THIOPAQ® reactor at the Budel Zink zinc smelter (Budeldorplein, The Netherlands). Methanogenesis was suppressed, while small amounts of acetate were produced. In contrast with the order of competitiveness expected, hydrogen appeared to be more readily consumed by homoacetogens than by methanogens.

The bacterial communities of both reactors used in the study of Weijma et al (15) were further investigated (chapter 5). The communities of both reactors were dominated by a limited number of species belonging to the genera *Desulfovibrio*, *Acetobacterium* and *Desulfomicrobium*. This relative abundance of heterotrophic SRB and homoacetogenic species confirmed the earlier conclusions by Weijma et al. (15) that a community of heterotrophic SRB and homoacetogenic microorganisms were responsible for the observed sulfate reduction and acetate production. Based on these findings it was hypothesized that both trophic groups were able to coexist because they were not limited by the same substrate (Chapter 5): homoacetogens were thought to be growth-limited by hydrogen, and heterotrophic SRB by acetate. 16S ribosomal RNA gene-targeted denaturing gradient gel electrophoresis (DGGE) analyses showed that, even though methanogenic activity was suppressed, methanogens had not been completely washed out of either reactor by the end of the experiment performed by Weijma et al. (15). Sequence analyses showed that the archaeal diversity was limited to a single species with 99% sequence identity to *Methanobacterium formicicum* strain Fcam. It was speculated that the better retention of sulfidogenic biomass in the reactor, compared to methanogenic biomass, played a role in the competition (15). However, a follow up study showed that methanogens were outcompeted at both short and long sludge retention times (2.6 and 19.8 days) indicating biomass retention may not play a role (5).

As an alternative to the selective washout of methanogenic archaea it was hypothesized that the growth of the methanogens may have been acetate limited resulting in a reduced ability to compete for hydrogen. (Chapter 5). To test this hypothesis we studied a more defined co-culture of a homoacetogen, a heterotrophic sulfate-reducer and a methanogen in a continuous system without biomass retention. To confirm our findings, we also determined the effect of acetate addition on the maximum growth rate (μ_{max}) of *Methanobacterium formicum* species.

Materials and methods

Cultures

Three model microorganisms representing the three trophic groups were used: the methanogenic archaeon *Methanobacterium formicum* strain JF-1 (DSMZ 2639), the heterotrophic sulfate reducer *Desulfovibrio gigas* strain SB1 (DSMZ 16681) and a highly enriched culture of a homoacetogenic *Acetobacterium* species designated HM1 (97% sequence identity to *Acetobacterium woodii*). *Desulfovibrio gigas* strain SB1 (DSMZ 16681) was previously isolated (Chapter 4) from the full-scale THIOPAQ reactor at the Budel zinc smelter (Budeldorplein, The Netherlands). The homoacetogenic culture was obtained from reactor R- studied by Weijma et al. (15) by serial dilution of crushed sludge in liquid medium followed by enrichment on solid medium using the roll tube technique.

Cultivation. For cultivation a bicarbonate buffered basal medium was used for all cultures (13). *Methanobacterium formicum* JF-1 was routinely cultured in 120-mL serum vials containing 50 ml of bicarbonate buffered medium with a H_2/CO_2 (4:1) gas phase of 1.7 bar supplemented with 4 mM acetate, 16 mg/L yeast extract, 200 mg/L peptone. The medium for *Desulfovibrio gigas* strain SB1 was supplemented with 20 mM sulfate and 2 mM acetate. The homoacetogenic culture was routinely cultured on fructose (10 mM) with a N_2/CO_2 (4:1) gas phase of 1.7 bar.

Growth rate determination of Methanobacterium formicum strain JF-1

Strain JF-1 was grown in 500-mL serum bottles containing 200 mL of bicarbonate buffered medium with a H_2/CO_2 (4:1) gas phase of 1.7 bar. Duplicate cultures were grown on either H_2/CO_2 as sole carbon and energy source or supplemented with 5 mM of acetate (sodium salt). The batches were inoculated with 10 ml of mid log phase second-generation cultures that were pre-grown on H_2/CO_2 as sole carbon and energy source. Experiments were conducted at pH 7.1 and a temperature of 30 ^oC.

Reactor set-up and operation

Reactor experiments were conducted using a completely stirred tank reactor (CSTR) with a liquid volume of 1 L and a gas volume of 1.3 L. A schematic diagram of the reactor set-up is given in figure 1. The reactor vessel (Fairmentec GmbH, Germany) was made of borosilicate glass, all the reactor internals including the lid were made of PEEK (Polyetheretherketone), and all tubing was made of butyl rubber. The reactor was equipped with a gas sparger, 3 flow spoilers and paddle leaf stirrer. An anaerobic sulfide reduced bicarbonate buffered medium (13) that contained 20 mM SO_4^{2-} (sodium salt) and no organic carbon source was used as the influent.

The gas lines were fitted with sample ports (SP) with butyl rubber seals. The reactor vessel was fitted with dip tube (DT) sample point. The medium and all equipment were sterilized by

autoclaving, and components were connected aseptically. Gas feeds were filtered using membrane filters (0.2 µm pore size). The reactor was inoculated with late exponential 50 mL cultures of *Desulfovibrio gigas* strain SB1, the homoacetogenic enrichment HM1 and *Methanobacterium formicicum* JF-1. The reactor was started as a batch reactor with a pressurized gas phase (1.4 bar) of H_2/CO_2 (4:1) and a stirrer speed of 500 rpm.

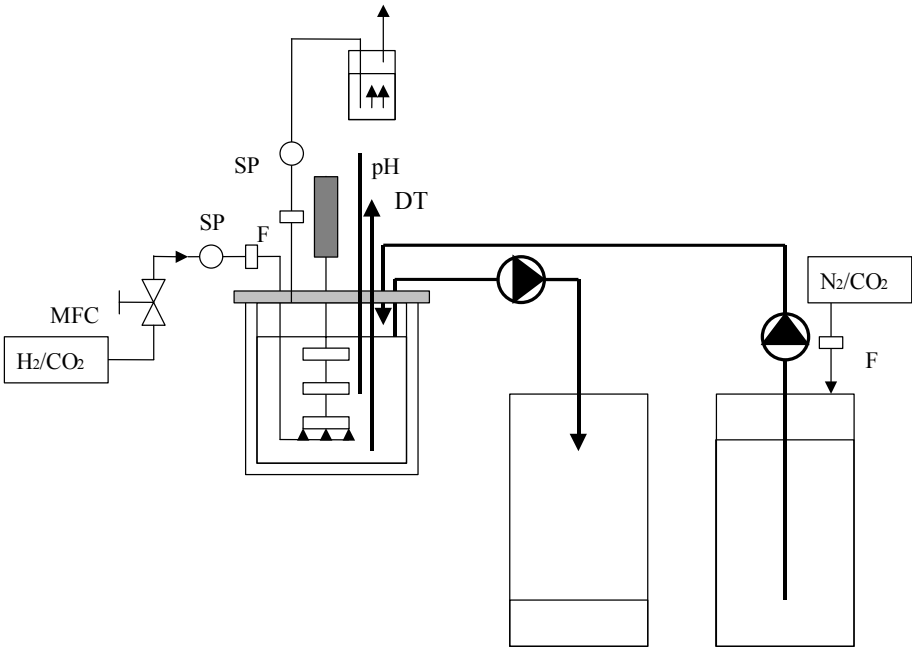

Figure 1 A schematic diagram of the reactor set-up. Abbreviations used: F (Filter), SP (Sample Port), DT (Dip Tube) MFC (Mass Flow Controller).

After three days the reactor was switched to continuous mode at a dilution rate of 0.18 day^{-1}. A mixture of H_2/ CO_2 (4:1) was fed at a flow rate of 1,678 $nL \cdot hr^{-1}$ (normal Liter) using an electronic mass flow controller (MFC) (Brooks Instrument 5850 TR). The reactor was operated at pH 7.1 and a temperature of 30 °C.

Sampling and Analyses

Acetate and sulfate were analyzed by high performance liquid chromatography (HPLC) and ion chromatography (IC) respectively, and hydrogen and methane by gas chromatography (GC) as described previously (11, 13). Biomass concentration was measured by determining the optical density at 660 nm (OD_{660}).

Results and discussion

The homoacetogenic enrichment HM1, *Desulfovibrio gigas* strain SB1 and *Methanobacterium formicicum* JF-1 were co-cultured under autotrophic conditions in a H_2 and CO_2 fed CSTR. The reactor was started in batch mode and was switched to continuous operation at a dilution rate of 0.18 day^{-1} after 3 days. This dilution rate was comparable to the sludge retention time of 1.7 days as used by Weijma et al (15). A stable OD_{660} was observed after approximately 12 days, after it had decreased from 0.09 at the end of batch mode to 0.02 by day 12 (Fig. 2).

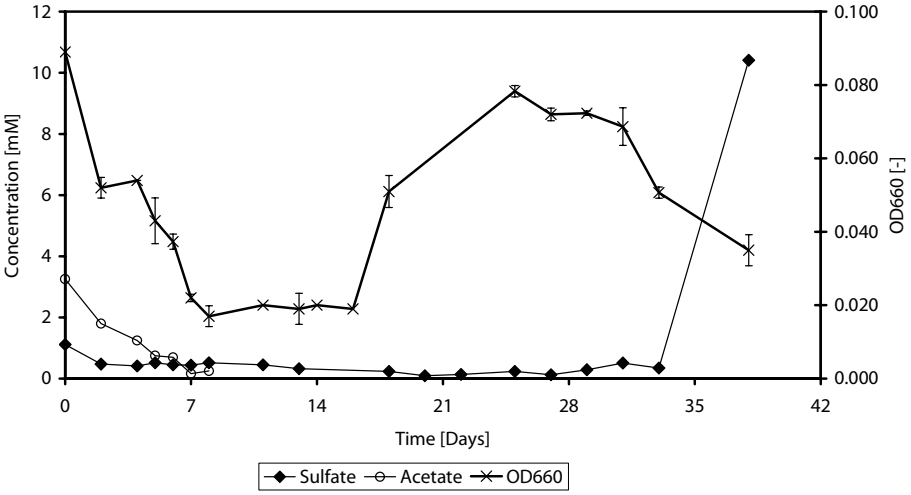

Figure 2 Acetate, sulfate and biomass concentration in the completely stirred tank reactor. OD_{660} values represent the means of triplicate determinations with error bars representing the standard deviation

Microscopic observation confirmed that both the homoacetogen and the heterotrophic sulfate reducer were present, and between days 7 and 13 this co-culture reduced 98 % of all sulfate in the influent. The effluent sulfate concentration ranged from 0.33 to 0.54 mM, acetate concentration ranged from 0.16 to 0.25 mM. By day 18 no methane could be detected in the vent gas and no cells of *M. formicicum* JF-1 were observed. Apparently, *M. formicicum* JF-1 was not able to compete with the homoacetogenic and sulfate reducing co-culture and washed out.

On day 18 an active culture of *M. formicicum* JF-1 was added directly to the CSTR. Following its addition, the OD inside the reactor increased sharply. Nevertheless, no methane was detected in the vent gas by day 25 and microscopic observation showed that *M. formicicum* JF-1 had not established within the reactor during this time. At the same time no cells of the homoacetogen *Acetobacterium* HM1 were observed indicating that this organism had also washed out. The increase in OD could only be attributed to an increased number of the heterotrophic sulphate

reducer. After day 31 the OD started to decrease sharply again and on day 38 an increase in the effluent sulfate concentration was observed indicating washout of the sulfate reducer.

The addition of the *M. formicicum* culture did not result in growth of this microorganism in the reactor. However, it did result in a washout of all 3 trophic groups from the reactor. The most likely reason for this was the fact that the *M. formicicum* culture that was added contained yeast extract, peptone and acetate. The culture had been grown in the presence of these organic components to obtain a dense culture. Addition of this culture introduced organic carbon sources to the system other than the acetate produced by the homoacetogen. This is likely to have disturbed the balance between the sulfate reducer and the homoacetogen causing an active competition for hydrogen.

Based on the earlier work by Badziong et al. (2) who showed that *Desulfovibrio* species grown on H_2, CO_2 and acetate obtain approximately 70% of their cellular carbon from acetate and 30% from carbon dioxide, Noguera et al. (6) proposed the following equation for cell synthesis by *Desulfovibrio* species:

$$2\ CH_3COO^- + HCO_3^- + 2H_2 + 3H^+ + NH_3 \rightarrow C_5H_7O_2N + 5H_2O$$

This equation assumes that the reducing equivalents that are necessary to reduce carbon dioxide and acetate to pyruvate for cell synthesis are provided by hydrogen. Pyruvate is assumed to be a key intermediate in the synthesis of cell mass. Even though most of the sulfate was already reduced, the large increase in OD caused by the growth of the sulfate reducer probably has resulted in an increased consumption of hydrogen and very likely an active competition for hydrogen with both the homoacetogen and the methanogen. The washout of the homoacetogen eventually led to washout of strain SB1, as it was depleted of a source of organic carbon.

The washout and inability of *M. formicicum* to establish itself in the reactor after the addition of day 18 cannot be attributed to its inability to compete with the homoacetogen for hydrogen, since it is likely that both the homoacetogen and the methanogen were outcompeted for hydrogen by the sulfate reducer. However, *M. formicicum* JF-1 was not able to compete with the homoacetogenic and sulfate reducing co-culture at the start of the experiment. During the experiment no wall growth was observed. Since retention of biomass in CSTR is not dependent on settling characteristics, selective washout of methanogens based on a poor retention can be excluded as the reason for methanogens not being able to compete with sulfate-reducers. This agrees with observations that methanogens are outcompeted irrespective of sludge retention times (5).

The observed effects of addition of the *M. formicicum* JF-1 culture, rich in organic carbon, suggest that the heterotrophic sulfate reducer was growth limited by acetate during the first 18 days. However, this may have also been the case for the methanogen. Even though methanogens are

known for their autotrophic growth on H_2 and CO_2, they display very poor growth under these conditions. Therefore, they are normally cultured on relatively organic rich media that contain acetate, yeast extract and other complex organic carbon sources (16). Growth kinetic parameters of methanogens on H_2 and CO_2 have so far only been determined using these relatively organic rich media (1, 10, 12). Under autotrophic conditions these growth kinetic parameters may very well be lower resulting in a poor ability to compete for hydrogen.

To determine whether the growth kinetics for growth on H_2/CO_2 of autotrophic methanogens are affected by acetate, the maximum growth rate of *M. formicicum* strain JF-1 was determined in the presence and absence of acetate. The average maximum growth rate μ_{max} without acetate was found to be 0.655 day^{-1} (SD = 0.167), versus 0.973 day^{-1} (SD = 0.273) in the presence of acetate. These results indicate that even though *M. formicicum* strain JF-1 was capable of autotrophic growth, growth kinetics were adversely affected by no carbon source being available.

Shauer and Ferry (12) previously determined the specific growth rate of *M. formicicum* strain JF-1 previously. They found a higher average specific growth of 1.968 day^{-1} with a 95% confidence interval of 1.176 -2.760 day^{-1}. Although they did not add acetate as a carbon source, the medium that was used contained yeast extract and trypticase as complex organic additives. Their experiments were also performed at a higher temperature of 37°C. This together with the use of an organic rich medium may explain the large differences in the specific growth rate between their study and ours. Acetyl-CoA is regarded to be a central intermediate in carbon assimilation in methanogens (3). Acetate, its precursor, can either be synthesized from CO_2 or via activation of 'external' acetate via acetate kinase and phosphotransacetylase. Activation of acetate would require 1 ATP less per mole of acetyl-CoA produced compared to synthesis from CO_2. As a consequence it may be expected that growth in the presence of an external source like acetate would be energetically more efficient.

Peters et al (9) found a maximum growth rate of 0.576 day^{-1} for the homoacetogen *Acetobacterium woodii* grown on hydrogen and carbon dioxide. This is comparable to the specific growth rate of *Methanobacterium formicicum* strain JF-1 that we found under autotrophic conditions. In the study of Weijma (15) the true SRT in the reactor fed with acetate (R+) and the reactor without acetate addition (R-) amounted to average values of 1.7 and 4.9 days, respectively. Consequently, the specific growth rate of the microorganisms in reactor R- would have been 0.204 day^{-1} and 0.588 day^{-1} for reactor R+. Based on the maximum growth rates obtained from our batch studies and an estimate of K_s values for *M. formicicum* (based on that of *Methanospirillum hungatei* strain JF-1 (10)), *A. woodii* would outcompete *M. formicicum* for hydrogen at these growth rates (figure 3). This would even be the case if *M. formicum* would have a similar K_s value as reported for the heterotrophic SRB *Desulfovibrio vulgaris* strain G11 (10) and a μ_{max} of 0.648 day^{-1} (Fig. 3)

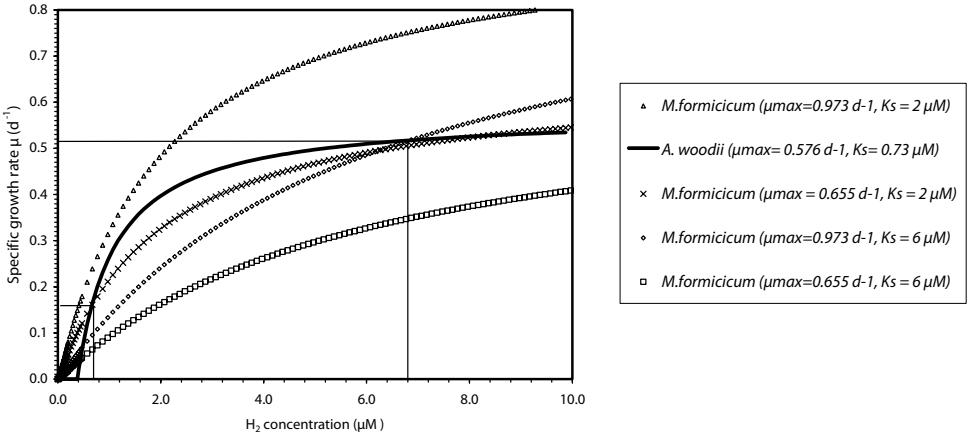

Figure 3 Specific growth rate of *Methanobacterium formicicum* JF-1 and *Acetobacterium woodii* on hydrogen*.

*Calculations are based on a modified Monod equation (8) to account for threshold concentrations;

$$\mu = \mu_{\max} \cdot \frac{S - S_t}{(S - S_t) + K_s}$$

K_s values for *Methanobacterium formicicum* JF-1 are based on the ranges given by Robinson and Tiedje (10) for growth of *Methanospirillum hungatei* JF-1 (6 μM) or *Desulfovibrio vulgaris* G11 (2 μM) at 37°C. Monod parameters for *Acetobacterium woodii* were obtained from Peters et al. (9) for growth at 30°C. Hydrogen thresholds, measured at 28 – 34 °C ,were obtained from Cord-Ruwisch et al. (4).

This simple model illustrates that *Acetobacterium woodii* would be able to outcompete *M. formicicum* strain JF-1 under the conditions observed in the experiments of Weijma et al. (15) and our CSTR experiment. Even though homoacetogens are reported to have a high hydrogen threshold and a low μ_{max}, they possess a high affinity (K_s), which gives them a kinetic advantage over methanogens at intermediate growth rates (0.15 - 0.50 day^{-1}).

Conclusions

A stable co-culture of the homoacetogen *Acetobacterium* strain HM1 and the heterotrophic sulfate reducer *Desulfovibrio gigas* strain SB1 was established in a CSTR fed with H_2/CO_2 and SO_4^{2-} as the sole substrates. This consortium was able to outcompete *Methanobacterium formicicum* JF-1. Addition of an organic carbon source resulted in the washout of the homoacetogen and an increased number of the sulfate reducer, illustrating that the co-culture between the homoacetogen and the heterotrophic sulfate reducer was possible because growth of the heterotrophic SRB was carbon limited.

Batch experiments conducted with *M. formicicum* strain JF-1 showed that the maximum specific growth rate on hydrogen was lower without acetate (0.655 day^{-1}, SD = 0.167 day^{-1}) than with acetate (0.973 day^{-1}, SD = 0.273 day^{-1}). A simple model based on Monod kinetics showed that *Acetobacterium woodii* would be able to outcompete *M. formicicum* strain JF-1 under the conditions applied in the CSTR experiment and the earlier experiments of Weijma et al. (15). Even though homoacetogens are reported to have a high hydrogen threshold and a low μ_{max} they posses a high affinity (K_s), which gives them a kinetic advantage over methanogens at intermediate growth rates (0.15 - 0.50 day^{-1}) at 30°C in environments with low or no organic carbon sources.

Acknowledgements

This research was supported by a grant from the E.E.T. program of the Dutch ministries of Economic Affairs, Education, Culture and Sciences and Housing, Spatial Planning and the Environment (EETK98028).

References

1. **Archer, D. B., and G. E. Powell.** 1985. Dependence of the specific growth rate of methanogenic mutualistic cocultures on the methanogen. Archives of Microbiology **141:**133-137.
2. **Badziong, W., and R. K. Thauer.** 1978. Growth yields of *Desulfovibrio vulgaris* (Marburg) growing on hydrogen plus sulfate and hydrogen plus thiosulfate as the sole energy sources. Archives of Microbiology **117:**209-214.
3. **Blaut, M.** 1994. Metabolism of methanogens. Antonie van Leeuwenhoek **66:**187-208.
4. **Cord-Ruwisch, R., H. J. Seitz, and R. Conrad.** 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Archives of Microbiology **149:**350-357.
5. **Esposito, G., J. Weijma, F. Pirozzi, and P. N. L. Lens.** 2003. Effect of the sludge retention time on H_2 utilization in sulphate reducing gas-lift reactors. Process Biochemistry **39:**491-498.

6. **Noguera, D. R., G. A. Brusseau, B. E. Rittmann, and D. A. Stahl.** 1998. A unified model describing the role of hydrogen in the growth of *Desulfovibrio vulgaris* under different environmental conditions. Biotechnology and Bioengineering **59:**732-746.

7. **Oude Elferink, S. J. W. H., A. Visser, L. W. Hulshoff Pol, and A. J. M. Stams.** 1994. Sulfate reduction in methanogenic bioreactors. FEMS Microbiology Reviews **15:**119-136.

8. **Pavlostathis, S. G., and E. Giraldo-Gomez.** 1991. Kinetics of anaerobic treatment: a critical review. Critical Reviews in Environmental Control **21:**411-490.

9. **Peters, V., P. H. Janssen, and R. Conrad.** 1998. Efficiency of hydrogen utilization during unitrophic and mixotrophic growth of *Acetobacterium woodii* on hydrogen and lactate in the chemostat. FEMS Microbiology Ecology **26:**317-324.

10. **Robinson, J. A., and J. M. Tiedje.** 1984. Competition for hydrogen between sulfate-reducing and methanogenic bacteria for hydrogen under resting and growing conditions. Archives of Microbiology **137:**26-32.

11. **Scholten, J. C. M., and A. J. M. Stams.** 1995. The effect of sulfate and nitrate on methane formation in a freshwater sediment. Antonie van Leeuwenhoek **68:**309-315.

12. **Shauer, N. L., and J. G. Ferry.** 1980. Metabolism of formate in *Methanobacterium formicicum*. Journal of Bacteriology **142:**800-807.

13. **Stams, A. J. M., J. B. Van Dijk, C. Dijkema, and C. M. Plugge.** 1993. Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Applied and Environmental Microbiology **59:**1114-1119.

14. **van Houten, B. H. G. W., K. Roest, V. A. Tzeneva, H. Dijkman, H. Smidt, and A. J. M. Stams.** 2006. Occurence of methanogenesis during start-up of a full-scale synthesis gas fed reactor treating sulfate and metal rich wastewater. Water Research **40:**553-560.

15. **Weijma, J., F. Gubbels, L. Hulshoff Pol, A. J. M. Stams, P. Lens, and G. Lettinga.** 2002. Competition for H_2 between sulfate reducers, methanogens and homoacetogens in a gas-lift reactor. Water Science and Technology **45:**75-80.

16. **Whitman, W. B., T. L. Bowen, and D. R. Boone.** 1991. The Methanogenic Bacteria, p. 719-767. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The Prokaryotes, 2nd edn. ed, vol. Vol. 1. Springer-Verlag, New York.

7

Summary and concluding remarks

Scope and objectives of the research

In the past decade, the application of the biological sulfur cycle in anaerobic wastewater treatment systems has increased markedly. The use of sulfate-reducing bioreactors to simultaneously remove both oxidized sulfur compounds and metals shows great potential to treat wastewaters generated as a result of flue gas scrubbing, mining activities and galvanic processes.

Even though earlier studies showed that methanogenesis did not play a dominant role in mesophilic synthesis gas fed lab-scale reactors (10, 11), pilot and full-scale installations have been faced with significant acetate and methane formation. In addition, no detailed information about the phylogenetic and functional composition of the microbial communities within these bioreactors was available prior of this study.

In order to understand and enable the effective control the performance of these bioreactors the research presented in this thesis aimed to increase the understanding of the microbial aspects of sulfate-reducing synthesis gas fed bioreactors giving greater insight into the competition for hydrogen between the different hydrogenotropic microorganisms present in the sludge. The dominant microorganisms in sludge communities were identified and temporal variations in the community structure were assessed. The effect of carbon dioxide and acetate addition on the competition for hydrogen and the sludge community was studied as well.

Microbial composition of the sludge community

In chapters 2, 3 and 5 the microbial communities of sludge obtained from lab-scale, pilot scale and full-scale synthesis gas fed gas lift reactors were identified. 16S rRNA gene analysis demonstrated that the bacterial communities were mainly dominated by the sulfate-reducing genera *Desulfovibrio* and *Desulfomicrobium*. Archaeal communities comprised populations belonging to the methanogenic genus *Methanobacterium*. Most probable number counts confirmed that heterotrophic sulfate-reducing bacteria were dominant (10^{11}-10^{12} cells/g VSS) compared to homoacetogens (10^{5}-10^{6} cells/g VSS) and methanogens (10^{8}-10^{9}cells/g VSS).

It was also demonstrated that even these mainly hydrogen fed sulfate-reducing bioreactors can sustain a diverse bacterial community, not limited to hydrogenotrophic sulfate-reducing bacteria (SRB). A number of abundant clones were found to be present that did not belong to known genera of SRB, but rather showed affiliation to the proteolytic microorganism *Proteiniphilum acetatigenes* and uncultured *Thermotogales* while other clones clustered within the Chloroflexi subphylum I as defined by Hugenholtz and co-workers *(3)*. Even though very little is known about the physiology of *Chloroflexi,* a cultured representative strain UNI-1T has been shown to grow on complex substrates like sugars and amino acids (9). This strain did not show growth on

hydrogen. As the ingoing wastewater did not contain complex organic compounds, these organisms could be involved in the degradation of dead cells in sludge. Okabe and co-workers (6) presented data that strongly suggested that the members of the phylum *Chloroflexi* preferentially utilized microbial products predominantly derived from biomass decay. The relative abundance of these organisms observed after the occurrence of a calamity with the full-scale reactor would agree with this putative role. Too much zinc containing wastewater was fed to the reactor, which resulted in an unknown free zinc concentration inside the reactor. The drop in performance suggests that toxic concentrations were reached, which would have resulted in substantial cell death and lysis. Under normal conditions, due to the relatively high growth rate in the reactors in the range of 0.14 to 0.25 d^{-1}, there will be a continuous production of dead cells allowing these putative scavengers to persist within the system.

Koizumi and co-workers have observed coexistence of SRB and Chloroflexi in the top sediment of a saline meromictic lake (5). They proposed a syntrophic relationship between SRB and Chloroflexi, where members of subphylum I might be initial degraders of macromolecules, providing fatty acids and hydrogen as electron donors for SRB. If this interaction also takes place in the Budel reactor, these intermediates might sustain a more diverse microbial community than just hydrogenotrophic SRB.

A large number of unique clones represented by a single RFLP pattern were found indicating a relatively diverse community. An unknown number of these unique clones are likely to be sulfate reducers considering the high rates of sulfate reduction of the sludge. Due to the short sludge retention time (4 to 7 days) competition for hydrogen is determined by Monod kinetics, not hydrogen threshold concentrations. As a consequence the fluctuations that frequently occur during operation at full scale with regards to feed rates, SRT and in particular hydrogen levels may enable multiple hydrogenotrophic SRB to be sustained. Based on conditions prevailing in the reactor, only a limited number will become dominant. This functional redundancy within the sulfate reducing microbiota could be an important factor in enabling a stable reactor performance to be maintained under process fluctuations.

Diversity and flexibility of microbial communities has been suggested to potentially enhance the robustness of reactors under varying operational conditions, such as metal and acidity shocks (4). Strain SB1, a hydrogenotrophic sulfate-reducing bacterium described in chapter 4 was found to be one of the most abundant hydrogenotrophic sulfate reducers in the Budel full-scale reactor after the occurrence of the calamity. Considering its relatively high growth rate of 1.9 - 2.8 d^{-1}, strain SB1 may have become abundant during a period of hydrogen excess after the drop in activity in the reactor. The presence of this fast growing sulfate-reducing bacterium in the sludge community may explain the fast recovery of the reactor after the calamity.

The temporal dynamics of the bacterial community in the full-scale reactor was also monitored over a period of 128 weeks (Chapter 3). The diversity found in the clone libraries appeared to

contrast with the results obtained with DGGE, as the DGGE fingerprints showed a limited number of dominant bands. This lower microbial diversity observed by DGGE might have been caused by several factors. The high relative abundance of a few taxa might have interfered with the amplification of less abundant species during PCR. A high number of faint bands, that were hardly distinguishable from the background, were visible on DGGE, supporting this possibility.
After a period of successive changes the community structure stabilized between week 41 and 101 with regard to the dominant bands. This period was characterized by stable operation of the reactor with only minor fluctuations in sulfate input rate, hydrogen concentration in the gas recycle, and feed gas rate and composition. After 101 weeks a shift in the bacterial community to only one predominant band was observed. This shift coincided with a period of a low hydrogen concentration in the gas recycle. The lowered hydrogen concentration is likely to have resulted in a stronger competition for hydrogen, and consequently would have lowered the diversity and/or relative abundance of the hydrogenotrophic community. It was speculated that the abundant microorganisms, which showed the highest level of sequence identity to *Desulfovibrio giganteus*, possess a high affinity for hydrogen, giving them a selective advantage at low hydrogen concentrations. Two dominant bands were observed over prolonged periods during the reactors operation. The band associated with clone BUD11, a member of the genus *Desulfovibrio,* became dominant during the stable period with a high hydrogen concentration in the gas-recycle (weeks 18 - 101). The band of *Desulfomicrobium* clone BUD03 clone was detectable over the longest period of time (121weeks) and remained prominent for a longer time during the period with a lower hydrogen concentration in the gas-recycle. These two microorganisms appear to have been important microorganisms for the overall performance of the Budel reactor.

Carbon Dioxide

Earlier studies on mesophilic synthesis gas fed lab-scale reactors showed that methanogenesis did not play a dominant role in (10, 11). However, methanogenesis was not rapidly suppressed during start-up of the full-scale Budel reactor, despite the predominance of sulfate-reducing bacteria (Chapters 2 and 3). Even though the fluctuations that occur frequently during operation at full scale may enable multiple hydrogenotrophic SRB to be sustained and increase process stability, it may also allow methanogenesis to persist. As result of these fluctuations a continuous state of hydrogen limitation will not be reached. Since Monod kinetic parameters for methanogens are relatively similar to those of SRB (7), methanogens are not readily outcompeted. Previous research had shown that methanogenesis did not play a dominant role in mesophilic synthesis gas fed lab-scale reactors (2, 10-12) and methanogens were outcompeted. A possible explanation for the discrepancy in observation between full-scale and

lab-scale would be that less fluctuations occur at lab-scale resulting in a more continuous state of hydrogen limitation.

An alternative strategy to suppress methanogenesis would be to limit the carbon dioxide feed to the reactor. Methanogens and homoacetogens depend on carbon dioxide for their energy metabolism, while heterotrophic SRB use bicarbonate only as a carbon source. They do not depend on bicarbonate for their energy metabolism and therefore their requirement for carbon dioxide is much lower. As carbon dioxide is not the main carbon source for the sulfate reducing microbiota, lowering the carbon dioxide concentration could favor the growth of these SRB over methanogens and homoacetogens. Hence, carbon dioxide could be a possible tool to control the competition for hydrogen.

The effect of the carbon dioxide feed rate on methane production in the full-scale reactor was monitored for 128 weeks (Chapter 3). The carbon dioxide concentration in the feed gas was gradually lowered from 20-22 % to 6-3 % (v/v) over a period of 4 weeks, resulting in a lowered carbon dioxide feed rate of 20-15 $Nm^3 \cdot hr^{-1}$ (Normal m^3: T = 293 K, p = 101.325 Pa). Before the carbon dioxide feed rate was lowered, the reactor had a high methanogenic activity of 46 $Nm^3 \cdot hr^{-1}$. From week 6 onwards the carbon dioxide feed rate was kept below 15 $Nm^3 \cdot hr^{-1}$ and by week 8 the methanogenic activity had decreased to 3 Nm^3 methane hr^{-1}. Between weeks 8 and 93, at a calculated hydrogen consumption rate of 2 $Nm^3 \cdot hr^{-1}$, less than 1% of the hydrogen supply was used for methanogenesis.

From week 88 the feed gas carbon dioxide concentration varied between 3.4 and 10.2 %, due to a problem with the gas absorber used for carbon dioxide removal from the feed gas. This caused an increased feed of carbon dioxide to the reactor. After a period of little methane production, an increase in methane production was detected from week 93 onwards. At a calculated hydrogen consumption rate of 132 $Nm^3\ hr^{-1}$ in week 103, approximately 25% of the supplied hydrogen was used for methanogenesis. DGGE profiles showed that after the carbon dioxide feed rate had been lowered initially, the archaeal profiles decreased in complexity and maintained a consistent profile up to week 128. These results suggest that the lowered carbon dioxide feed decreased the archaeal diversity. Nevertheless, the Archaea were not washed out completely. After the rate of methanogenesis had increased after 93 weeks, no obvious change in diversity was detected. The increase in the rate of methanogenesis also suggests that the archaeal community was carbon dioxide limited between week 13 and week 95. This is also reflected by the decrease in diversity observed by DGGE, as the competition for a single growth limiting substrate could be expected to decrease diversity. Limiting the carbon dioxide feed proved to be a good tool to control the methanogenic activity, although it did not result in a complete washout of methanogens from the reactor.

Acetate

The *Desulfovibrio* and *Desulfomicrobium* species that were identified as the dominant sulfate-reducers are heterotrophic and require an organic carbon source like acetate for growth. In both full and pilot scale systems acetate had been added as a carbon source. However, under autotrophic conditions SRB would depend on the production of acetate by homoacetogenic bacteria present in the sludge. In case of acetate-limited sulfate reduction, the growth kinetics of the SRB would thus be determined by the growth kinetics of the homoacetogenic bacteria. Alternatively, autotrophic SRB might outcompete heterotrophic sulfate reducers. Many methanogens, however, can grow autotrophically with hydrogen and carbon dioxide and do not require an external carbon source. In that case, methanogens may outcompete both heterotrophic sulfate-reducers and homoacetogens.

Based on the order of competitiveness autotrophic methanogens should be able to outcompete heterotrophic SRB for hydrogen if organic carbon sources are not available (1, 7). Weijma et al. (12) have studied this situation using two lab-scale gas-lift reactors. Contrary to what was expected their results showed that methanogenesis was suppressed, while small amounts of acetate were produced. Hydrogen was more readily consumed by homoacetogens than by methanogens.

The results presented in chapter 5 showed that the bacterial communities of both lab-scale reactors were indeed dominated by a limited number of microorganisms mainly belonging to the genera *Desulfovibrio*, *Acetobacterium* and *Desulfomicrobium*. This confirmed the earlier hypothesis by Weijma et al. (2002) that there was cooperative interaction between heterotrophic SRB and homoacetogens. We hypothesized that both trophic groups were able to coexist because they were not limited by the same substrate: homoacetogens being hydrogen limited, while heterotrophic SRB were acetate limited. Furthermore the growth of the methanogens may also have been acetate limited, resulting in a reduced ability to compete for hydrogen.

To test whether a consortium of a heterotrophic sulfate-reducing bacteria and homoacetogens were able to coexist and compete effectively with methanogens, three model organisms were co-cultured in a completely stirred tank reactor fed with H_2/CO_2 and SO_4^{2-} as the sole substrates (Chapter 6). A stable co-culture of the homoacetogen *Acetobacterium* strain HM1 and the heterotrophic sulfate reducer *Desulfovibrio gigas* strain SB1 was established. This consortium was able to outcompete *Methanobacterium formicicum* JF-1. Addition of an organic carbon source resulted in the washout of the homoacetogen and an increased abundance of the sulfate reducer, illustrating that the co-culture between the homoacetogen and the heterotrophic sulfate reducer was possible because growth of the heterotrophic SRB was carbon limited.

Batch experiments conducted with *M. formicicum* strain JF-1 showed that the maximum specific growth rate on hydrogen was lower without acetate (0.655 day^{-1}, SD = 0.167 day^{-1}) than with

acetate (0.973 day^{-1}, SD = 0.273 day^{-1}). A simple model based on Monod kinetics showed that *Acetobacterium woodii* would be able to outcompete *M. formicicum* strain JF-1 under the conditions applied in the CSTR experiment and the earlier experiments of Weijma et al. (2002). Even though homoacetogens are reported to have a high hydrogen threshold and a low μ_{max} they posses a high affinity (K_s), (8) which gives them a kinetic advantage over methanogens at intermediate growth rates (0.15 -0 .50 day^{-1}) at 30°C in environments with low or no organic carbon sources.

Conclusions

The results presented in this thesis give more insight in the microbial composition, and processes within synthesis gas fed bioreactors treating sulfate rich wastewaters and the factors that affect the competition for hydrogen. Some of these insights may prove to be useful for optimization and control of these bioreactors at full-scale:

- Due to the relatively short sludge retention time hydrogen threshold concentrations are not reached, instead Monod kinetic parameters control the competition for hydrogen in the systems.
- A continuous state of hydrogen limitation may not be reached as a result of the fluctuations that occur at full-scale and as a result suppression of methanogenesis may be a slow process.
- Limiting the carbon dioxide feed appears to be a very effective tool to control methanogenesis although it does not lead to complete removal of all methanogens from the system.
- The capacity of hydrogenotrophic methanogens to compete for hydrogen appears to affected by the availability of an organic carbon source. Limiting the feed of an organic carbon source to a minimum, may provide another tool to control methanogenesis

In general a more rigid control of the sludge retention time and the hydrogen, carbon dioxide and acetate feed to the sulfidogenic reactor would minimize or prevent the occurrence of methanogenesis.

References

1. **Cord-Ruwisch, R., H. J. Seitz, and R. Conrad.** 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Archives of Microbiology 149:350-357.

2. **Esposito, G., J. Weijma, F. Pirozzi, and P. N. L. Lens.** 2003. Effect of the sludge retention time on H_2 utilization in sulphate reducing gas-lift reactors. Process Biochemistry 39:491-498.

3. **Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace.** 1998. Novel division level of bacterial diversity in a Yellowstone hot spring. Journal of Bacteriology 180:366-376.

4. **Kaksonen, A. H., J. J. Plumb, P. D. Franzmann, and J. A. Puhakka.** 2004. Simple organic electron donors support diverse sulfate-reducing communities in fluidized-bed reactors treating acidic metal- and sulfate-containing wastewater. FEMS Microbiology Ecology 47:279-289.

5. **Koizumi, Y., H. Kojima, and M. Fukui. 2004.** Dominant microbial composition and its vertical distribution in saline meromictic lake Kaiike (Japan) as revealed by quantitative oligonucleotide probe membrane hybridization. Applied and Environmental Microbiology. 70:4930-4940.

6. **Okabe, S., T. Kindaichi, and T. Ito.** 2005. Fate of 14C-labeled microbial products derived from nitrifying bacteria in autotrophic nitrifying biofilms. Applied and Environmental Microbiology 71:3987-3994.

7. **Oude Elferink, S. J. W. H., A. Visser, L. W. Hulshoff Pol, and A. J. M. Stams.** 1994. Sulfate reduction in methanogenic bioreactors. FEMS Microbiology Reviews 15:119-136.

8. **Peters, V., P. H. Janssen, and R. Conrad.** 1998. Efficiency of hydrogen utilization during unitrophic and mixotrophic growth of *Acetobacterium woodii* on hydrogen and lactate in the chemostat. FEMS Microbiology Ecology 26:317-324.

9. **Sekiguchi, Y., T. Yamada, S. Hanada, A. Ohashi, H. Harada, and Y. Kamagata.** 2003. *Anaerolinea thermophila* gen. nov., sp. nov. and *Caldilinea aerophila* gen. nov., sp. nov., novel filamentous thermophiles that represent a previously uncultured lineage of the domain Bacteria at the subphylum level. International Journal of Systematic and Evolutionairy Microbiology 53:1843-1851.

10. **van Houten, R. T.** 1996. Biological sulfate reduction with synthesis gas. Ph.D. thesis. Wageningen Agricultural University, Wageningen, Netherlands.

11. **van Houten, R. T., L. Hulshoff Pol, and G. Lettinga.** 1994. Biological sulfate reduction using gas-lift reactors fed with hydrogen and carbon dioxide as energy and carbon source. Biotechnology and Bioengineering. 44:586-594.

12. **Weijma, J., F. Gubbels, L. Hulshoff Pol, A. J. M. Stams, P. Lens, and G. Lettinga.** 2002. Competition for H_2 between sulfate reducers, methanogens and homoacetogens in a gas-lift reactor. Water Science and Technology. 45:75-80.

Dutch summary / Nederlandse samenvatting

Onderzoeksgebied en doelstellingen van deze studie

In de afgelopen tien jaar is het gebruik van de biologische zwavelcyclus in afvalwaterzuivering aanzienlijk toegenomen. De toepassing van sulfaatreducerende bioreactoren voor de gelijktijdige verwijdering van geoxideerde zwavelverbindingen en metalen bezit een groot potentieel voor de behandeling verschillende soorten afvalwater, zoals mijnafvalwater en afvalwater dat vrijkomt bij galvanische processen of het ontzwavelen van rookgassen.

Ondanks dat eerdere studies aantoonden dat methanogenese geen rol van betekenis speelde in mesofiele laboratoriumschaalreactoren (10, 11), deed zich zowel op proefschaal als praktijkschaal toch aanzienlijke methanogenese en zelfs acetaatvorming voor in reactoren die gevoed werden met synthesegas. Eveneens was er aan het begin van deze studie weinig gedetailleerde informatie wat betreft de fylogenetische en functionele samenstelling van de microbiële gemeenschappen in deze bioreactoren.

De studie beschreven in dit proefschrift richtte zich op de microbiële aspecten van deze sulfaatreducerende bioreactoren, met als doel meer inzicht in de competitie om waterstof tussen verschillende waterstofconsumerende micro-organismen te krijgen en een effectieve controle van deze reactoren mogelijk te maken. Het onderzoek richtte zich op zowel praktijkschaal-, proefschaal- en laboratoriumschaalreactoren. Bij aanvang van deze studie werd een praktijkschaalreactor opgestart voor de behandeling van zink- en sulfaatrijk afvalwater van de Zinifex Budel Zink zinkfabriek (Budel-Dorplein, Nederland). De dominante micro-organismen in het slib werden geïdentificeerd en de temporale verschillen in de slibsamenstelling werden bestudeerd. Tevens is de slibsamenstelling van een proefschaalreactor voor de behandeling van mijnafvalwater bestudeerd (Kennecott, Utah, Verenigde Staten) en twee labschaalreactoren (sectie Milieutechnologie, Wageningen Universiteit), die werden gebruikt om de rol van acetaat te bestuderen tijdens de competitie om waterstof. Naast de rol van acetaat richtte de studie in dit proefschrift zich ook op de rol van koolstofdioxide tijdens de competitie om waterstof.

Microbiële slibsamenstelling

In hoofdstukken 2, 3 en 5 werd de microbiële slibsamenstelling van slib afkomstig van de laboratoriumschaal-, proefschaal- en praktijkschaalreactoren beschreven. Analyse op basis van het 16S rRNA gen toonde aan dat de Bacteriële slibsamenstelling gedomineerd werd door de sulfaatreducerende genera *Desulfovibrio* en *Desulfomicrobium*. De Archaea maakten deel uit van het genus *Methanobacterium*. Dit genus bestaat uit uitsluitend methanogenen. Most probable number schattingen met het slib van de praktijk- en proefschaalreactoren bevestigden dat heterotrofe sulfaatreducerende bacteriën dominant waren (10^{11}-10^{12} cellen/g VSS) in vergelijking met homoacetogenen (10^{5}-10^{6} cellen/g VSS) en methanogenen (10^{8}-10^{9}cellen/g VSS). Daarnaast werd aangetoond dat deze hoofdzakelijk waterstofgevoede reactoren een diversere microbiële gemeenschap huisvesten dan waterstofconsumerende sulfaatreduceerders, methanogenen en

homoacetogenen. Een aantal kloons toonde verwantschap met onder andere het proteolitische micro-organisme *Proteiniphilum acetatigenes*, niet gecultiveerde *Thermotogales* soorten en Chloroflexi subphylum I zoals gedefinieerd door Hugenholtz et al. *(3)*. Er is nog weinig bekend over de fysiologie van deze Chloroflexi. Een gecultiveerde stam UNI-1T groeit op complexe substraten zoals suikers en aminozuren, maar niet op waterstof (9). Aangezien het ingaande afvalwater geen complexe organische verbindingen bevat en groei op waterstof niet waarschijnlijk lijkt, zouden deze micro-organismen mogelijk van dood celmateriaal kunnen leven. Okabe et al. (6) maakten aannemelijk dat Chloroflexi bij voorkeur groeiden op microbiële producten afkomstig van de afbraak van celmateriaal. Dit zou een verklaring kunnen zijn voor de relatieve toename van deze soorten na een calamiteit met de praktijkreactor. Tijdens deze calamiteit werd er teveel zinkhoudend afvalwater naar de reactor gestuurd en werd het slib blootgesteld aan een onbekende concentratie vrij zink. Een sterke afname van de sulfaatreductie in de reactor duidde erop dat de concentratie toxisch was, wat in een aanzienlijke hoeveelheid dood celmateriaal tot gevolg zou hebben gehad. Onder normale condities zal door de relatief hoge groeisnelheid in deze reactoren (14 tot 0.25 dag^{-1}) ook een relatief hoge productie van dood celmateriaal zijn waardoor deze micro-organismen zich kunnen handhaven in de reactor.

Koizumi et al. vonden sulfaatreducerende bacteriën en Chloroflexi in de bovenste laag van een brak sediment in een sterk gestratificeerd meer (5). Zij stelden een syntrofe relatie tussen de sulfaatreduceerders en Chloroflexi voor. Hierin zouden subphylum I Chloroflexi verantwoordelijk zijn voor de initiële afbraak van organisch materiaal. De tijdens de afbraak gevormde producten zoals vetzuren en waterstof zouden als elektronendonoren voor sulfaatreduceerders kunnen dienen. Indien deze syntrofe interactie ook in synthesegas gevoede sulfaatreducerende bioreactoren plaatsvindt, zouden deze producten tot gevolg kunnen hebben dat de sulfaatreducerende micro-organismen niet uitsluitend beperkt zijn tot het gebruik van waterstof. De aanwezigheid van andere elektronendonoren zou ook een hogere diversiteit tot gevolg kunnen hebben dan men op basis van uitsluitend waterstof zou mogen verwachten.

Op basis van restrictie-analyse werd een groot aantal onbekende kloons gevonden met een uniek patroon. Gezien de hoge sulfaatreducerende capaciteit van de praktijkreactor zal een aantal van deze onbekende kloons waarschijnlijk sulfaatreduceerders zijn. Door de relatief korte slibleeftijd van 4 tot 7 dagen zullen waterstof threshold concentraties niet gehaald worden. De competitie om waterstof zal het resultaat zijn van de Monod kinetische eigenschappen van de aanwezige micro-organismen. Tijdens de bedrijfsvoering op praktijkschaal traden er voortdurend verschillen op in de bedrijfsvoering wat betreft slibleeftijd, gastoevoer en afvoer met als gevolg dat de waterstofconcentratie in de reactor moet hebben gefluctueerd. Deze fluctuerende waterstofconcentratie kan het mogelijk maken dat micro-organismen met verschillende Monod kinetische eigenschappen zich zouden kunnen handhaven in de reactor. Op basis van de heersende omstandigheden in de reactor zullen maar een beperkt aantal micro-organismen

dominant worden, terwijl anderen in lagere aantallen aanwezig blijven. Deze diversiteit wat betreft sulfaatreduceerders zou een belangrijke rol kunnen spelen bij het handhaven van een stabiele sulfaatreductie onder fluctuerende bedrijfsvoeringomstandigheden. Diversiteit en de daarmee samenhangende flexibiliteit van microbiële ecosystemen zouden kunnen bijdragen aan stabiliteit robuustheid van bioreactoren, bij bijvoorbeeld calamiteiten zoals sterke veranderingen in de pH of blootstelling aan verhoogde metaalconcentraties (4).

Stam SB1, een heterotrofe waterstofconsumerende sulfaatreduceerder beschreven in hoofdstuk 4, was een van de dominante sulfaatreduceerders in de praktijkschaalreactor op het tijdstip van isolatie na een calamiteit. Door de sterke afname van de sulfaatreductie ontstond een periode van relatieve waterstofovermaat. Gezien de relatief hoge groeisnelheid van 1.9 - 2.8 dag^{-1} van stam SB1 zou deze situatie er toe geleid kunnen hebben dat stam SB1 dominant werd. De aanwezigheid van dit soort opportunistische snelgroeiende sulfaatreduceerders zou verantwoordelijk kunnen zijn voor het snelle herstel van de reactor na de calamiteit.

De verschillen in de bacteriële samenstelling van het slib werden gedurende een periode van 128 weken gevolgd (Hoofdstuk 3). De diversiteit die gevonden werd op basis van clone libraries leek in contrast te staan met die op basis van denaturing gradient gel electrophoresis DGGE. DGGE analyse gaf maar een beperkt aantal dominante banden te zien. Dit verschil wordt waarschijnlijk veroorzaakt door het feit dat de dominantie van een beperkt aantal soorten de amplificatie van minder dominante soorten zal bemoeilijken tijdens PCR. Een groot aantal erg lichte banden waren zichtbaar op de gels die amper van de achtergrond te onderscheiden waren. Een tweede reden voor het waargenomen verschil in diversiteit werd veroorzaakt doordat de gebruikte universele primers twee tot drie afwijkende basenparen hadden met de primerlocatie van een aantal soorten die met behulp van kloneren gevonden waren. Deze verschillen zorgden voor een slechte amplificatie van deze soorten tijdens de PCR-reactie.

Op basis van DGGE analyse stabiliseerde de bacteriële samenstelling van de praktijkschaalreactor zich tussen week 41 en 101 wat betreft de dominante soorten. Deze periode komt overeen met een stabiele periode in de bedrijfsvoering met betrekking tot sulfaatbelasting, de gassamenstelling en de gas- en afvoer. Vanaf week 101 vond er een verschuiving in de bacteriële samenstelling plaats naar slechts één dominante band. Deze verschuiving vond plaats in periode waarin de waterstofconcentratie erg laag was. Deze sterk verhoogde dominantie van één soort is waarschijnlijk het gevolg van een verhoogde selectie druk ten gevolge van de lage waterstofconcentratie. De dominante soort werd geïdentificeerd als een *Desulfovibrio giganteus* stam. Het lijkt aannemelijk dat deze stam een hoge affiniteit voor waterstof bezit. Gedurende de periode van 120 weken waren er twee dominante banden voor langere periodes zichtbaar. Een band, die overkwam met die van kloon BUD11, een *Desulfovibrio* soort, werd dominant gedurende een periode met een hoge waterstofconcentratie (weken 18 - 101). De andere band die overeenkwam met *Desulfomicrobium* kloon BUD03 was het langst zichtbaar (121 weken). Deze

twee micro-organismen zijn waarschijnlijk belangrijk geweest voor de sulfaatreducerend activiteit van de praktijkschaalreactor.

Koolstofdioxide

Eerdere studies met labschaalreactoren toonden aan dat methanogenese geen rol van betekenis speelde onder mesofiele condities (2, 10-12). Echter tijdens het opstarten van de praktijkschaalreactor vond er aanzienlijke methanogenese plaats. Ondanks dat het slib gedomineerd werd door sulfaatreducerende bacteriën werd methanogenese niet snel door sulfaatreductie onderdrukt (Hoofdstukken 2 en 3). Hoewel de fluctuaties in bedrijfsvoeringomstandigheden waarschijnlijk een diverse sulfaatreducerende gemeenschap mogelijk maken, is het waarschijnlijk ook de oorzaak van de langzame onderdrukking van methanogenese. Door deze fluctuaties is er geen voortdurende staat van waterstoflimitatie. Aangezien de Monod kinetische eigenschappen van methanogenen niet sterk van die van sulfaatreduceerders verschillen (7), zal methanogenese niet snel onderdrukt worden onder fluctuerende omstandigheden. Het verschil tussen de observaties op labschaal en praktijkschaal zou hierdoor verklaard kunnen worden aangezien de bedrijfsvoering op labschaal strikter te handhaven is. Een voortdurende staat van waterstoflimitatie is makkelijker te handhaven.

Een alternatief voor waterstoflimitatie zou het beperken van de koolstofdioxidetoevoer naar de reactor kunnen zijn. Methanogenen en ook homoacetogenen zijn afhankelijk van koolstofdioxide voor hun energievoorziening en als koolstofbron. Sulfaatreduceerders gebruiken koolstofdioxide alleen als koolstofbron en hebben daardoor een veel lagere behoefte voor koolstofdioxide. Het verminderen van de koolstofdioxidetoevoer zou daarom een mogelijkheid zijn om methanogenese te beperken.

Het effect van het verlagen van de koolstofdioxidetoevoer naar de praktijkschaal reactor is gedurende een periode van 128 weken gevolgd (Hoofdstuk 3). De koolstofdioxideconcentratie in de toevoer werd verlaagd van 20-22 % naar 6-3 % (v/v) over een periode vier weken. De koolstofdioxidetoevoer werd hierdoor 20-15 $Nm^3 \cdot uur^{-1}$ (Normaal m^3: T = 293 K, p = 101.325 Pa). Voor deze verlaging bedroeg de methanogene activiteit 46 $Nm^3 \cdot hr^{-1}$. Vanaf week 6 was de koolstofdioxidetoevoer lager dan 15 $Nm^3 \cdot uur^{-1}$ en in week 8 was de methanogene activiteit gedaald naar 3 $Nm^3 \cdot hr^{-1}$. Tussen week 8 en 93 bedroeg de waterstofconsumptie voor methanogenese minder dan 1% van de totale toevoer op basis van een berekende waterstofconsumptie van 2 $Nm^3 \cdot hr^{-1}$. Vanaf week 88 fluctueerde de koolstofdioxideconcentratie tussen de 3,4 and 10,2 % in de gastoevoer. Vanaf week 93 was er een toename in de methanogene activiteit waarneembaar. In week 103 bedroeg de waterstofconsumptie voor methanogenese 132 $Nm^3\ hr^{-1}$; ongeveer 25 % van de waterstoftoevoer.

DGGE analyse gaf een afname in de diversiteit van de Archaea naar slechts twee banden te zien nadat de koolstofdioxidetoevoer naar de reactor was verlaagd. Dit twee banden patroon

veranderde niet na de toename van de koolstofdioxidetoevoer in week 88 en bleef tot en met week 128 waarneembaar. Dit wijst erop dat de methanogene gemeenschap waarschijnlijk koolstofdioxide gelimiteerd was tussen week 13 en 88. Het verlagen van de koolstofdioxidetoevoer zorgde dus voor een verlaging van de diversiteit en een sterke onderdrukking van methanogenese maar resulteerde niet in een volledige verwijdering van alle methanogenen uit de reactor.

Acetaat

Dominante sulfaatreduceerders in de reactoren behoorden tot de genera *Desulfovibrio* en *Desulfomicrobium*. Deze sulfaatreduceerders hebben naast koolstofdioxide een organische koolstofbron nodig voor groei. Zowel op praktijk- als proefschaal werd er een organische koolstofbron zoals acetaat toegevoegd. In het geval dat er geen acetaat meer zou worden toegevoegd zouden deze sulfaatreduceerders niet meer kunnen groeien, of zouden zij afhankelijk worden van eventuele acetaatproductie door de groei van homoacetogenen. De groeikinetiek van sulfaatreduceerders zou hierdoor afhankelijk zijn van die van homoacetogenen. Een andere mogelijkheid zou zijn dat heterotrofe sulfaatreduceerders vervangen zouden worden door autotrofe soorten. Vele waterstofconsumerende methanogenen zijn autotroof en hebben voor hun groei geen organische koolstofbron nodig. Op basis van bekende kinetische parameters zouden methanogenen de competitie om waterstof kunnen winnen van homoacetogenen of autotrofe sulfaatreduceerders in deze situatie (1, 7). Weijma et al. (12) hebben deze situatie onderzocht op labschaal met behulp van twee labschaalreactoren en hun resultaten toonden aan dat methanogenese werd onderdrukt en er sulfaatreductie plaatsvond en er ook kleine hoeveelheden acetaat werden geproduceerd.

De dominante micro-organismen in deze reactoren behoorden tot de genera *Desulfovibrio*, *Acetobacterium* en *Desulfomicrobium* (Hoofdstuk 5). Dit bevestigde de eerdere hypothese van Weijma et al., dat er een coöperatieve interactie tussen heterotrofe sulfaatreduceerders en homoacetogenen plaatsvond. Als hypothese voor deze interactie tussen beide groepen werd voorgesteld dat ze door verschillende substraten gelimiteerd werden: homoacetogenen door waterstof en heterotrofe sulfaatreduceerders door acetaat. Als hypothese voor de observatie dat homoacetogenen de competitie om waterstof wonnen van methanogenen werd voorgesteld dat de beschikbaarheid van acetaat het competitieve vermogen van methanogenen beïnvloedt.

Om deze hypothesen te testen werden drie modelorganismen samen gecultiveerd in een volledig gemengde reactor (CSTR) die gevoed werd met waterstof, koolstofdioxide en sulfaat als de enige substraten (Hoofdstuk 6). Een stabiele cocultuur van de homoacetogeen *Acetobacterium* strain HM1 en de heterotrofe sulfaatreduceerder *Desulfovibrio gigas* stram SB1 was mogelijk waarbij *Methanobacterium formicicum* JF-1 uitspoelde. Het toevoegen van een organische koolstofbron resulteerde in het uitspoelen van de homoacetogeen en een toename van de sulfaatreduceerder.

Dit bevestigde de hypothese dat deze cocultuur mogelijk was doordat de sulfaatreduceerder door acetaat gelimiteerd was.

Groei-experimenten met *M. formicicum* strain JF-1 toonden dat de maximale groeisnelheid lager was wanneer deze methanogeen autotroof werd gegroeid (0.655 day^{-1}, SD = 0.167 day^{-1}), in vergelijking met groei in aanwezigheid van acetaat (0.973 day^{-1}, SD = 0.273 day^{-1}). Een simpel model op basis van Monod kinetiek gaf aan dat *Acetobacterium woodii* de competitie om waterstof zou winnen van *M. formicicum* stam JF-1, onder zowel de condities in het CSTR experiment als die van de experimenten van Weijma et al. (2002). Ondanks dat homoacetogenen een hoge waterstof threshold concentratie hebben en een lage maximale groeisnelheid μ_{max} beschikken ze over een lage affiniteit voor waterstof (K_s) (8). Hierdoor zijn ze in het voordeel in vergelijking met methanogenen tussen groeisnelheden van 0.15 -0 .50 dag^{-1} bij een temperatuur van 30°C in ecosystemen met weinig of geen organische koolstofbronnen.

Conclusies

De onderzoeksresultaten in dit proefschrift geven meer inzicht in de microbiële samenstelling en microbiële processen in sulfaatreducerende bioreactoren die gevoed worden met synthesegas. Een aantal van deze observaties zou gebruikt kunnen worden om de bedrijfsvoering van deze reactoren te optimaliseren:

- Door de relatief korte slibleeftijden worden waterstof threshold concentraties niet bereikt. De competitie om waterstof zal het gevolg zijn van Monod kintiek parameters van de aanwezige micro-organismen.
- Door voortdurende fluctuaties in de bedrijfsvoering zal waterstof niet voortdurend in limiterende hoeveelheden aanwezig zijn. Dit kan er toe leiden dat de methanogenese maar zeer langzaam onderdrukt wordt door sulfaatreductie.
- Het beperken van de koolstofdioxidetoevoer blijkt een effectief middel om methanogenese te beperken. Het leidt echter niet tot een volledige verwijdering van alle methanogenen.
- De capaciteit van methanogenen om waterstof te gebruiken wordt beïnvloed door de aanwezigheid van een organische koolstofbron. Het gelimiteerd toevoegen van een organische koolstofbron zou een mogelijk middel kunnen zijn om methanogenese te beperken.

Een striktere controle van de slibleeftijd en de waterstof- en koolstofdioxidetoevoer en een beperkte toevoeging van acetaat zou methanogenese kunnen beperken of voorkomen.

Literatuur

1. **Cord-Ruwisch, R., H. J. Seitz, and R. Conrad.** 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Archives of Microbiology **149:**350-357.

2. **Esposito, G., J. Weijma, F. Pirozzi, and P. N. L. Lens.** 2003. Effect of the sludge retention time on H_2 utilization in sulphate reducing gas-lift reactors. Process Biochemistry **39:**491-498.

3. **Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace.** 1998. Novel division level of bacterial diversity in a Yellowstone hot spring. Journal of Bacteriology **180:**366-376.

4. **Kaksonen, A. H., J. J. Plumb, P. D. Franzmann, and J. A. Puhakka.** 2004. Simple organic electron donors support diverse sulfate-reducing communities in fluidized-bed reactors treating acidic metal- and sulfate-containing wastewater. FEMS Microbiology Ecology **47:**279-289.

5. **Koizumi, Y., H. Kojima, and M. Fukui.** 2004. Dominant microbial composition and its vertical distribution in saline meromictic lake Kaiike (Japan) as revealed by quantitative oligonucleotide probe membrane hybridization. Applied and Environmental Microbiology. **70:**4930-4940.

6. **Okabe, S., T. Kindaichi, and T. Ito.** 2005. Fate of 14C-labeled microbial products derived from nitrifying bacteria in autotrophic nitrifying biofilms. Applied and Environmental Microbiology **71:**3987-3994.

7. **Oude Elferink, S. J. W. H., A. Visser, L. W. Hulshoff Pol, and A. J. M. Stams.** 1994. Sulfate reduction in methanogenic bioreactors. FEMS Microbiology Reviews **15:**119-136.

8. **Peters, V., P. H. Janssen, and R. Conrad.** 1998. Efficiency of hydrogen utilization during unitrophic and mixotrophic growth of *Acetobacterium woodii* on hydrogen and lactate in the chemostat. FEMS Microbiology Ecology **26:**317-324.

9. **Sekiguchi, Y., T. Yamada, S. Hanada, A. Ohashi, H. Harada, and Y. Kamagata.** 2003. *Anaerolinea thermophila* gen. nov., sp. nov. and *Caldilinea aerophila* gen. nov., sp. nov., novel filamentous thermophiles that represent a previously uncultured lineage of the domain Bacteria at the subphylum level. International Journal of Systematic and Evolutionairy Microbiology **53:**1843-1851.

10. **van Houten, R. T.** 1996. Biological sulfate reduction with synthesis gas. Ph.D. thesis. Wageningen Agricultural University, Wageningen, Netherlands.

11. **van Houten, R. T., L. Hulshoff Pol, and G. Lettinga.** 1994. Biological sulfate reduction using gas-lift reactors fed with hydrogen and carbon dioxide as energy and carbon source. Biotechnology and Bioengineering. **44:**586-594.

12. **Weijma, J., F. Gubbels, L. Hulshoff Pol, A. J. M. Stams, P. Lens, and G. Lettinga.** 2002. Competition for H_2 between sulfate reducers, methanogens and homoacetogens in a gas-lift reactor. Water Science and Technology. **45:**75-80.

Acknowledgements / Dankwoord

De tocht zit erop. Terugkijkend is de tocht toch wat langer geworden en anders gelopen dan oorspronkelijk gepland. Promoveren lijkt wat dat betreft net mountainbiken. Vol goede moed van start, zo goed mogelijk voorbereid, met een redelijk idee van hoe je gaat rijden en wat je ongeveer tegen zult komen en vooral nieuwsgierig naar het onbekende. Eenmaal onderweg kom je er achter dat sommige bergen in het echt net wat hoger en steiler zijn dan ze op papier lijken, of dat je af en toe de verkeerde afslag neemt, waardoor je aardig wat extra kilometers voor niets lijkt te hebben gedaan. En af en toe kan het mis gaan en maak je de onvermijdelijke schuiver of ga je 'over the bar'. Maar natuurlijk ook de dingen waar je het voor doet; de momenten dat het lekker loopt, je nieuwe dingen ziet en merkt dat je vooruit gaat en in staat bent om dingen te doen die je eerst niet voor mogelijk hield. Mountainbiken doe je niet alleen en hetzelfde geldt voor promoveren. Je hebt mensen nodig die ervoor zorgen dat je de tocht kunt doen, je de juiste adviezen geven en je steunen als het zwaar is en tegenzit en je materiaal draaiende houden. En het is stukken leuker als je mensen om je heen hebt die dezelfde tocht doen en ook delen in de lol en het plezier, en voor je het weet kom je onderweg ook nog leuke nieuwe mensen tegen.

Allereerst wil ik Fons bedanken; zonder jou was dit boekje er zeker niet gekomen. Bedankt voor alle steun, advies en vertrouwen, ook al leek het er op een gegeven moment op dat het misschien heel anders zou aflopen. Je hebt heel wat dingen achter de schermen geregeld die dit boekje mogelijk hebben gemaakt. Ontzettend bedankt hiervoor. Ik wil ook Antoon en Hauke heel erg bedanken voor alle advies, steun en interesse. Antoon, jou enthousiasme voor onderzoek werkte aanstekelijk en de link met het project van Kees bleek een goede keus. Ook al was je slechts in het begin bij het project betrokken, je interesse in de gang van zaken daarna heb ik zeer gewaardeerd. Hauke, ook al was je in het begin niet bij het project betrokken, je bent ontzettend belangrijk voor me geweest, vooral tijdens het schrijfwerk van dit boekje. Bedankt voor je advies en vaak erg snelle reacties en ook voor het feit dat je de taak van co-promotor op je wilde nemen. Willem ook al was je niet zo direct bij mijn project betrokken, bedankt voor je regelmatige interesse en suggesties.

Ik heb met heel veel plezier rondgelopen bij MicFys en wil iedereen in de wisselende bezetting daarvoor van harte bedanken. In het bijzonder voor het begrip en de steun die ik heb gehad in de periode dat het minder goed ging. Dat heeft ontzettend veel voor me betekend. Mijn 'roomies' Caroline, Miriam en in het eerste uur ook nog Frank. Bedankt voor alle raad, daad en gezelligheid. Anne Meint, John en Kees bedankt voor de uiterst vermakelijke avonden 'Eufraat en Tigris', vooral mede door Anne Meints geniale strategische zetten. En ook het regelmatige mountainbiken, waarbij John zelfs gek genoeg was om me te volgen in het downhill en freeride gebeuren op en vooral vanaf Duitse hellingen. Iets wat ik ontzettend mis ook al woon ik nu in een

van de mountainbike hotspots in de wereld. Anne Meint, dankjewel dat je ook de taak van paranimf op je wilde nemen. Wim D. heel erg bedankt voor de goede samenwerking, input en al het werk dat je in mijn project gestoken hebt. Het maakt een groot en zeer belangrijk deel van dit boekje uit, ik ben daarom ook blij dat je paranimf wilde zijn. Dit boekje bevat ook het werk van de studenten die ik mede heb begeleid als AIO: Vezela, Armand, Roel en ook Martine (ook al was dat niet voor mijn project). Bedankt voor alle inzet en de prettige samenwerking.

Ook alle anderen binnen het Laboratorium voor Microbiologie hartelijk bedankt voor de leuke tijd en alle hulp. De Moleco's heel erg bedankt voor het Vlaamse Reus bezoek en alle andere vormen van de 'hè gezellig'-heid. Kees, Carien en Meta, bedankt voor de nodige afleiding in de vorm van een potje Kolonisten. Kees, erg bedankt voor het op sleeptouw nemen in het begin en voor alle werk en niet werkgebonden 'interacties'. Hans ook heel erg bedankt voor alle hulp. Wim R., bedankt voor alle technische en IT ondersteuning. Francis en Nees, bedankt voor het vaak last-minute afhandelen van alle administratieve en financiële rompslomp (zoals het mede mogelijk maken van mijn trip naar Canada) en Jannie voor het reilen en zeilen van de autoclaven en de keuken.

Het onderzoek in dit boekje was onderdeel van een groter EET-project. Henk, Cris, Wiel, Look en Jan, dank jullie wel voor de prettige samenwerking, de discussies en ideeën. Het onderzoek in dit proefschrift heeft een sterk milieutechnologische inslag. Dit leverde dan ook de nodige gezamenlijke meetings met de sectie Milieutechnologie en Paques op, zoals S-lunches en de zwaveldagen. Bedankt voor de discussies, input, goede sfeer tijdens de meetings. Gatze bedankt voor het zo af en poolshoogte nemen tijdens meetings en het 'planten' van het idee van mijn AIO-schap na mijn afstudeervak. Waarschijnlijk had ik het zonder deze aanmoediging niet gedaan. Alle (oud) milieutechnologen bedankt voor alle vormen van gezelligheid zoals het frequente Loburg-bezoek op vrijdagen.

Iedereen van Haarweg 143 heel erg bedankt, ook al ontgroei je het afdelingsleven als je geen student meer bent, het is toch heel lang een fijn thuis geweest. Sander, dankjewel voor de vriendschap en support, net zoals Arthur, Vincent en Maarten door al de jaren. De Hassertjes, you know who you are, ook erg bedankt voor het regelmatige drankgelach, dat helaas door de jaren en toegenomen onderlinge afstand minder frequent geworden is nu. Judith, ook al zat je andere kant van de Atlantische Oceaan, je bent toch erg belangrijk geweest bij het tot stand komen van dit boekje. De wereld ziet er ineens heel anders uit vanaf de bodem van de Grand Canyon en je steun en relatieveringsvermogen had ik niet willen missen.

A large part of this thesis was written at the same time I was working at the University of Wales, Bangor. I would like to thank Barrie Johnson and Kevin Hallberg for their support and flexibility. Also a big thanks to the other people at BART, 'Shep', Owen, Richy, Stewart, Naoko, and especially 'KC' who was also going through finishing the PhD process. Thanks for support and the really nice time.

Ma en pa, en Heidi, Henk, Mark en Cor voor alle hulp en steun van het thuisfront. Het 'studeren' zit er nu eindelijk op. Misschien dat ik jullie nu ook weer wat vaker te zien krijg nu ik dit eindelijk afgerond heb, ook al woon ik nu op dat eilandje voor de Nederlandse kust.

And finally, Joan. Officially you were not a PhD supervisor during this project, but in reality it felt like having a third supervisor and mental coach on 24 hr standby. Your help and input during the writing of this thesis has been large (allthough not as large as certain individuals at BacGen suspect) **;)** . However, you have been much, much more than that. Thanks, for all your love, support and care and helping me to finish what I started. It was not easy moving to another country, finding and combining a job with finishing this thesis and trying to have a life. But I would not have wanted to do it any other way.

Iedereen heel erg bedankt, cheers!
Bernd

This research was supported by a grant from the E.E.T. program of the Dutch ministries of Economic Affairs, Education, Culture and Sciences and Housing, Spatial Planning and the Environment (EETK98028), and done in cooperation with Paques Biosystems B.V. (Balk, Netherlands) and Zinifex Budel B.V.

Dit onderzoek werd uitgevoerd met subsidie van het Programma E.E.T. (Economie, Ecologie en Technologie) een gezamenlijk initiatief van de Ministeries van Economische Zaken, Onderwijs, Cultuur en Wetenschappen en Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer (EETK98028, Nieuwe waterhuishouding van een zinkfabriek) en uitgevoerd in samenwerking met Paques Biosystems B.V. (Balk, Netherlands) and Zinifex Budel B.V.

Curriculum Vitae

Bernardus, Henricus, Gerardus, Wilhelmina (Bernd) van Houten werd op 7 april 1974 in 's-Hertogenbosch geboren en groeide op 'tussen de rivieren' in Kerkdriel in de Bommelerwaard. Na het behalen van zijn gymnasiumdiploma in 1993 aan het Jeroen Bosch College in 's-Hertogenbosch, begon hij de studie Milieutechnologie aan de HAS Den Bosch. Gedurende deze studie liep hij stage bij Kiwa in Nieuwegein, TNO in Apeldoorn en Ingenieursbureau Oranjewoud B.V. in Almere en sloot die studie 1997 in de richting 'Schone Technologie' met een afstudeeropdracht bij DMV Campina in Veghel.

Op zoek naar meer diepgang begon hij als 'doorstromer' de studie Milieuhygiëne in Wageningen, waarbij de nadruk op milieutechnologie en microbiologie lag. Gedurende een afstudeervak bij de sectie Milieutechnologie van Wageningen Universiteit bestudeerde hij het effect van accumulatie van α-amylase in de vloeistoffase op de hydrolysesnelheid van aardappelzetmeel in anaërobe bioreactoren. Gefascineerd door de anaërobe microbiologie deed hij vervolgens een afstudeervak bij het Laboratorium voor Microbiologie op het gebied van reductieve dehalogenering van gechloreerde verbindingen.

Na het behalen van zijn diploma startte in januari 2000 als AIO bij het Laboratorium voor Microbiologie op het onderzoek beschreven in dit proefschrift. In december 2004 verhuisde hij naar Aberystwyth in Wales en gedurende april 2005 tot april 2006 was hij werkzaam als research officer in het Bangor Acidophile Research Team (BART) aan de University of Wales, Bangor waar hij onderzoek deed aan compost bioreactors en wetlands.