# Microbial community analysis in sludge of anaerobic wastewater treatment systems

Integrated culture-dependent and culture-independent approaches

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# Abstract

The need for clean water is increasing and anaerobic wastewater treatment can be used as a cost-effective solution for purification of organically polluted industrial waste streams. This thesis presents results from microbiological investigations of several full-scale and lab-scale anaerobic wastewater treatments systems. Anaerobic wastewater treatment has gained popularity and is now one of the key technologies in environmental biotechnology. However, knowledge of the microbial community structure – function relationships is limited. A combination of cultivation-dependent and cultivation-independent techniques can be used to improve this knowledge. In this thesis, batch serial dilution incubations from a methanol-fed lab-scale thermophilic (55°C) methanogenic bioreactor indicated that syntrophic interspecies hydrogen transfer-dependent methanol conversion is at least equally important as direct methanogenesis in this lab-scale reactor. A direct methanol-utilizing *Methanomethylovorans hollandica*-related strain was detected up to a $10^8$-fold dilution, while *Thermodesulfovibrio* relatives and *Methanothermobacter thermoautotrophicus* strains were found till $10^9$–fold dilutions in the presence of $H_2/CO_2$. Microbial diversity was further evaluated in two expanded granular sludge bed reactors fed with increasing oleic acid loading rates. The archaeal community in the reactor inoculated with granular sludge stayed quite stable and active, whereas the relative abundance of *Methanosaeta*-like organisms gradually decreased in the reactor inoculated with suspended sludge when oleate loads were increased to 8 kg of chemical oxygen demand $m^{-3}$ $day^{-1}$. *Desulfomicrobium* and *Methanobacterium* were found to dominate the start-up of a full-scale synthesis gas fed gas-lift reactor treating metal and sulphate rich wastewater. Most Probable Number (MPN) counts confirmed that heterotrophic sulphate reducing bacteria (SRB) 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). Methanogens can still persist in sulphate-reducing bioreactors with short sludge retention time, since competition for hydrogen is determined by Monod kinetics and not by hydrogen threshold values. The microbial community in a full-scale upflow anaerobic sludge blanket reactor treating paper mill wastewater operated at 37°C was relatively stable over a period of 3 years as indicated by a high similarity (>75%) of denaturing gradient gel electrophoresis profiles of 16S ribosomal RNA gene fragments. Batch incubations at different temperatures resulted in microbial community changes. While the archaeal community composition differed significantly between incubations at 45 and 55°C, the bacterial composition changed between 37 and 45°C. Overall the bacterial community was dominated by *Firmicutes* (68% of the clones) and *Delta-Proteobacteria* (17% of the clones). A sequential degradation of first butyrate and then propionate at 37°C was linked to strong presence of *Syntrophomonas* sp. and *Desulfobulbus propionicus*, respectively. MPN series allowed estimating the number of micro-organisms per ml sludge that could use propionate without sulphate ($10^9$), propionate and sulphate ($10^5$), butyrate without sulphate ($10^8$), butyrate with sulphate ($10^5$), glucose ($10^9$) and $H_2/CO_2$ ($10^{10}$). *Archaea* were mainly dominated by *Methanosaeta*, but also *Crenarchaeota*-relatives were identified. Bacterial clone sequences were related to a variety of different known species, with expected functions in anaerobic digestion like fermentative bacteria, syntrophic short chain fatty acids oxidisers and SRB. However, about 80% of the clones was similar to sequences in the database without close cultured relatives, but many of these appeared to be present in anaerobic environments. It is important to

improve knowledge of these unknown micro-organisms and fast accurate monitoring and identification could be instrumental in realising this. Therefore, a pilot macro-array was developed and tested. It appeared that combining probes generated by PCR amplification of the V1 and V6 variable regions of the 16S rRNA gene provided accurate differentiation of closely related organisms. The integrated application of molecular and cultivation dependent analyses of microbiota structure and function of a broad variety of anaerobic wastewater treatment systems described in this thesis has been used to improve insight of the ecophysiology in such reactors. Some general commonalities of anaerobic systems have been found, but also system-specific characteristics. This provides potential identification of general and system-specific indicator populations, allowing improved diagnostics and reactor predictability.

# Content

# Preface and thesis outline

The research presented in this thesis was financed by the Technology Foundation, STW, applied science division of NWO, the Netherlands, and entitled: 'Molecular monitoring of microbial diversity in anaerobic wastewater treatment systems'. Anaerobic wastewater treatment is a commonly applied technology for the purification of industrial wastewater with high organic matter content. Relatively small, high-rate reactors with biomass retention are applied for an efficient conversion of organic compounds into methane and carbon dioxide. The Upflow Anaerobic Sludge Blanket (UASB) reactor is the most common anaerobic reactor type, but new, more efficient reactors with biomass retention have been developed. The microbial composition of anaerobic bioreactors is rather complex. Besides conventional culture-dependent microbiological methods culture-independent molecular biological methods, mostly targeting 16S ribosomal RNA and the encoding gene, can be used nowadays to investigate the microbial composition of anaerobic bioreactors. The research described in this thesis mainly focused on fatty acid-degrading communities which form the core of all methanogenic bioreactors. Syntrophic micro-organisms rely on other organisms, such as sulphate reducing bacteria (SRB) or methanogens, for the removal of their metabolic products. These SRB and methanogens benefit from the products produced by syntrophs, which results in a delicate balance and fierce competition. Improved knowledge of these organisms and their interactions is important for wastewater treatment reactor stability and control.

**Chapter 1** gives an overview of current knowledge of the ecophysiology of micro-organisms in anaerobic wastewater treatment systems. A short description of different applications of anaerobic wastewater treatments is given, followed by a description of tools and techniques to study micro-organisms in such ecosystems, including culture-dependent and especially molecular approaches that can be used to provide insight into microbial community structure – function relationships that govern wastewater treatment processes. Advantages and disadvantages of the various approaches are discussed. A summary of known cultivated key micro-organisms in anaerobic wastewater treatment plants is given, followed by a detailed description of published molecular inventories of the microbial diversity in such systems. Furthermore, **chapter 1** provides an outlook on emerging integrated approaches, stressing the need for concerted applications of innovative culture-dependent as well as culture-independent techniques, pivotal to achieve a better understanding and predictability of anaerobic wastewater treatment processes and reactor performance.

Culture-dependent and culture-independent techniques have been applied to study the bacterial and archaeal population dynamics in anaerobic sludge, incubated at different temperatures with different feed mixtures, as described in **chapter 2**. The biochemical results of the incubations were compared with the microbial community shifts using Denaturing Gradient Gel Electrophoresis (DGGE) fingerprinting and subsequent cloning and sequencing analysis (324 bacterial clones analysed). With this approach the identity of microbes could be linked to their function in the mixed communities.

In **chapter 3** micro-organisms linked to direct and indirect methane formation from methanol in a lab-scale thermophilic (55°C) bioreactor were investigated. Reactor sludge was disrupted and serial dilutions were incubated in specific growth media containing methanol and possible intermediates of methanol degradation as substrates.

Dominant micro-organisms in the different dilutions were identified by 16S rRNA-gene sequence analysis. Furthermore, dilution PCR was used to determine the relative abundance of *Archaea* and *Bacteria* in all investigated samples.

In other lab-scale reactors the influence of granular and suspended sludge as inoculum was investigated. Bacterial and archaeal diversity was evaluated in two expanded granular sludge bed reactors fed with increasing oleic acid loading rates up to 8 kg of chemical oxygen demand $m^{-3}$ $day^{-1}$ as described in **chapter 4**. During operation, the sludge in both reactors was segregated in two layers: a bottom settled one and a top floating one. Both layers in the two reactors were microbiologically characterised.

The start-up of a full-scale synthesis gas fed gas-lift reactor treating metal and sulphate rich wastewater was investigated and presented in **chapter 5**. Most Probable Number (MPN) counts were used to determine the abundance of heterotrophic SRB, homoacetogens and methanogens. Furthermore, 16S rRNA gene analysis was applied to identify the dominant microbial groups, and an explanation for the co-existence of SRB and methanogens was given.

To get insight into the microbial community of a full-scale upflow anaerobic sludge blanket reactor treating paper mill wastewater, conventional microbiological methods were combined with 16S rRNA gene analyses, as described in **chapter 6**. Particular attention was paid to micro-organisms able to degrade propionate or butyrate in the presence or absence of sulphate. Serial enrichment dilutions allowed estimating the number of micro-organisms per ml sludge and quantitative RNA dot-blot hybridisation revealed the ratio *Archaea*/*Bacteria*. The microbial community composition was further characterised by 16S rRNA-gene-targeted DGGE fingerprinting, and via cloning and sequencing of the most dominant amplicons from the bacterial and archaeal patterns.

**Chapter 7** describes the integrated application of more extensive bio-molecular and cultivation-based experiments to further improve the knowledge on complex microbial community structure - function relationships in the full-scale anaerobic paper-mill wastewater treatment system addressed in previous chapters 2 and 6. MPN series allowed estimating the number of micro-organisms per ml sludge that could use propionate with and without sulphate, butyrate with and without sulphate, glucose and $H_2/CO_2$. Cloning of 16S rRNA genes, screening of clones with amplified ribosomal DNA restriction analysis and partial sequencing revealed the expected high species richness. From this clone library a set of 121 abundant and not closely related clones was nearly completely sequenced. This information was used to develop and test a pilot macro-array to provide the means for high throughput microbial community analysis tools. Such tools could prove useful for microbial diagnostics in environmental biotechnology towards an improved ability to predict functionality and failure of anaerobic reactor systems.

Finally, the results obtained in the different chapters are discussed. In general, a combination of culture-dependent and culture-independent microbiological techniques has been used to get insight of the ecophysiology of anaerobic bioreactors. Since these bioreactors were mainly methanogenic, expected methanogenic *Archaea* such as *Methanosarcinales* (mainly *Methanosaeta*) and *Methanobacteriales*, with known functions were detected, but also members of the *Crenarchaeota* have been found.

Further research is needed to elucidate the functional role of these *Crenarchaeota* in anaerobic wastewater treatment. Besides expected fermenting bacteria, syntrophic short chain fatty acids oxidisers and SRB, many bacterial phylotypes have been detected with unknown function. Advanced molecular techniques have been applied for rapid monitoring and identification of the microbes present. Still, integrated multidisciplinary research approaches are needed to understand and control useful (and less-wanted) microbes in anaerobic environments, such as engineered wastewater treatment systems.

# Chapter 1

## Microbial ecophysiology of anaerobic wastewater treatment systems - State of the art and approaches towards improved understanding of structure-function relationships

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## Abstract

Anaerobic wastewater treatment has gained popularity and is now one of the key technologies in environmental biotechnology. Nevertheless, while system design has improved and efficient conversions are achieved, fundamental background knowledge on the ruling biological processes is often lacking. This paper gives an overview of current knowledge of microbial ecophysiology in anaerobic wastewater treatment systems and focuses mainly on cultivation-independent molecular techniques that can be used for improved understanding of microbial community structure – function relationships that govern wastewater treatment processes. First a short overview of different applications of anaerobic wastewater treatments is given, followed by a description of tools and techniques for the study of micro-organisms in such ecosystems, including culture-dependent and molecular approaches. Advantages and disadvantages of the various approaches are discussed. A summary of known cultivated key micro-organisms in anaerobic wastewater treatment plants is given, followed by a detailed description of published molecular inventories of the microbial diversity in these systems. With cultivation-independent studies many unknown micro-organisms have been detected. Comparison of these studies showed that anaerobic wastewater treatment systems are in general dominated by low-GC Gram-positives, the *Cytophaga-Flexibacter-Bacteroides* (CFB) group, and *DeltaProteobacteria*. *Methanosarcinales* (mainly *Methanosaeta*) dominate usually the archaeal community, but also *Methanobacteriales* and unexpected *Crenarchaea* are regularly found in anaerobic wastewater treatment systems. Finally, an outlook is provided on emerging integrated approaches, stressing the need for concerted applications of innovative culture-dependent as well as culture-independent techniques. The knowledge on structure and functionality of bioreactor microbiota that can be expected from such novel strategies will be pivotal to a better understanding and predictability of anaerobic wastewater treatment processes and reactor performance.

## Anaerobic wastewater treatment

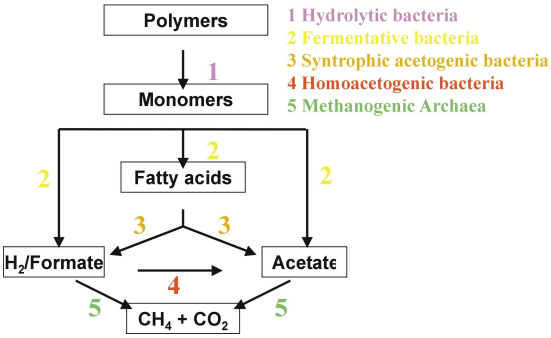
Anaerobic wastewater treatment is considered the most cost-effective solution for organically polluted industrial waste streams [van Lier *et al.* 2001], and has gained interest due to increasing energy prices and more stringent legislation for the discharge of industrial wastewater, since the 1970's [Lettinga *et al.* 1995]. Anaerobic treatment processes are known for the unique ability to convert highly objectionable wastes into useful products [McCarty 2001]. Treatment of industrial wastewater in anaerobic bioreactors has grown especially in importance since the introduction of the Upflow Anaerobic Sludge Blanket (UASB) reactor about 25 years ago. In an UASB reactor the wastewater is pumped from the bottom into the reactor, and is purified while passing a bed of biomass (sludge), which is retained in the reactor by self-immobilization of anaerobic micro-organisms into densely packed granules [Lettinga *et al.* 1980]. Sedimentation of these granules prevents the micro-organisms from being washed out, while the short inter-microbial distances create short diffusion distances resulting in high conversion rates. Even though a variety of additional anaerobic bioreactor designs has now been developed, UASB reactors are worldwide still the most common [Franklin 2001]. Due to improved performance and economic efficiency, however, Expanded Granular Sludge Blanket (EGSB) and Internal

Circulation (IC) reactors are currently replacing more conventional UASB systems [Franklin 2001]. EGSB systems have a comparable design as UASB reactors, but contain an expanded granular sludge bed allowing more circulation and interaction between the organic compounds and the micro-organisms in the sludge granules. Presently, more than 70% of the anaerobic reactors worldwide are UASB or EGSB [Franklin 2001]. Anaerobic wastewater treatment systems can operate at different temperatures and convert a broad variety of wastes. First mainly applied in food- and related industries, anaerobic digestion is also massively used in the treatment of sewage, and in the pulp and paper industry. Recently it is also more frequenctly applied to waste streams from chemical and petrochemical industries [Macarie 2000]. The following review will describe several of these anaerobic wastewater treatment systems and the microbial communities responsible for the actual anaerobic conversions. Approaches to study the microbiota present in the anaerobic wastewater treatment systems, and their actual metabolic activity, are discussed and finally an outlook is provided on emerging integrated approaches, stressing the need for concerted applications of innovative culture-dependent as well as culture-independent techniques. The knowledge on structure and functionality of bioreactor microbiota that can be expected from such novel strategies will be important for improved understanding and predictability of anaerobic wastewater treatment processes and reactor performance.

## Methanogenic wastewater treatment systems

In methanogenic environments, organic compounds are degraded in the absence of light and inorganic electron acceptors such as oxygen, sulphate and nitrate, and only bicarbonate and protons act as terminal electron acceptors [De Bok *et al.* 2004, Stams *et al.* 2006]. Different physiological microbial groups are active in methanogenic bioreactors to mineralize (complex) organic material to methane and carbon dioxide (Fig. 1a) [Gujer & Zehnder 1983]. Biopolymers like proteins, carbohydrates, nucleic acids and fats are first hydrolyzed to mono- and oligomers, and then fermented to products which can be used by methanogens directly (acetate, hydrogen, formate) and to reduced organic compounds such as propionate, butyrate, long-chain fatty acids, alcohols, lactate and succinate [Gujer & Zehnder 1983]. Acetogenic bacteria oxidize higher fatty acids anaerobically to acetate, $CO_2$, $H_2$ and formate [Gujer & Zehnder 1983]. Acetogenic bacteria can also oxidize lactate and ethanol to acetate, but fermentation of these compounds to e.g. acetate and propionate is also possible [Laanbroek *et al.* 1982]. Succinate can be decarboxylated to propionate [Schink & Pfennig 1982]. Acetate, formate and $H_2$ are converted by methanogens to methane and $CO_2$ [Schink 1997]. Volatile fatty acids are known to be important intermediates in the degradation of organic matter under methanogenic conditions. About 70% of the reducing equivalents formed in the anaerobic digestion process are transferred via acetate to methane [Gujer & Zehnder 1983]. Since 4 $H_2$, but only 1 acetate, are required to produce 1 $CH_4$, the contribution of $H_2$ to methanogenesis during anaerobic degradation of carbohydrates can maximally be 33% of the total $CH_4$ formed [Conrad 1999]. Propionate and butyrate are key intermediates in the mineralization of complex organic matter. The complete oxidation of propionate and butyrate can account for 20 to 43% of the total methane formation, depending on the type of digester and the nature of the organic compounds [Mackie & Bryant 1981].

a.

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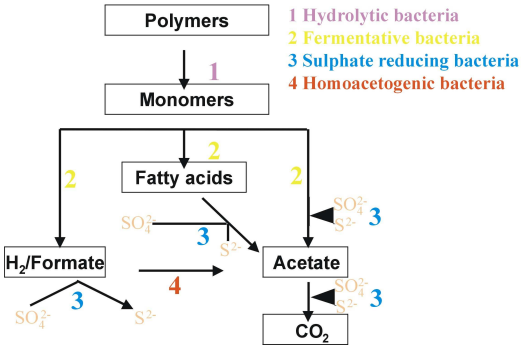



Figure 1: Anaerobic degradation of organic compounds under methanogenic (a) and suphidogenic (b) conditions.

## Sulphidogenic wastewater treatment systems

Sulphate reducing bacteria (SRB) are able to couple the oxidation of organic compounds and $H_2$ to sulphate reduction (Fig. 1b). SRB are a polyphyletic group of often physiologically versatile organisms that are not restricted to sulphate reduction [Castro *et al.* 2000]. In anaerobic bioreactors, SRB can compete with methanogens and homoacetogens for common substrates. In the presence of sulphate, SRB compete with methanogens and acetogens for compounds like formate and $H_2$, and propionate and butyrate, respectively [Oude Elferink *et al.* 1994, Colleran *et al.* 1995, Stams *et al.* 2005]. There is a change from mainly methanogenic conversion to mainly sulphidogenic conversion of organic compounds like methanol in the temperature range from 37 to 65ºC [Rintala & Lettinga 1992, Visser *et al.* 1993, Weijma *et al.* 2000]. Besides the temperature, the COD/$SO_4^{2-}$ ratio also influences the competition between methanogenesis and sulphidogenesis. At a COD/$SO_4^{2-}$ ratio (g/g) lower than 0.67, in theory all the organic material can be degraded via sulphate reduction [Harada *et al.* 1994]. Even in methanogenic reactors, SRB can be present in relatively high numbers [Raskin *et al.* 1996]. It is known that several SRB have a fermentative and acetogenic metabolism [Widdel & Hansen 1992]. This could explain the fast response of methanogenic ecosystems after the addition of sulphate. Many other factors, including pH, medium composition, hydrogen sulphides ($H_2S$) concentration and growth kinetics can influence the competition between SRB and other microbial groups.

One of the major problems to be overcome in anaerobic treatment is toxicity of components in various industrial wastewaters, both in terms of discharge into the environment, and with respect to impaired reactor performance. SRB convert sulphate to $H_2S$, which, like other gaseous sulphur compounds (e.g. volatile organic sulphur compounds such as dimethyl sulphide and mercaptans), are toxic, corrosive and odorous. Hence their formation has to be avoided, in contrast to carbon ($CO_2$/$CH_4$) and nitrogen ($N_2$) gaseous end products [Vallero *et al.* 2003]. Driven by this prerequisite, also microbial conversions of the sulphur cycle were successfully applied in the last two decades. In addition, microbial conversion techniques were developed in a range of environmental biotechnological processes to avoid pollution by sulphurous compounds, heavy metals, xenobiotics and nitrogen compounds with clear opportunities for recovery of useful compounds [Vallero *et al.* 2003, Van Houten *et al.* 2006]. Sulphuric compounds also impair process performance, and studies under both mesophilic and thermophilic conditions showed that granular sludge is less

inhibited by $H_2S$ than suspended sludges at low and neutral pH, whereas the inhibition is very similar at high pH values [Visser *et al.* 1996].

## High temperature wastewater treatment systems

While currently most systems are operated under mesophilic conditions, there is increasing interest in high temperature wastewater treatment. Especially when the effluent wastewater from industrial processes is discharged at high temperatures, wastewater treatment at thermophilic conditions has advantages. There is no energy loss to lower the temperature of the wastewater and growth rates of micro-organisms might increase, so that higher conversion rates can be obtained, but maintenance requirements may also increase, resulting in low sludge production rates and formation of granular sludge [Mulder *et al.* 2001]. However, as different types of wastewaters are treated and different treatment systems are used, it is hardly possible to judge whether mesophilic or thermophilic treatment is more appropriate. From an engineering point of view, a thermophilic process is defined as any process that is operated at a temperature exceeding 45ºC [LaParra & Alleman 1999]. Also, thermophiles are defined as micro-organisms that grow at temperatures above 45ºC. However, some organisms with a wide temperature range for growth, extended over the mesophilic and thermophilic temperature range are found [Wiegel 1990]. Temperature changes can be expected to cause a shift in microbial composition of mixed cultures, because of differences in optimal growth temperatures and growth temperature ranges. A clear example is the rapid shift between methanogenesis and sulphate reduction in UASB reactors with sulphate as electron acceptor, caused by temperature increase from the mesophilic to thermophilic ranges [Rintala & Lettinga 1992, Visser *et al.* 1993, Weijma *et al.* 2000].
Most high temperature wastewater treatment reactors are inoculated with seed sludge from mesophilic sources. This fits with the idea that moderate thermophiles are ubiquitously present in the environment [Sonnleitner & Fiechter 1983]. However, when bioreactors were compared that were inoculated with sludge from a mesophilic activated sludge plant and operated in parallel at different temperatures, a decrease in species richness with increasing temperatures was observed [Konopka *et al.* 1999, LaParra *et al.* 2000]. It is not surprising that a mesophilic inoculum contains more mesophilic micro-organisms than thermophiles and it may be expected that more extreme environmental conditions will reduce the microbial diversity. Operation at elevated temperatures also affects the thermodynamics of key processes. At thermophilic conditions, reactions dependent on the $H_2$ partial pressure, such as the oxidation of propionate and butyrate, are possible at higher $H_2$ concentrations in the biogas, because of lower Gibbs free energy changes [Zinder 1990]. $H_2$ formation becomes energetically more favourable at higher temperatures, whereas $H_2$ consumption by methanogens becomes less favourable [De Bok *et al.* 2004]. Studies showed that at high temperatures (> 70ºC) approximately 95% of the total acetate conversion could be attributed to syntrophic acetate oxidation [Van Lier 1995, Ahring *et al.* 1995].

## Low temperature wastewater treatment systems

Recently, some research on anaerobic wastewater treatment at psychrophilic conditions has been conducted. Several industrial, but also municipal wastewater streams are discharged at low temperatures. Furthermore, at many geographical sites wastewater treatment systems have to operate at low ambient temperatures. Increasing the temperature of the wastewater would require a considerable energy input, which might not be necessary as efficient wastewater treatment at low temperatures has been demonstrated. However, information on, for example, granulation or the impact of seasonal temperatures on the long-term microbial stability of the process remains scarce [O'Flaherty *et al.* 2006]. Comparable to the micro-organisms found at thermophilic conditions, several micro-organisms found at psychrophilic conditions showed an extended temperature range for growth from psychrophilic to mesophilic, and reactors operated at psychrophilic conditions contain mainly psychrotolerant biomass [Lettinga *et al.* 1999, McHugh *et al.* 2003b, Connaughton *et al.* 2006]. The feasibility of high-rate anaerobic reactor systems for cold wastewaters depends primarily on the quality of the seed sludge in the reactors used and its development under psychrophilic conditions, the nature of the organic pollutants in the wastewater, and the reactor configuration, especially its capacity to retain viable sludge [Lettinga *et al.* 1999]. EGSB systems show impressive practical prospects, particularly for very low strength wastewaters (COD < 1 g/l) and for lower temperatures (around 10ºC) [Lettinga 1995]. Propionate-oxidation proved to be most sensitive in a psychrophilic anaerobic treatment and is directly related to the activity of acetoclastic methanogens [Lettinga *et al.* 1999]. Voolapalli and Stuckey (1999) also showed a strong relation between these micro-organisms. Furthermore, there are indications that psychrophilic methane production proceeds mainly via the hydrogen pathway [Connaughton *et al.* 2006].

## Culture-dependent approaches

Culture-dependent approaches are used for the retrieval and detailed investigation of micro-organisms present in ecosystems. Ideally also the activity and function of micro-organisms can be investigated by cultivation and/or by *in situ* measurements. Physiological and biochemical properties can be used for the identification and characterization of micro-organisms. Detailed physiological studies, which are often important in ecological research, almost always require cultivation of relevant micro-organisms [Briones & Raskin 2003]. Cultivation techniques have seen amazing improvements in the last decennia, allowing the cultivation of numerous, previously uncultured, micro-organisms. The development of anaerobic cultivation techniques, for example the roll tube method [Hungate 1969] and mass culture of hydrogen-oxidising methanogens [Bryant *et al.* 1968], has resulted in the isolation of a great number of organisms from anaerobic wastewater treatment systems. More recently, Valentine *et al.* (2000) developed a cultivation device for maintaining $H_2$ at sub-nanomolar concentrations, providing a method for the study of interspecies hydrogen transfer by externally fulfilling the thermodynamic requirement for low $H_2$ concentrations. It is also possible to propagate defined co-cultures of syntrophic micro-organisms, and although it is complicated to study the metabolism of these micro-organisms separately, these techniques have facilitated the generation of knowledge about complicated microbial assemblages [Boone & Bryant 1980]. The

number of microbial isolates has increased considerably, in particular because of improved knowledge of microbial metabolism as well as characteristics of ecological niches at which specific populations are naturally occurring. This enabled researchers to design better isolation strategies. Also, information about the genetic potential of uncultivated organisms derived from genomic or metagenomic sequences can be used to predict metabolic interdependencies and nutritional requirements [Tyson & Banfield 2005]. Another important driving force in the development and successful application of novel cultivation approaches is the growing interest of industry in exploiting the metabolic capacity of yet unknown micro-organisms and their enzymes (bioprospecting [Keller & Zengler 2004]). For example, various processes for high cell-density cultivation have been developed, which made the optimisation of formation of bioproducts possible [Park 2004]. Conventional isolation and cultivation methods are very sensitive techniques to detect micro-organisms that easily grow in pure culture and that can be cultivated on selective media. Theoretically each viable cell can be multiplied and may be detected as a colony on a selective agar plate, and by combining different cultivation techniques, it is possible to detect and identify very low numbers of micro-organisms [Akkermans *et al.* 1994]. The fraction of cells capable of multiplication (cultivability) on solid media can be quantified as colony forming units (CFU) [Bruns *et al.* 2003]. Samples of naturally occurring microbial communities are used as inoculum for laboratory-prepared growth media that are designed to select a small subset of the initial community and therefore an enrichment of certain micro-organisms is achieved. Since many micro-organisms prefer liquid media, most probable number (MPN) dilution series are often used for the quantification and enrichment of target populations. The highest dilutions of MPN series with detectable growth provide enrichments or even pure cultures of abundant, but fastidious bacteria which are missed in conventional enrichment trials, since dominant organisms are frequently overgrown by accompanying species which are less abundant, but grow faster under laboratory conditions. However, a principal limitation of the MPN method is that only a small number of parallel dilution series can be processed for each sample, which limits the number of potential isolates and causes a large statistical uncertainty. To overcome these limitations a novel approach for high throughput cultivation was developed. With a MicroDrop® micro-dispenser system droplets of 150-200 pl are created at the nozzle of a glass micropipette by means of a computer-driven piezo transducer, and are dispensed automatically at predetermined positions with the aid of a XYZ-positioning system, so that the inoculation of 96 samples takes less than one minute. Bruns *et al.* (2003) compared the MicroDrop technique with the MPN technique and found that the MicroDrop technique tends to yield cultivation efficiencies which are a factor of 2.7 ($r^2 = 0.570$, level of significance $P < 0.001$) lower. This may be due to an underestimation of cultivable bacteria by the MicroDrop technique and/or from an overestimation by the MPN method, caused by high surface tension and shear forces or clumps of micro-organisms respectively. However, the MicroDrop technique makes high throughput cultivation possible, since many cultures can be started in parallel. In another study microbial cells were encapsulated in gel microdroplets for massive parallel microbial cultivation under low nutrient flux conditions, followed by flow cytometry to detect microdroplets containing microcolonies [Zengler *et al.* 2002]. Gel microdroplets separate micro-organisms from each other while still allowing the free flow of metabolites and signalling molecules between different microcolonies. Therefore, this method might be also applicable for the analysis of interactions between different micro-organisms under *in situ* conditions [Zengler *et al.* 2002]. Straightforward

techniques like the micromanipulator or a Fluorescence Activated Cell Sorter (FACS) can also be used for physical separation of micro-organisms from mixed cultures [Fröhlich & König 1999, Park *et al.* 2005].
Nevertheless, ideal growth conditions and growth requirements are not very well known for most micro-organisms. The validity of measurements conducted on microbial communities removed from their original field setting is uncertain; because it is not sure that conditions imposed on the native micro-organisms (post-sampling and incubation) have not quantitatively or qualitatively altered these populations and their physiological reactions [Madsen 2005]. Therefore, cultivation will always be selective for certain micro-organisms and will be normally not comparable to the natural ecosystem from which the sample is derived (e.g. high concentrations of nutrients). Only 27 out of 53 bacterial phyla contain previously cultivated micro-organisms, with many phyla represented by only a few isolates and some phyla containing only one described species [Keller & Zengler 2004, Rappé & Giovannoni 2003]. Current inventories list about 8200 validly classified species of prokaryotes. The inability to isolate and cultivate many types of microbes has long limited the range of micro-organisms that are available for further analysis. Although the vast majority of microbes resist cultivation by traditional methods, it has been proposed that many more strains could be isolated using novel imaginative approaches. However, it is well known that many micro-organisms do not grow under conditions routinely used in the laboratory, and growth conditions of most micro-organisms are actually not known. Besides unknown growth requirements, stress caused by the cultivation procedures or obligatory interactions of micro-organisms with each other may be additional reasons that hamper cultivation. Still, the ability to study individual strains from different environments under laboratory conditions is often essential to obtain insight in their metabolic function. If methods are found both to reveal the form of microbial interdependencies and to simulate them in the laboratory, cultivation of targeted strains can be achieved [Tyson & Banfield 2005]. The combination of methods for the direct description of microbial communities with traditional methods for enrichment and isolation of important strains is, and will stay powerful in future research.

## Culture-independent approaches

Although cultivation of micro-organisms is important and researchers have dedicated themselves to this task for several decades; most micro-organisms have only been detected with culture-independent techniques. There are now many major groups of Bacteria and Archaea known only from sequences directly retrieved from environmental samples, and, until these organisms can be cultivated, the only means of understanding their role in the environment is through culture-independent characterization linked to determination of *in situ* metabolic activity [Gray & Head 2001]. Although most micro-organisms are not uncultivable, unfortunately, growth conditions of most of them are not yet known. This is not only due to methodological limitations, but also due to a lack of taxonomic knowledge. It is difficult to study the diversity of a group of micro-organisms when it is not understood how to categorize or identify the species present [Kirk *et al.* 2004]. The recognition of the limitation to isolate a major part of the microbiota resulted in the development of a novel research area called ‘Molecular Microbial Ecology’ [Akkermans *et al.* 1996]. The difference between this and classical microbial ecology is that microbial ecosystems are now

being studied as a whole using culture-independent approaches [Zoetendal *et al.* 2004a]. The field of molecular microbial ecology is defined as the application of molecular technology, typically based on comparative nucleic acid sequence analysis, to identify specific micro-organisms in a particular environment, to assign functional roles to these micro-organisms, and to assess their significance or contribution to environmental processes independent from cultivation [Zoetendal *et al.* 2004b]. Microbial diversity describes complexity and variability at different levels of biological organisation [Torsvik & Ovreas 2002]. It encompasses genetic variability within taxa (species), and the number (richness) and relative abundance (evenness) of taxa and functional groups (guilds) in communities. Molecular techniques provide an excellent method for the rapid and quantitative monitoring of microbes in their communities and allow the investigation of spatial and temporal community changes within microbial ecosystems [Sekiguchi *et al.* 2001b, O'Flaherty *et al.* 2006].

One of the most basic culture-independent microbiological techniques is microscopy, pioneered by Antonie van Leeuwenhoek (1632-1723). Although the first use was limited by microscope magnification, nowadays micro-organisms can be morphologically studied with a whole range of microscopes (light and fluorescence microscopes, scanning and transmission electron microscopes (SEM and TEM), atomic force microscopes (AFM) and confocal laser scanning microscopes (CLSM). However, limited morphological variation renders the identification and differentiation of an estimated 1 – 10 million different species in the world merely based on structural features impossible [Pace 1996].

With the help of specialized and powerful molecular techniques, specific microbial population dynamics and activities can be monitored. Microbial ecologists now have at their disposal a raft of complementary culture-independent methods that can be used to investigate microbial function, even *in situ* [Gray & Head 2001]. Cultivation might be neither necessary nor the most effective approach for understanding of the roles of the incredibly vast number of micro-organisms in the natural environment where comprehensive genomic information can be obtained and it could be possible to rely upon cultivation-independent methods to investigate physiology and activity directly [Tyson & Banfield 2005]. It has been shown that ecological interactions are an essential element to be considered in studying microbial physiology and that functional genomics approaches can be used to complement classical microbiological methods for this purpose [Johnson *et al.* 2006]. Although quantification is still difficult, several ambitious attempts to achieve this are conducted. Significant biases are potentially introduced at various stages of molecular ecological approaches that make quantification difficult. Already during nucleic acid extraction, differences in lyses efficiency for different microbial populations can skew their representation in the nucleic acid pool [Vaughan *et al.* 2000]. The degree of cell lysis therefore should be determined independently, which can be done by microscopic enumeration of cells in an environmental sample before and after lysis treatment [Theron & Cloete 2000]. Typically, a direct cell lysis approach to DNA/RNA extraction and purification may be dissected into the following conceptual steps: washing the material to remove soluble components that may impair manipulation of subsequently isolated nucleic acids; disruption of cells in the material to release DNA or RNA; separation of the DNA or RNA from solids; and isolation and purification of the released nucleic acids for downstream molecular procedures [Moré *et al.* 1994].

Most culture-independent approaches for detection, identification and quantification make use of ribosomal RNA (rRNA) and the corresponding gene as a phylogenetic marker. Ribosomal RNA is present in all organisms and exhibits sufficient sequence

variation to distinguish between different species, while providing regions of conservation at different levels of taxonomic resolution. Molecular tools based on 16S rRNA gene identification have revolutionised microbial ecology [Dabert *et al.* 2002]. A limitation of the rRNA-based techniques, however, is the development of primers and probes, since specific probes are designed based on the comparison between sequences present in the database [Dabert *et al.* 2002]. Another limitation of the use of the 16S rRNA gene is that it only gives an indication about the presence and abundance of micro-organisms (see also http://rrndb.cme.msu.edu [Klappenbach *et al.* 2001]), but not about their activity. Simultaneous monitoring of process performance and variations in microbial community structure can solve this limitation. It also has been observed that the amount of rRNA per cell is roughly proportional to the metabolic activity of the cell, although this is not universal for all micro-organisms and can vary with growth phase, since some microbial groups have a persistent high level of ribosomes, even in starving cells [Amann *et al.* 1995, Farrelly *et al.* 1995, Flärdh *et al.* 1992, Vaughan *et al.* 2000, Wagner 1994b].

PCR-based approaches

Polymerase Chain Reaction (PCR) is the basic technique in most molecular laboratories. With the advent of PCR [Saiki *et al.* 1988], a method became available to study genetic information in an efficient way. With specific primers, perfect amplification of virtually all nucleotide sequences is possible. Functional genes from different micro-organisms can be amplified, but for molecular identification of microbes the 16S rRNA gene is mostly used. At a length of approximately 1500 nucleotides, 16S rRNA genes contain sufficient information for reliable phylogenetic analysis [Amann *et al.* 1995]. An advantage of the 16S rRNA sequences is the existence of a large database with currently about 500,000 (> 300 bp) small subunit rRNA sequences (www.arb-silva.de), which can be used for the design of primers [Ludwig *et al.* 2004]. Since the 16S rRNA gene has conserved regions, with the same sequence in many micro-organisms, and variable regions, which sequences can be species specific, amplification and hence detection at different levels of taxonomic specificity can be achieved. The use of conserved 16S rRNA sequences also allows detection of previously unknown and uncultured micro-organisms. However, only limited conclusions regarding the biogeography and ecophysiology of uncultured bacteria can be drawn from the mere presence or absence of their 16S rRNA sequences in an ecosystem [Jaspers & Overmann 2004]. Moreover, it is known that PCR can cause biases, for example by preferential amplification of certain sequences [Head *et al.* 1998]. During exponential amplification of the mixture of DNA templates, ratio discrepancies between the amplified 16S rRNA gene fragments and the original mixture, caused by differences in *Taq* polymerase activity with varying G+C-content may occur. While primers with 70% similarity to target sequences from pure cultures are sufficient for successful annealing and amplification, poor complementarity of ‘universal’ primers, especially at the 3’ end, will result in under representation of these sequences in the final PCR product [Baker *et al.* 2003, Polz & Cavanaugh 1998, Stern & Holland 1993]. Furthermore, chimeric sequences and other PCR-generated artefacts may arise during amplification, especially from complex microbial communities with several similar phylotypes [Hugenholtz & Huber 2003, Osborne *et al.* 2005]. A Ligase Chain Reaction (LCR) can have greater specificity than PCR, since two oligonucleotides are used for each DNA strand and are ligated together to form a single primer [Barany 1991a/b]. LCR uses both a DNA ligase enzyme and a DNA polymerase to perform the reaction.

Real-time- or quantitative PCR is based on the continuous monitoring of changes of fluorescence in the PCR tube during PCR and, in contrast to the conventional end-point detection PCR, quantification occurs during the exponential phase of amplification [Malinen *et al.* 2003]. Thus, the bias often observed in the PCR template-to-product ratios can be largely avoided [Suzuki & Giovannoni 1996]. Real-time PCR was developed in 1992 and made use of ethidium bromide and a fiberoptic cable connected to a spectrofluorometer [Higuchi *et al.* 1992]. Nowadays a broad spectrum of detection chemistries and machines with advanced software are available (see for a recent review Valasek & Repa (2005)). When compared to dot-blot hybridization, real-time PCR has superior sensitivity and is also more convenient and less expensive for the quantification of selected bacterial populations [Malinen *et al.* 2003]. Quantification of rRNA that is isolated directly from the ribosomes may be used to reveal the metabolically most active members of a bacterial community.
Other quantitative PCR approaches are competitive (RT-)PCR and Most Probable Number (MPN) PCR. With competitive PCR a specific standard of known concentration is added at different concentrations to the target followed by PCR amplification. The difference in size between the target and the standard allows discrimination and subsequent quantification on an agarose gel. The principle of MPN-PCR is similar to MPN cultivation for the quantification of micro-organisms. Target DNA is diluted until extinction and used as template for PCR using species or group specific primers. The products from the MPN PCR are also analysed by agarose gel electrophoresis, which is one of the weak points of these quantitative PCR approaches, since agarose gels are not highly discriminative resulting in a low resolution. Truly quantitative information using molecular methods can only be obtained if cell lyses and extraction efficiency, as well as biases in the PCR step are under strict experimental control [Petersen & Dahllöf 2005]. Also the earlier mentioned disadvantages of PCR end-point detection play a role in competitive (RT-)PCR and MPN PCR quantitative approaches.

Denaturing (temperature) gradient gel electrophoresis of PCR amplicons

Denaturing Gradient Gel Electrophoresis (DGGE), or the nowadays less used Temperature Gradient Gel Electrophoresis (TGGE), is based on the melting behaviour of double stranded DNA fragments. The melting behaviour, mostly described as the melting temperature (i.e. the temperature, where a double-stranded nucleic acid fragment dissociates), is dependent on the nucleotide composition of a fragment. Even in the case of a single-nucleotide substitution, fragments will potentially melt at a different temperature. By adding a GC-rich tail (GC-clamp) to one of the primers for the amplification, a fragment is produced that will partially melt, when it is electrophoresed in a denaturing gradient polyacrylamide gel. The GC-clamp will remain double stranded and the resulting fork-like structure causes the fragment essentially to stop migrating. This process will occur at a different position in the gel, when one or more basepair(s) are substituted or deleted. Therefore, mutations can be detected. Normally a urea and formamide gradient is applied in DGGE and a temperature gradient in TGGE. A certain concentration of urea/formamide combined with e.g. 60ºC has the same effect as a much higher temperature without the presence of urea/formamide. Muyzer *et al.* (1993) first applied DGGE to analyse complex bacterial communities. DGGE fingerprinting is an excellent and effective method to follow changes of microbial communities in time and space. In addition, DGGE is well suited for monitoring of complex communities, focusing on phylotypes for which the occurrence and/or the relative frequency are affected by any environmental change

[Akkermans *et al.* 2000, Fromin *et al.* 2002]. By inter-sample comparison, dominant shifts in population dynamics can be studied in more detail. The intensity of an individual band is a semi-quantitative measure for the relative abundance of this sequence in the community [Vaughan *et al.* 2000]. It has been reported that DGGE or TGGE are sensitive enough to detect organisms that constitute up to 1% of the total microbial community [Muyzer *et al.* 1993, Zoetendal *et al.* 1998]. This means that only the most dominant bacteria will be represented in the profiles when domain-specific primers are used, while with group-specific primers also minor microbial groups can be followed by DGGE analysis. For example, Heuer *et al.* 1997 generated DGGE profiles with primers specific for *Actinomycetes* and Boon *et al.* (2002) used multiple group-specific primer pairs for the generation of a set of DGGE fingerprints characterising microbiota in different wastewater treatment plants. With improvements of statistical software, similarity indices can be calculated, and cluster analysis of community profiles can be performed. Gafan *et al.* (2005), for example, analysed DGGE profiles in three different ways: bacterial diversity by using the Shannon-Wiener index; hierarchical cluster analysis, expressed as a dendrogram; and individual DGGE bands and their intensities were compared using logistic regression analysis. Fromin *et al.* (2002) provided an excellent overview of currently used statistical analysis methods for DGGE data. In addition, Internal Standards in Molecular Analysis of Diversity (ISMAD) might be especially useful in comparative ecological and eco-toxicological DGGE experiments where differences between treatments from the same original community are studied and where changes due to treatment effects are sought [Petersen & Dahllöf 2005]. The use of ISMAD during PCR-DGGE makes it possible to analyse whether differences in bacterial abundance and diversity are due to differences in the original samples, or due to biases from experimental variability between samples introduced during the DNA extraction, PCR and DGGE [Petersen & Dahllöf 2005]. However, sometimes limitations in DGGE resolution can occur, since a single band does not always represent a single strain [Sekiguchi *et al.* 2001a] and this might compromise the results of DGGE identification markers, as developed for example by Keyser *et al.* (2006) for identification of methanogens in UASB granules, and diversity estimators. Recently, a Denaturing High-Performance Liquid Chromatograph (DHPLC) method was used and compared with DGGE for the analysis of natural bacterial communities, and DHPLC analysis produced profiles of the community that described the composition and abundance of bacterial species [Barlaan *et al.* 2005]. The major advantage of DHPLC compared with gel-based approaches includes the use of automated instrumentation and convenience of analysis, especially the fraction collection of peaks for DNA isolation and sequencing. Sequencing of DGGE bands directly or via a cloning step, is important to identify the micro-organisms behind the banding profiles.

Single strand conformation polymorphism based approaches

Another technique for the detection of differences in DNA sequences using separation by electrophoresis is Single Strand Conformation Polymorphism (SSCP). Comparable to D(T)GGE, this technique was first developed in clinical research for the detection of known or novel polymorphisms of point mutations in DNA [Orita *et al.* 1989]. Single-stranded DNA is separated in a polyacrylamide gel based on differences in mobility caused by their secondary structure. Even when DNA fragments are of equal size and no denaturant is present, folding and hence mobility will be dependent on the DNA sequences [Lee *et al.* 1996]. By the use of a variable part of the 16S rRNA gene

(for example the V3 region) from an environmental sample, each SSCP peak corresponds to a distinct microbial sequence, indicating the presence of a microbial strain or species retrieved from the sample [Leclerc *et al.* 2001, Lee *et al.* 1996]. Lee *et al.* (1996) reported that SSCP can be applied to separate 16S rRNA gene fragments which are amplified from complex bacterial assemblages, and that it was sensitive enough to detect a bacterial population that accounted for less than 1.5% of a bacterial community. In general, SSCP has the same limitations as DGGE. Some single-stranded DNA fragments can form more than one stable conformation and therefore one sequence may be represented by multiple bands [Tiedje *et al.* 1999]. However, SSCP does not require a GC-clamp or the construction of gradient gels and is therefore potentially more simple and straightforward than DGGE [Schwieger & Tebbe 1998]. Hori *et al.* (2006a) compared DGGE and SSCP based on the V3-V4 16S rRNA gene region and concluded that SSCP was superior compared to DGGE in detecting the bacterial dynamics of a methanogenic bioreactor. If one PCR primer is labelled with a fluorescent dye the detection can be automated and heteroduplex formation can be avoided. Furthermore, probes can be easily used for further detection and identification of cetain bands.
Denaturing gradient electrophoresis and SSCP fingerprinting patterns do not reflect only a tiny fraction of the real diversity, but correspond to a representation of the whole microbial community, when not only the number of visible bands or peaks are considered, but when the whole picture, including background is analysed [Loisel *et al.* 2006].

Restriction analysis based approaches

A large number of molecular techniques make use of specific nucleic acid-modifying enzymes, initially purified and characterised from micro-organisms. The thermo-stable *Taq* DNA polymerase, used for PCR, is a good example, but also restriction enzymes are widely used. Restriction enzymes recognize specific DNA sequences and cut in a reproducible way. The combination of PCR and restriction can, for example, be used for enhanced amplification of minor DNA templates [Green & Minz 2005]. Unwanted or dominant DNA templates can be amplified in a first round of PCR, the produced double stranded products cut by restriction enzymes, resulting in the digested template no longer being available for PCR amplification [Green & Minz 2005].
In Amplified Ribosomal DNA Restriction Analysis (ARDRA), the ribosomal RNA gene is amplified by PCR and digested into specific fragments by restriction enzymes (usually with 4-bp recognition sites). After the incubation with restriction enzymes, fragments are separated by high resolution gel electrophoresis, resulting in specific patterns from different sequences. ARDRA can be used for rapid comparison of rRNA genes [Laguerre *et al.* 1994, Moyer *et al.* 1994]. The typical analysis of restriction digests for isolates or clones is performed on agarose gels, while for community analysis the potentially large number of fragments can be resolved by using polyacrylamide gels to produce a community-specific pattern [Martinez-Murcia *et al.* 1995], but new high resolution matrices are nowadays available as well.
Terminal Restriction Fragment Length Polymorphism (T-RFLP) is another derived fingerprinting technique and makes use of restriction enzymes as well, but only terminal restriction fragments (T-RF) are detected and used for qualitative and quantitative analysis [Liu *et al.* 1997]. T-RFLP employs PCR, in which one of the two primers is fluorescently labelled at the 5'-end, for the amplification of a specific region of the 16S rRNA gene or for the amplification of functional genes. After

amplification, PCR products are cleaved with a site-specific restriction endonuclease to obtain genetic fingerprints of microbial communities or a specific product from a single micro-organism and the T-RF's are precisely measured by using an automated DNA sequencer. Size markers bearing a different fluorophore can be included in every sample and complex communities can result in 60-80 unique T-RF [Marsh 1999]. The areas under the peaks of the obtained electropherograms indicate the relative proportions of the fragments. Several online tools have been developed for the performance of *in silico* hydrolysis of 16S rRNA gene sequences and recently an ARB-implemented tool was developed to predict the terminal restriction fragments of aligned small-subunit rRNA gene or functional gene sequences [Abdo *et al.* 2006, Ricke *et al.* 2005]. T-RFLP is a powerful tool for assessing the diversity of complex microbial communities and for rapid comparison of the community structure and diversity of different ecosystems. Like with DGGE, however, data obtained by T-RFLP should be cautiously interpreted, since microbial populations that are not numerically dominant are not represented, because the template DNA's from these populations represent a small fraction of the total community DNA and consequently the species diversity of the microbial community is underestimated [Liu *et al.* 1997]. Furthermore, determination of similarities of complex bacterial communities from T-RFLP profiles generated by a single restriction enzyme may lead to erroneous conclusions. Hence, it is better to use multiple restriction enzymes individually, generating multiple data sets. Osborne *et al.* (2006) introduced the variable threshold method for analysis of such multiple datasets, allowing more confident conclusions about the similarities of complex microbial communities.

Cloning and sequencing approaches

For proper phylogenetic characterization of micro-organisms, the sequence of the marker gene used should be determined. Comparative sequencing of the 16S rRNA gene has become by far the most commonly used measure of environmental diversity [Vaughan *et al.* 2000]. Ideally the whole genome should be sequenced and actually this is done more often nowadays. Even the metagenome of a whole community can be subjected to cloning and sequencing [Green Tringe *et al.* 2005, Tyson *et al.* 2004, Venter *et al.* 2004]. However, sequencing of whole community genomes is not practical (and economical), because most communities comprise at least hundreds to thousands of species [Torsvik *et al.* 2002]. Pyrosequencing [Ronaghi *et al.* 1996] has been used to estimate the total diversity in soils and resulted in the detection of more than 50,000 OTU's [Roesch *et al.* 2007].

Ribosomal RNA sequences can be obtained by PCR amplification either from the encoding genes or directly from rRNA. In the case of direct amplification from rRNA, the sensitive reverse transcription-polymerase chain reaction (RT-PCR) has to be employed [Vaughan *et al.* 2000]. Cloning consists in this case of ligation of the amplified rRNA (gene) fragments into a plasmid, usually with antibiotic resistance genes, and the transformation of competent *E. coli* host cells with the resulting vector. The 16S rRNA sequences that are naturally amplified in the obtained transformants can be screened, e.g. with (a combination of) ARDRA and DGGE, before isolation of the plasmids. The 16S rRNA inserts of these plasmids can be sequenced, for example with the Sanger dideoxy method [Sanger *et al.* 1977]. Sequence comparisons of nucleic acids isolated from complex microbial ecosystems can be used to provide molecular characterization, while at the same time providing a classification system, which predicts natural evolutionary relationships [Pace 1997, Woese *et al.* 1990]. Several programmes for determining the most similar sequence are available at

various internet sites (for example Genbank: http://www.ncbi.nlm.nih.gov/BLAST/ [McGinnis & Madden 2004]; or the RDP database: http://rdp.cme.msu.edu/ [Maidak *et al.* 2001, Cole *et al.* 2005]) and databases containing sequences that are aligned according to their secondary structure paradigm are available (e.g. ARB [Ludwig *et al.* 2004]). By the use of these programmes phylogenetic relationships of micro-organisms can be investigated. Furthermore, Kemp & Aller (2004a) suggested exploration of clone libraries in progressive stages by calculating phylotype richness estimators, before characterising a new subset of clones, until these estimators stabilise. They also developed a simple webbased tool for these calculations (http://www.aslo.org/lomethods/free/2004/0114a.html) [Kemp & Aller 2004b]. However, the diversity and prevalence of individual variants within environmental bacterial populations has not been extensively explored, and so questions regarding the ecological importance of genotypic variation remain unanswered [Thompson *et al.* 2005]. Cloning is also not without bias, but sequence analysis of 16S rRNA genes has become a standard procedure in the identification of isolates and it is now impossible to adequately describe microbial communities without 16S rRNA sequence data [Leser *et al.* 2002, Zoetendal *et al.* 2004a].

Fluorescence *in situ* hybridisation based approaches

Phylogenetically based oligonucleotide probes can be used directly for quantification via Fluorescence *In Situ* Hybridisation (FISH) and Dot-Blot hybridization. FISH is a quantitative method on a cell by cell basis and was first developed in the late 1980s with radioactive labelled oligonucleotides, but soon fluorescently labelled oligonucleotide probes were demonstrated to allow detection of individual cells *in situ* much better [Giovannoni *et al.* 1988, De Long *et al.* 1989]. This made whole-cell hybridisation with rRNA-targeted probes a suitable tool for determinative, phylogenetic, and environmental studies in microbiology [Amann *et al.* 1990]. Nowadays it is even possible to detect up to seven microbial groups simultaneously with so called Rainbow-FISH [Sunamura & Maruyama 2006]. Variables that influence the sensitivity and reproducibility of the *in situ* hybridisation technique include: the effects of cell fixation on target RNA preservation and accessibility to probes; the type and quality of probes; the efficiency of probe-target hybrid formation; the stability of hybrids formed *in situ* during post hybridisation treatments; the method of detection of hybrids; and the background noise masking the hybridisation signal [Pernthaler & Amann 2004]. Optimal fixation should result in good probe penetration, retention of the maximal level of target RNA, and maintenance of cell integrity and morphologic detail [Motor & Göbel 2000]. The accessibility of rRNA has been systematically investigated [Fuchs *et al.* 1998 & 2001, Behrens *et al.* 2003] and recently it has been shown that a rational probe design strategy, involving $\Delta G^0_{overall}$, hybridisation kinetics, and fluorophore quenching, resulted in no truly inaccessible target regions in the 16S rRNA of *E. coli* [Yilmaz *et al.* 2006]. Cells of different species have different ribosome contents ranging roughly between $10^3$ and $10^5$ ribosomes per cell and even for one strain, cellular rRNA contents can vary significantly (at least over one order of magnitude), since they are directly correlated with the growth rate [Amann *et al.* 1995, DeLong *et al.* 1989]. However, the relative rRNA abundance should represent a reasonable measurement of the relative physiological activity of the respective population, since it is the product of the number of detected cells and the average rRNA content [Amann *et al.* 1995, de Vries *et al.* 2004, Wagner 1994b]. FISH is a good technique for identification of specific micro-organisms in complex communities and one of the best methods suited

for quantification of these micro-organisms *in situ*, while maintaining structural information. Probes for FISH have to be chosen wisely and in a nested approach to ensure the correct enumeration and identification [Schmid *et al.* 2005]. Recently, a method for simultaneous FISH of mRNA and rRNA in environmental bacteria was published, which facilitates the simultaneous identification, activity detection and assessment of biogeochemical impact of individual organisms *in situ* [Pernthaler & Amann 2004]. Such developments are possible by increasing the signal intensity, and Zwirglmaier published a comprehensive review on this topic, focussing mainly on two widely recognised varieties, tyramide signal amplification and multiply labelled polynucleotide probes [Zwirglmaier 2005]. In principle, five objectives of microbial ecological studies can be addressed with FISH: bypass cultivation problems; obtain information on community structure by using varying sets of probes; accurately enumerate target populations; identify sub-populations in natural systems and locate their niche; and determine the *in situ* cellular rRNA content (as a metabolic fitness measurement) [Vaughan *et al.* 2000]. Without automation, however, FISH is very laborious and only a few probes can be used per analysis. Flow Cytometry (FCM)-based approaches could overcome this limitation, and several studies have demonstrated the use of FCM in association with FISH [Davey & Kell 1996, Porter *et al.* 1996, Wallner *et al.* 1995]. As an extension to FCM, Fluorescent Activated Cell Sorting (FACS) can be used to separate different populations from a mixed community, allowing for the physical enrichment or isolation even of yet uncultured organisms that can be used for subsequent molecular genetic studies and cultivation. Park *et al.* (2005) used this technique for the analysis of activated-sludge and Yellowstone Lake hydrothermal vent samples and detected numerous bacterial species, including previously unknown species, not readily detectable in the original sample due to low relative abundance. However, by the application of a μ-flow cytometric analysis system Gerdts and Luedke (2006) used slower flow rates which made it possible to collect much weaker fluorescence signals. Moreover, by using probes indicating the integrity and metabolic activity status of a microbial cell, phylogenetic information can be potentially linked to viable status [Ben-Amor *et al.* 2002; Ben-Amor *et al.* 2005; Vaughan *et al.* 2005]. A challenge, however, for the application of FCM to anaerobic sludge granules is the need for single cells, since clustered cells (e.g. granules) are very difficult to disrupt. If, however, these problems can be solved, the quantitative potential is enormous.

Dot-blot hybridisation based approaches

A disadvantage of PCR based approaches is that they do not always provide unbiased quantitative data because of amplification biases. Quantification of a certain 16S rRNA sequence type relative to the total 16S rRNA content of a given sample can be obtained by dot-blot hybridization of a directly isolated nucleic acid mixture with universal and specific oligonucleotide probes [Amann *et al.* 1995, Raskin *et al.* 1994a]. For this purpose, total RNA is isolated from a sample, immobilised on a membrane and hybridised with labelled oligonucleotide probes. The relative abundance can be calculated as a ratio of the amount of specific probe bound to a given sample to the amount of hybridised universal probe [Amann *et al.* 1995]. When radioactively labelled oligonucleotide probes were used in a dot-blot assay, rRNA sequences with a relatively low abundance between 0.1 and 1% could be quantified [Amann *et al.* 1995]. Although quantification is very accurate, these data of relative rRNA abundance can not be directly translated into cell numbers, since cells of different species have different ribosome contents ranging roughly between $10^3$ and

$10^5$ ribosomes per cell [Amann *et al.* 1995]. However, because PCR or other amplification procedures are not involved, the quantification is very accurate, and many oligonucleotide probes have been developed, validated and successfully used in the past 15 years. Recently, many of those probes have been collected in an interactive web-based database, ProbeBase (http://www.microbial-ecology.net/probebase/) [Loy *et al.* 2003]. Essentially all types of samples can be used for quantitative dot-blot hybridisation, which makes it the method of choice in those systems which are difficult for FISH (e.g. patchy environments) [Amann & Ludwig 2000]. Furthermore, von Wintzingerode *et al.* (1999) prepared specific PCR-made probes from a selection of bacterial clones to screen their bacterial clone library via dot-blot hybridisation.

Phylogenetic micro-arrays

DNA micro-arrays (also called DNA chips, gene chips or biochips) typically consist of thousands of immobilised DNA fragments (PCR product, oligonucleotides or other DNA fragments) present on a surface, such as coated glass slide or membrane [Ye *et al.* 2001]. It can be considered as a reverse traditional dot-blot, since the identity of the spotted probes is known and the sample is labelled. Labelled sample nucleic acids will mark the exact positions on the array where hybridisation occurred. The micro-array experiment output consists of a list of hybridisation events, indicating the presence or the relative abundance of specific DNA or RNA sequences present in the sample. Micro-arrays are already widely used for the detection of transcriptional profiles (expression arrays) or the similarities and differences of genetic contents among different micro-organisms, and they can be used to subtype (fingerprint strains relative to the reference strain) bacterial isolates and for the identification of new diagnostic genetic markers [Call *et al.* 2003]. Also mutation detection and the search of polymorphisms are done with micro-arrays, but the detection and identification of high numbers of different microbes, especially from complex microbial communities in environmental samples with micro-arrays is still very challenging. An annotated selection of World Wide Web sites relevant to environmental microbiological micro-arrays was recently published [Wackett 2006] as well as a number of excellent reviews [Letowski *et al.* 2003, Blaškovič & Barák 2005, Loy & Bodrossy 2006, Wagner *et al.* 2007]. The rRNA is still the most widely used marker gene, but a number of higher resolution and/or non-universal (i.e. focused on a narrower group of microbes) genes are also being used as markers [Bodrossy and Sessitsch 2004, Bodrossy *et al.* 2006]. By using consensus primers to amplify and label targets, it is possible to differentiate a large number of organisms, provided that sufficient polymorphism exists within the amplified region [Nicolaisen *et al.* 2005].

Use of micro-arrays for determinative studies provides several advantages over conventional hybridisation formats. Thousands of different oligonucleotides can be immobilised on a single array, allowing the simultaneous detection of a great variety of different micro-organisms in a single sample. There is a low-sample-requirement, and automation and high-throughput analysis with micro-arrays can be realised easily. Micro-arrays, used in combination with high spatial resolution *in situ* measurements of concentrations of inorganic and organic ions and molecules, pH, and redox potential, could allow linkage of gene expression or protein production to specific organisms and processes [Tyson & Banfield 2005]. Furthermore, DNA micro-arrays are promising for the quantification of microbial genes and therefore highly suited for molecular ecology studies [Cho & Tiedje 2002, DeSantis *et al.* 2005, Palmer *et al.* 2007, Rajilic-Stojanovic 2007]. Direct detection and identification of rRNA from

environmental samples, without PCR amplification is possible [Small *et al.* 2001, Koizumi *et al.* 2002, Denef *et al.* 2003, El Fantroussi *et al.* 2003, Peplies *et al.* 2004, Kelly *et al.* 2005]. Two enzymatic signal amplification systems that have been employed are enzyme-linked fluorescence (ELF-97; Molecular Probes) and tyramide signal amplification (TSA) [Call *et al.* 2003]. The sensitivity of microbial diagnostic micro-arrays is usually defined as the lowest relative abundance of the target group detectable within the analysed community and is in the range of 1-5% [Bodrossy and Sessitsch 2004]. Currently the method of choice is the synergistic combination of micro-array and real-time PCR, in which the screening is performed by micro-arrays and the precise quantification and high-throughput screening of selected target sequences is achieved by real-time PCR [Klein 2002].

Although DNA micro-arrays have many advantages and are very promising, many optimisations have to be done. Adapting micro-array hybridisation for use in environmental studies faces, besides quantification, several challenges associated with specificity and sensitivity [Zhou and Thompson 2002, Cook and Sayler 2003]. Discrimination between perfect and mismatch binding between the probes on the array and the genetic content of the targets is needed [Liu *et al.* 2001, Urakawa *et al.* 2002, Urakawa *et al.* 2003]. In some studies oligonucleotide probes were designed to have almost identical melting temperatures, but there are indications that present models are only capable of predicting *T*m values of free probes and not those of surface-bound probes [Bodrossy *et al.* 2003, Franke-Whittle *et al.* 2005]. Thermal dissociation curves for each probe-target duplex and application of multiple probes for specific targets on the DNA micro-array help to avoid false positive detection [El Fantroussi *et al.* 2003, Liu *et al.* 2001, Urakawa *et al.* 2002, Urakawa *et al.* 2003]. Understanding the principles governing nucleic acid hybridisations of short probe-target duplexes is necessary and fundamental for the application of micro-array technology to routine environmental microbiology because it would facilitate the design of proper probes, minimize the changes of non-specific hybridisation, and improve the ability for confident interpretation of micro-array data [Urakawa *et al.* 2002]. Urakawa *et al.* found for example that besides the formamide concentration in the hybridisation buffer (stringency of the hybridisation buffer), the position and type of mismatch are important for discrimination between perfect match and mismatch hybridisation [Urakawa *et al.* 2002]. He *et al.* (2005) investigated the effects of probe-target identity, continuous stretch (length of probe part without any mismatch), mismatch position and free energy on the design of 50-mer and 70-mer probes and then experimentally compared the designed probes to establish probe design criteria. They found that a combination of similarity (85%), stretch (15 bases), and free energy (- 30 kcal/mol) was able to exclude all non-specific probes for 50-mer probes and a combination of similarity (85%), stretch (20 bases), and free energy (- 40 kcal/mol) for 70-mer probes [He *et al.* 2005]. The criteria for 50-mer oligonucleotides were recently reevaluated and a combination of 90% similarity, 20 bases stretch and a free energy of – 35 kcal/mol were found to be predictive of probe specificity [Liebich *et al.* 2006]. With this necessary background information and availability of three dimensional structures for large amount of probe binding and direct dissociation monitoring (e.g. polyacrylamide gel patches [Yershov *et al.* 1996, Guschin *et al.* 1997] or porous metal oxide [Beuningen *et al.* 2001]) and high-density micro-arrays (e.g. Affymetrix GeneChips [Brodie *et al.* 2006, Brodie *et al.* 2007, DeSantis *et al.* 2005, DeSantis *et al.* 2007, Wilson *et al.* 2002a, Wilson *et al.* 2002b]), the routine use of this new technology is within reach, however, data handling and interpretation of data are still challenging. Also bead-based methods for multiplexed identification and

quantification with a flow cytometer have great potential [Spiro *et al.* 2000, Wireman *et al.* 2006]. Completely integrated miniature systems, so called 'laboratories-on-a-chip', will further improve microbial monitoring by achieving detection and identification within minutes at the single-cell level [Liu & Zhu 2005]. This will deepen the understanding of microbial community structure and diversity and correlate these to the spatial distribution of micro-organisms.

## Key functional groups in microbial sludge communities

Although micro-organisms are present almost everywhere and responsible for chemical transformations in the biosphere, our understanding of the composition of the interactions and dynamics within microbial ecosystems is limited. While the general processes occurring in anaerobic biological wastewater treatment plants, such as hydrolysis, fermentation, acetogenesis, methanogenesis and sulphidogenesis are well understood (Fig. 1), and equipment for general anaerobic biodegradability testing is available [Guwy 2004], the microbial community responsible for these conversions is often considered as a black box [Head *et al.* 1998, Dabert *et al.* 2002]. This fact is not due to an underestimation of the biological component, but rather is caused by the limitations of available methods for the isolation, identification and physiological characterisation of micro-organisms involved.
Many biogeochemical processes are not catalysed by individual micro-organisms, but instead by cooperating populations (consortia) [Madsen 2005]. Effective conversion of organic matter into methane for example requires the concerted metabolic activities of physiologically different microbial populations. Oxidation of volatile fatty acids is a key process in methane formation. In the absence of sulphate, propionate and butyrate conversion are thermodynamically only possible at a low partial hydrogen pressure and a low formate concentration [Boone *et al.* 1989, Harmsen *et al.* 1996, Schink 1997]. These conditions are met in syntrophic consortia of acetogens and methanogens, where the acetogens oxidise propionate and butyrate to acetate, $H_2$ and/or formate that subsequently are used by the methanogens (Fig. 1, Tables 1 & 2).

Table 1: Methanogenic orders [Karakashev *et al.* 2005].

| Order | Physiology |
|---|---|
| *Methanopyrales* | Hydrogenotrophic; hyperthermophilic |
| *Methanobacteriales* | Hydrogenotrophic; mesophilic or thermophilic |
| *Methanococcales* | Hydrogenotrophic; mesophilic or thermophilic |
| *Methanomicrobiales* | Hydrogenotrophic; mesophilic |
| *Methanosarcinales* | Strict aceticlastic (*Methanosaetaceae*), aceticlastic or hydrogenotrophic (*Methanosarcinaceae*); mesophilic or thermophilic |

Table 2: Isolated and characterized syntrophic micro-organisms.

| Substrate | Strain | 16S rRNA accession no | Reference |
|---|---|---|---|
| Acetate | *Clostridium ultunense* | Z69293 | Schnürer *et al.* 1996 |
| Acetate | *Thermoacetogenium phaeum* | AB020336 | Hattori *et al.* 2000 |
| Acetate | *Candidatus Contubernalis alkalaceticum* | DQ124682 | Zhilina *et al.* 2005 |
| Propionate | *Syntrophobacter wolinii* | X70905 | Boone *et al.* 1980 / Harmsen *et al.* 1993 |
| Propionate | *Syntrophobacter fumaroxidans* | X82874 | Harmsen *et al.* 1995 & 1998 |
| Propionate | *Syntrophobacter pfennigii* | X82875 | Wallrabenstein *et al.* 1995b / Harmsen *et al.* 1995 |
| Propionate | *Syntrophobacter sulfatireducens* | AY651787 | Chen *et al.* 2005 |
| Propionate | *Smithella propionica* | AF126282 | Liu *et al.* 1999 |
| Propionate | *Desulfotomaculum thermocisternum* | U33455 | Nilsen *et al.* 1996 |
| Propionate | *Desulfotomaculum thermosapovorans* | Y11575 | Fardeau *et al.* 1995 / Stackebrandt *et al.* 1997 |
| Propionate | *Desulfotomaculum thermobenzoicum*, subspecies *thermosyntrophicum* | AY007190 | Plugge *et al.* 2002a |
| Propionate | *Pelotomaculum thermopropionicum* | AB035723 | Imachi *et al.* 2000 & 2002 |
| Propionate | *Pelotomaculum schinkii* | X91169 & X91170 | De Bok *et al.* 2005 |
| Propionate | *Pelotomaculum* strain FP | AB159557 & AB159558 | Unpublished (Imachi *et al.* / De Bok *et al.* 2005) |
| Butyrate | *Syntrophomonas bryantii* | M26491 | Stieb & Schink 1985 / Zhao *et al.* 1990 / Wu *et al.* 2006b |
| Butyrate | *Syntrophomonas wolfeii* subspecies *wolfeii* | M26492 | McInerney *et al.* 1981 / Zhao *et al.* 1990 |
| Butyrate | *Syntrophomonas wolfeii* subspecies *metylbutyratica* | DQ449033 | Wu *et al.* 2007 |
| Butyrate | *Syntrophomonas sapovorans* | AF022249 | Roy *et al.* 1986 / Hansen *et al.* 1999 |
| Butyrate | *Syntrophomonas saponavida* | AF022248 | Lorowitz *et al.* 1989 / Hansen *et al.* 1999 / Wu *et al.* 2007 |
| Butyrate | *Syntrophomonas curvata* | AY290767 | Zhang *et al.* 2004 |
| Butyrate | *Syntrophomonas curvata* strain GB4-38 | AY536889 | Zhang *et al.* 2005 |
| Butyrate | *Syntrophomonas curvata* strain SB9-1 | AY643536 | Zhang *et al.* 2005 |
| Butyrate | *Syntrophomonas erecta* subspecies *erecta* | AY536889 | Zhang *et al.* 2005 |
| Butyrate | *Syntrophomonas erecta* subspecies *sporosyntropha* strain Y4-1 | DQ112186 | Wu *et al.* 2006a |
| Butyrate | *Syntrophomonas erecta* subspecies *sporosyntropha* strain 5-3-z | DQ086234 | Wu *et al.* 2006a |
| Butyrate | *Syntrophomonas cellicola* | DQ288691 | Wu *et al.* 2006b |
| Butyrate | *Syntrophomonas zehnderi* | DQ898277 | Sousa *et al.* 2007 |
| Butyrate | *Syntrophothermus lipocalidus* | AB021305 | Sekiguchi *et al.* 2000 |
| Butyrate | *Thermosyntropha lipolytica* | X99980 | Svetlitchnyi *et al.* 1996 |
| Butyrate | *Algorimarina butyrica* | AY851292 | Kendall *et al.* 2006 |
| Benzoate | *Syntrophus buswellii* strain GA | X85131 | Ausburger & Winter 1995 / Wallrabenstein *et al.* 1995a |
| Benzoate | *Syntrophus buswellii* strain SB | | Hopkins *et al* 1995 |
| Benzoate | *Syntrophus gentianae* | X85132 | Szewzyk & Schink 1989 / Wallrabenstein *et al.* 1995a |
| Benzoate | *Syntrophus aciditrophicus* | U86447 | Jackson *et al.* 1999 |
| Benzoate | *Sporotomaculum syntrophicum* | AB076610 | Qiu *et al.* 2003 |
| Glycolate | *Syntrophococcus sucromutans* | AF202264 | Krumholtz & Bryant 1986 |
| Glycolate | *Syntrophococcus glycolicus* | X99706 | Friedrich *et al.* 1996 |
| Glutamate | *Gelria glutamica* | AF321086 | Plugge *et al.* 2002b |
| Alcohol / lactate | *Tepidanaerobacter syntrophicus* | AB106353 | Sekiguchi *et al.* 2006 |
| Phthalate | *Pelotomaculum terephthalicum* strain JT | AB091323 | Qiu *et al.* 2004 / Qiu *et al.* 2006 |
| Phthalate | *Pelotomaculum isophthalicum* strain JI | AB232785 | Qiu *et al.* 2006 |
| Palmitate | Strain MPA | AB232558 | Hatamoto *et al.* 2007 |

Syntrophic interactions are symbiotic relationships between two partners that depend entirely on each other for energy conservation and growth [Stams 1994, Schink 1997]. Short chain fatty acids are important intermediates in the anaerobic degradation process. Propionate and butyrate oxidation to acetate and $CO_2$ are energetically very unfavorable reactions and syntrophic interaction with methanogens or SRB is needed to make this oxidation feasible [Schink 2002, Schink & Stams 2002]. The Gibbs free energy change is relatively low, resulting in only small amounts of energy available for growth and this small amount of energy has to be shared by the partner organisms. The amount of energy released in syntrophic propionate and butyrate oxidation is at the limit of what is necessary for energy conservation and growth. It appears that maintenance energies for syntrophic organisms may be significantly lower than those for other bacteria, perhaps because they are adapted to conditions of perpetual energy stress [Adams *et al.* 2006]. Because of the obligate syntrophy, examination of pure cultures is often not possible and kinetic data from defined co-cultures and mixed multi-species consortia are difficult to interpret. Moreover, the acetogenic bacteria of syntrophic cultures are mostly not able to grow on plates or in agar shake cultures.

$H_2$-production and utilization can profoundly influence the course of fermentations in anaerobic ecosystems [Hungate 1967]. The regulatory role of $H_2$ is affected by interspecies $H_2$-transfer [Ianotti *et al.* 1973]. The special feature of interspecies hydrogen transfer is that hydrogenotrophic organisms can create conditions for obligate $H_2$-producers to perform catabolic oxidations which would not have been energy yielding in the absence of hydrogenotrophs. At slightly elevated $H_2$ concentrations the reactions from which these $H_2$-producers obtain their energy become endergonic. As a consequence, the obligate $H_2$-producers stop their activities at elevated $H_2$ concentrations, while fermentative organisms under such conditions can alter their flow of electrons towards the formation of more reduced metabolites [Dolfing 1986]. In clustered balanced microbial consortia like granular anaerobic sludge, the distances between the various organisms are small enough to explain the high biodegradation rates through interspecies $H_2$-transfer [Grotenhuis 1992, Stams 1994]. Hence, the cooperation between different metabolic groups mentioned above is a prerequisite for a complete conversion of biodegradable organic matter into methane and $CO_2$.

Bacteria capable of syntrophic oxidation of propionate are members of the delta-Proteobacteria and the low-GC-Gram-positives, while known syntrophic butyrate oxidizers group only with the low-GC-Gram-positives (Table 2 & Fig. 3). Syntrophic bacteria do not constitute monophyletic groups, since many other metabolic types are grouped in between and do not show any tendency to transfer electrons to partner organisms. Syntrophy can hardly be assumed to be associated with a single evolutionary trait and syntrophy actually appears to be a lifestyle that is developed and perfected by a range of different organisms to varying extents [Schink & Stams 2002].

High rates of methane formation in UASB and EGSB reactors are achieved because they contain granular sludge with a high biomass density and short intermicrobial distances. Previous research indicated that the architecture of granular sludge is highly structured (see also Fig. 2) [Fang *et al.* 1995, Harmsen *et al.* 1996, MacLeod *et al.* 1990, Sekiguchi *et al.* 1999, Wu *et al.* 1991]. McHugh *et al.* (2003a) gave an overview of proposed models and theories for granule formation. In addition, microscopic studies indicated the presence of non-staining regions within the sludge granule [Harmsen *et al.* 1996, Sekiguchi *et al.* 1999, Wu *et al.* 1991]. Although these could be caused by dead organisms or inorganic compounds, it is more likely that

they indicate void regions or micro-channels. These channels could facilitate diffusive transport of substrates, nutrients and by-products within the granule [Wu *et al.* 1991]. Large biopolymers enter the granule through the outer layer, where dominating Bacteria ferment them. Degradation then continues towards the centre of the granule. Here, the interwoven communities of Archaea and Bacteria complete this process leading to syntrophic methane production. This is in turn released via the micro-channels. Together with other unknown factors, long chains formed by the dominant Archaea, *Methanosaeta*, might maintain the structural integrity of the granule. Within this structure also sulphate reducers can be active. Besides the syntrophic interactions, there should be a fierce competition between methanogens, sulphate reducers and other microbial groups.

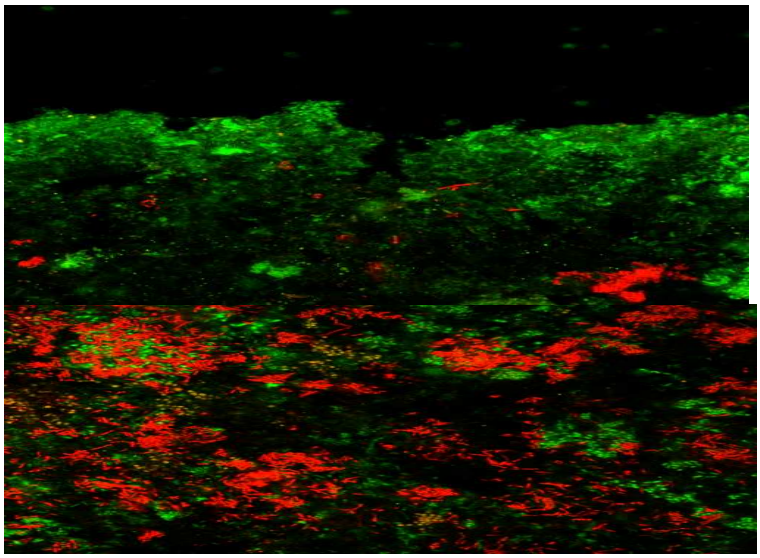


Fig 2: Layered structure of granule visualised with FISH. Bacteria (green) on the outside and Archaea (red) more to the inside, interwoven with bacteria.

*Syntrophobacter wolinii* was the first propionate oxidizer, which was obtained from a methanogenic enrichment of an anaerobic municipal sewage digester in a defined co-culture with a *Desulfovibrio* sp. [Boone & Bryant 1980]. *Syntrophomonas wolfei* was the first characterized syntrophic butyrate-oxidizing bacterium [McInerney *et al.* 1981]. Only a limited number of syntrophs has been characterized to date, because they are slow growing and it is difficult to isolate them as a result of their syntrophic lifestyle (Table 2). While some syntrophic organisms have only been described in co-culture with a hydrogenotrophic micro-organism, others have not been conserved or phylogenetically characterised, and are lost for further studies [Mountfort & Bryant 1982, Barik *et al.* 1985, Shelton & Tiedje 1984, Eichler & Schink 1985, Henson & Smith 1985, Lee & Zinder 1988, Wu *et al.* 1992, Imachi *et al.* 2006]. A number of specific 16S rRNA-targeted probes have been developed and used for the quantification of syntrophs by FISH and membrane-based hybridisation [Harmsen *et al.* 1995, Hansen *et al.* 1999].

Methanogens are active at the end of the anaerobic food chain and specialized in the degradation of a very limited number of substrates (see also Table 1). Hydrogenotrophic methanogens *Methanobacterium bryantii* [Bryant *et al.* 1967] and *Methanobrevibacter arboriphilus* [Zeikus & Henning 1975] utilize only $H_2/CO_2$, while e.g. *Methanospirillum hungatei* and *Methanobacterium formicicum* can use $H_2/CO_2$ and formate [Schauer *et al.* 1982]. Acetoclastic *Methanosaeta* spp. [Huser *et al.* 1982] only use acetate. The most versatile methanogens are *Methanosarcina* spp.

which can use $H_2/CO_2$, acetate, methanol, methylated amines and pyruvate [Jones 1991]. Raskin *et al.* (1994a) published group specific probes, which are frequently used for the characterisation of methanogenic communities. Several authors have evaluated, optimised and amended this set of probes, since many new 16S rRNA gene sequences are available nowadays and the probes were initially designed for *ex situ* membrane hybridisation [Crocetti *et al.* 2006, Jupraputtasri *et al.* 2005]. Furthermore, Hori *et al.* (2006b) applied real-time PCR for the detection and quantification of some methanogens.

SRB are a metabolically versatile and polyphyletic group of micro-organisms [Castro *et al.* 2000]. Castro *et al.* divided sulphate reducing prokaryotes (SRP) into 4 groups (Table 3) and provided a comprehensive overview of available group-specific probes and PCR primers. Most probes tested target Gram-negative mesophilic SRB, however, and probes for other phylogenetic groups have yet to be tested [Castro *et al.* 2000]. More than a decade ago, Devereux *et al.* published some extensively validated probes for SRB [Devereux *et al.* 1992]. Lipski *et al.* (2001) gave recently an extensive overview of probes for SRB and many other species and groups relevant in wastewater treatment and related biotechnologies.

Table 3: Four groups of sulphate reducing prokaryotes [Castro *et al.* 2000].

| Group | Some genera |
|---|---|
| Gram-negative mesophilic SRB (*Desulfovibrionaceae* & *Desulfobacteriaceae*) | *Desulfomicrobium*<br>*Desulfomonas*<br>*Desulfovibrio*<br>*Desulfohalobium*<br>*Desulfonatronum*<br>*Desulfobulbus*<br>*Desulfobacter*<br>*Desulfobacterium*<br>*Desulfococcus*<br>*Desulfosarcina*<br>*Desulfomonile*<br>*Desulfonema*<br>*Desulfobotulus*<br>*Desulfoarculus*<br>*Desulfobacula*<br>*Desulfospira*<br>*Desulfocella*<br>*Desulfobacca*<br>*Desulfacinum*<br>*Desulforhabdus*<br>*Desulfocapsa*<br>*Desulforhopalus*<br>*Desulfofustis* |
| Gram-positive spore-forming SRB | *Desulfotomaculum*<br>*Desulfosporosinus* |
| Bacterial thermophilic SRB | *Thermodesulfobacterium*<br>*Thermodesulfovibrio*<br>*Thermodesulforhabdus* |
| Archaeal thermophilic suphate reducers | *Archaeoglobus* |

Based on their oxidative capacity, SRP can be subdivided into two groups: genera that oxidize organic compounds completely to $CO_2$, and those, which carry out incomplete oxidation, usually to acetate as end product [Widdel 1988]. Complete oxidizers include *Desulfobacter, Desulfobacterium, Desulfonema, Desulfosarcina, Archaeoglobus* and *Desulforhabdus* and are able to reduce sulphate autotrophically with $H_2/CO_2$ [Widdel & Hansen 1992]. Incomplete oxidizers include *Desulfomicrobium, Desulfobulbus, Desulfobotulus, Thermodesulfobacterium* and the majority of isolates within the heterotrophic sulphate reducing genera *Desulfovibrio* and *Desulfotomaculum* [Widdel 1988].

## Molecular sludge inventories

Composition and structure

It is important to study microbial diversity not only for basic scientific research, but to understand the link between diversity and community structure and function and hence performance of biotechnological processes [Kirk *et al.* 2004]. Several studies have been published that made use of molecular, cultivation-independent techniques to reveal the microbial composition of anaerobic sludge to different extents (Table 4). Fostered by these efforts, there is now a much broader phylogenetic framework of organisms that are commonly found in anaerobic wastewater treatment systems than was previously available through cultivation-based approaches (Fig. 3). One of the first more extensive studies was performed using immunologic methods, and revealed a significant increase in methanogen diversity in mesophilic granules 4 months after a temperature shift from 38 to 55°C [Visser *et al.* 1991]. At the same time the relative abundance of methanogens increased, while the total amount of micro-organisms remained at a constant level of around $10^{10}$ cells per gram of dry weight [Visser *et al.* 1991]. The number of *Methanobrevibacter smithii* PS, *Methanospirillum hungatii* JF1 and *Methanosarcina thermophila* TM1 decreased, while *Methanosaeta concilii*, and especially *Methanobrevibacter arboriphilus* AZ and *Methanobacterium thermoautotrophicum* deltaH significantly increased at the higher temperature [Visser *et al.* 1991]. It was suggested that the latter two populations were dormant at low numbers until the conditions for their growth became favourable by the increase in temperature [Visser *et al.* 1991].

Fluorescent *in situ* hybridization (FISH) and dot-blot hybridization have been the most commonly used molecular techniques to reveal the microbial diversity of sludge communities in the past. Most studies have focused on methanogenic Archaea, but also SRB and occasionally other populations were investigated. This focus on a limited number of phylogenetic and/or functional groups can probably be explained by their role in the anaerobic food web, but also because of the availability of extensive validated probe sets for SRBs and methanogens [Devereux *et al.* 1992, Raskin *et al.* 1994a]. While yielding very useful, quantitative data, this approach is limited by the fact that novel organisms can not be detected unless their rRNA sequences match with probes that are typically designed based on known sequence information. Usefulness of the previously mentioned probes for methanogens was shown by slot blot hybridization and FISH [Raskin *et al.* 1994b]. *Methanosarcina* spp., members of the order *Methanobacteriales*, and *Methanosaeta* spp. were the most abundant methanogens present in acetate-fed laboratory chemostats, solid-waste digesters, and sewage sludge digesters, respectively [Raskin *et al.* 1994b]. In a large survey, Raskin *et al.* investigated the methanogenic and SRB community structure of

21 full-scale anaerobic sewage sludge digesters and found that *Methanosarcinales* (38-68%, mainly *Methanosaeta*) and *Methanomicrobiales* constituted the majority of the methanogens, while *Methanobacteriales* and *Methanococcales* played a relatively minor role [Raskin *et al.* 1995]. *Desulfovibrio*, ranging from approximately 3-17% (average 7.6%) of the total 16S ribosomal RNA (rRNA), and *Desulfobulbus* spp., 2-13% (average 5.5%) of the total 16S rRNA, were the dominant SRB populations, whereas *Desulfobacter* and *Desulfobacterium* spp. were present at lower levels (typically below 2% and 0.2% of total 16S rRNA, respectively) [Raskin *et al.* 1995]. In another study, Raskin *et al.* investigated the competition and coexistence of methanogens and SRB by comparing the microbial community structure and function before and after a major sulphate perturbation [Raskin *et al.* 1996]. Methanogenic reactors contained up to 25% methanogens (approximately 16% *Methanobacteriales* and 5% *Methanosarcinales*) as well as about 20% SRB (approximately 16% *Desulfovibrio* and 3% *Desulfobacterium*), even though sulphate was not present in the influent of this reactor [Raskin *et al.* 1996]. After sulphate was added to the influent, sulphate reduction was observed without lag-phase, and SRB levels increased to 30-40% (first *Desulfovibrio* and *Desulfobacterium* increased to approximately 26% and 8%, then decreased to 20.5% and 3.5%, and finally reached 35% and 4.5%, respectively) [Raskin *et al.* 1996]. Methanogenesis decreased immediately, and relative abundance of methanogens gradually decreased to approximately 8% after several months of operation at sulphidogenic conditions [Raskin *et al.* 1996]. On the other hand, a shift to sulphate-free medium in a sulphidogenic reactor did not cause a rapid shift to methanogenesis, and methane production and a substantial increase in the levels of methanogens was observed only after approximately 50 days following the perturbation [Raskin *et al.* 1996]. Levels similar to those observed in the previously methanogenic reactors were reached after three months of operation without sulphate [Raskin *et al.* 1996]. Harmsen *et al.* found a strong decrease of Bacteria (from 40% in the inoculum to 3% after three months) and a subsequent slight increase of Archaea to 55% in a methanogenic reactor, while a sulphidogenic reactor showed a surprising bacterial decrease from 40% to 15% and an increase of Archaea from 30% to 50% of DAPI stained cells [Harmsen *et al.* 1996]. However, in the methanogenic reactor the number of syntrophic organisms increased and the number of *Desulfobulbus* spp. remained equal, whereas *Desulfobulbus* spp. increased rapidly to almost 50% of all Bacteria present in the sulphidogenic reactor [Harmsen *et al.* 1996]. A strong decrease of bacteria from 20.3% ± 4.0% to 3.4% ± 0.2% was also found by Raskin *et al.* in laboratory chemostats, 5 months after changing the feed from glucose to acetate, while Archaea increased from 76.3% ± 15.8% to 85.8% ± 5.8% [Raskin *et al.* 1994b]. Methanogens and SRB were followed during the start-up of a mesophilic (37°C) and a thermophilic (55°C) continuously mixed bench-top reactor, and methanogenic population dynamics correlated well with the measured performance [Griffin *et al.* 1998]. *Methanosaeta* species were the most abundant methanogens in the inoculum, mesophilic anaerobic sewage sludge and cattle manure, but, as acetate built up, their levels decreased rapidly, which was paralleled by an increase in *Methanosarcina* species (up to 11.6% and 4.8% of total rRNA in mesophilic and thermophilic digesters, respectively) [Griffin *et al.* 1998]. *Methanobacteriaceae* were the most abundant hydrogenotrophic methanogens in both digesters, but their levels were higher in the thermophilic digester [Griffin *et al.* 1998]. While the most abundant SRB present in the digesters were members of the family *Desulfovibrionaceae*, they accounted for less than 2% of total rRNA and it seemed that SRB were not able to compete effectively with methanogens [Griffin *et*

*al.* 1998]. Merkel *et al.* found *Methanosaeta* dominating (> 90%) a totally mixed anaerobic pilot-scale reactor (33°C, approximately 250 liter, varying VFA mix as feed), while *Methanosarcina* were found to account for less than 1% of the archaeal subpopulation [Merkel *et al.* 1999]. SRB were found to fluctuate between 7-20% of the total cell counts and after increasing the sludge retention time from 25 to 100 days between 2-5% [Merkel *et al.* 1999]. Because sulphate content was low in the influent (< 0.2 mM/l), SRB were presumed to perform a fermentative and acetogenic metabolism [Merkel *et al.* 1999]. A substantial SRB population (1-5%) was also found by Gonzalez-Gil *et al.* in a full-scale EGSB reactor (570 $m^3$) with approximately 5.5 mg/l sulphate in the influent [Gonzalez-Gil *et al.* 2001].

One of the first molecular inventories of anaerobic sludge microbiota, based on rRNA gene-targeted PCR and partial sequencing, was published in 1994 [Ng *et al.* 1994]. Although this was a revolutionizing strategy at that time, only 27 sequences of less than 100 bp were retrieved because of technical limitations. Since anaerobic sludge digesters treat mixed feedstock under non-sterile conditions and are inoculated with organisms associated with plants and animals, including humans, and with organisms from terrestrial aquatic sources, the microbial community of the digester contents might be expected to be extremely heterogeneous. In addition, the microbial populations of digesters involved in the hydrolysis and fermentation of biopolymers are possibly not extremely specialized, and the conversions are carried out by bacteria of genera common to many habitats [Ng *et al.* 1994]. This proposed large diversity was confirmed in a large molecular inventory of sludge communities [Godon *et al.* 1997]. The community structure of an anaerobic reactor was established by analysis of 579 partial rRNA gene sequences (about 500 bp), and a total of 146 Operational Taxonomic Units (OTU's) defined by > 96% sequence similarity were found, comprising 133 bacterial, 6 archaeal and 7 eukaryal OTU's [Godon *et al.* 1997]. The distribution of the 133 OTU's into the bacterial phyla was: 46% low-GC Gram-positives, 16% in the *Cytophaga-Flexibacter-Bacteroides* (CFB) group, 17% of *Proteobacteria*, and 21% in other phyla (high-GC gram-positive, Green-Non-Sulphur bacteria (GNS), *Spirochaetes*, *Synergistes*, *Planctomyces-Chlamidia*, and non-affiliated) [Godon *et al.* 1997]. Remarkably, none of the bacterial SSU rRNA sequences had less than 3% divergence from sequences present in the databases at that time [Godon *et al.* 1997]. The number of archaeal OTU's was restricted to 6, and only 3 of them composed already more than 90% of the archaeal clone library. They were closely related to *Methanobacterium formicicum*, *Methanosarcina barkeri*, and *Methanosarcina frisius*, while the other OTU's were atypical and related to *Crenarchaea* and *Thermoplasma* spp. [Godon *et al.* 1997]. Eucarya present in the anaerobic wastewater treatment system were related to yeast (46%), amoebae (16%) and *Tritrichomonas foetus* (37%) [Godon *et al.* 1997]. Members of all taxa expected with known function, e.g., *Bacteroides*, *Eubacterium*, *Clostridium*, delta-*Proteobacteria* (SRB), *Syntrophomonas*, and methanogens, but also several genera whose functions were unknown in anaerobic wastewater treatment systems, including *Spirochaetes*, GNS, *Planctomyces*, *Synergistes*, non-methanogenic Archaea and Eucarya were found [Godon *et al.* 1997]. The bacterial 16S rRNA gene diversity of cultivable populations retrieved from the same anaerobic digester was compared with the molecular inventory via the same OTU definition [Delbès *et al.* 1998]. Only 20 OTU's were isolated, but there was not a large discrepancy with those found in the molecular inventory of Godon *et al.* [Delbès *et al.* 1998, Godon *et al.* 1997]. While only one isolate (a *Fusobacterium* sp.) was obtained that was not found with molecular techniques, no isolates were obtained from the *Planctomyces-Chlamydia*,

*Spirochaetes*, *Synergistes* and GNS groups [Delbès *et al.* 1998]. The reactor community was also monitored during the following two years, indicating rapid significant shifts in the bacterial composition, while the archaeal community was relatively stable [Zumstein *et al.* 2000]. Curtis *et al.* (2002) calculated that the bacterial richness of this anaerobic digester might be over 9000 taxa.

Sekiguchi *et al.* (1998) compared the microbial composition of granular sludge from mesophilic (35°C) and thermophilic (55°C) anaerobic lab-scale reactors treating sucrose/propionate/acetate-based artificial wastewater. With an OTU definition of completely (100%) identical sequences, 115 partial (500-600 bp) sequences from the mesophilic reactor and 110 partial (500-600 bp) sequences from the thermophilic reactor were grouped in 36 and 24 OTU's, respectively [Sekiguchi *et al.* 1998]. Besides methanogenic Archaea (19%) (almost solely *Methanosaeta concilii*), 27% of the total number of clones from the mesophilic reactor grouped in the delta subclass of *Proteobacteria* (SRB and syntrophic strains), 14% grouped with GNS bacteria, 10% with *Planctomyces* and relatives, 8% with *Cytophaga*/*Flexibacter*/*Bacteroides*, 5% with *Thermodesulfovibrio*, 7% with low GC Gram-positive bacteria (e.g. *Clostridium acetobutylicum*-related) and 4% with high GC Gram-positive bacteria (related to *Propionibacterium*) [Sekiguchi *et al.* 1998]. From the thermophilic reactor mainly (22% of the total number of clones) methanogenic Archaea clones were obtained, which were related to *Methanosaeta thermophila* (20 clones) and *Methanobacterium thermoformicicum* (4 clones) [Sekiguchi *et al.* 1998]. Similar to the mesophilic sludge, also the thermophilic sludge contained many (18%) GNS bacteria, while *Thermodesulfovibrio yellowstonii*-related organisms were significantly more abundant (19%) in the thermophilic sludge, as well as low GC Gram-positive bacteria (16%), like *Thermosyntropha lipolytica* and other syntrophic propionate-oxidizer-related species, and *Planctomyces* relatives (13%) [Sekiguchi *et al.* 1998]. *Synergistes*, belonging to the green sulphur bacteria, were only present in low numbers, while *Spirochaetes*-relatives were only detected in the mesophilic reactor. With FISH the presence of *Desulfobulbus* species in the mesophilic sludge was confirmed, but only in the outer layer of granules and *Syntrophobacter*-related species were confirmed and detected inside the granules [Sekiguchi *et al.* 1999]. Like Sekiguchi *et al.* (1999) also Santegoeds *et al.* (1999) found methanogens (probably *Methanosaeta*) in the core of granules, whereas SRB (mostly *Desulfovibrio* and *Desulfobulbus* in methanogenic-sulphidogenic aggregates, and *Desulfosarcina* and/or *Desulfococcus* in sulphidogenic granules) were mainly present in the outer shell. With a probe specific for GNS bacteria, filamentous cells were detected, mainly in the outermost layer of thermophilic sludge granules and only a few inside the granules of mesophilic and thermophilic sludge granules. The mesophilic sludge granules, however, were also covered with filamentous organisms, but did not hybridise with the specific GNS bacteria probe, suggesting that these are phylogenetically different from those observed in thermophilic granules [Sekiguchi *et al.* 1999]. In more indepth studies Sekiguchi *et al.* (2001c) and Yamada *et al.* (2005) revealed that these filamentous organisms probably metabolize primary substrates in wastewater, such as carbohydrates, since these cells were observed mainly on the surface of the sludge granules and that they seem to be important for granulation. *In situ* hybridization with probes for uncultured organisms does not only uncover the *in situ* morphology of specific populations, but also implies possible *in situ* metabolic functions from their spatial location. Such data will provide further insight into the structure and function of microbial consortia and give valuable information for isolation and cultivation of currently uncultivable microbes [Sekiguchi *et al.* 1999].

Wu *et al.* (2001) defined OTU's as clones with a unique electrophoretic position on DGGE, resulting in 5 archaeal and 15 bacterial OTU's from a terephthalate-degrading anaerobic granular sludge system. The majority of archaeal clones was most closely related to *Methanosaeta concilii* (81.7%), while other clones formed two OTU's related to *Methanospirillum* and *Methanogenium*. Most bacterial clones grouped in the delta subclass of *Proteobacteria* (78.5%), and also GNS bacteria (7.5%) and *Synergistes* (0.9%) were found. In a thermophilic anaerobic hybrid reactor treating terephthalate Chen *et al.* (2004) found mainly *Methanosaeta thermophila* (93% of archaeal clones) and a *Methanospirillum* related to the one found by Wu *et al.* (2001). Bacterial clones were obtained from two time points, covering an undefined perturbation and showed only moderate similarity (six phylotypes), while 30 phylotypes were unique for the sampling times. Most bacterial phylotypes were related to low GC Gram-positive bacteria and also close relatives of the candidate division OP5 and *Cytophaga*/*Flexibacter*/*Bacteroides* were found often [Chen *et al.* 2004].

Collins *et al.* (2003) investigated the microbial biomass composition of a mesophilic full-scale citric acid wastewater treatment system. By ARDRA 13 archaeal and 18 bacterial OTU's were identified from the respective 96-clone libraries. Five bacterial OTU's were identified as chimera. The other bacterial clones grouped with Gram-positive organisms (57%), delta-*Proteobacteria* (24%), *Cytophaga*/*Flexibacter*/*Bacteroides* (12%), and beta-*Proteobacteria* (7%). Eight archaeal OTU's represented euryarchaeotal methanogens, in particular *Methanosarcinales*, and remarkably, five archaeal OTU's, accounting for 56% of the archaeal clones, represented close relatives of *Crenarchaeota*. More recently, Roest *et al.* (2005) also found *Crenarchaeota*-relatives in a full-scale mesophilic paper mill wastewater treatment reactor.

The methanogenic community structures of six anaerobic sludges were examined by McHugh *et al.* (2003c). While ARDRA revealed in total 18 different OTU's, sequence analysis showed strong similarities between several of the OTU's. All clones were assigned to the class *Euryarchaeota*, and 111 (56%) *Methanosaeta*-like clones were found among the 200 analyzed. Especially a psychrophilic lab-scale reactor was dominated by *Methanosaeta* (75%), but their presence in all tested sludges, irrespective of wastewater type or operating temperature, indicated the importance of this organism for stable and efficient operation of an anaerobic bioreactor. *Methanobacteriales*-members were found in five of the six sludges (24% of clones analysed). In another study the archaeal diversity in 44 anaerobic digesters was determined and not more than 23 different genetic variants were detected [Leclerc *et al.* 2004]. Each digester harboured between two to nine archaeal sequences, all in the *Euryarchaeaota* subdomain, and with only one of them corresponding to a putative acetate-utilizing species, which was in 84% of the digesters *Methanosaeta concilii* [Leclerc *et al.* 2004]. In 73% of the digesters, sequences were detected that were closely related to 16S rRNA gene clone vadinDC06, located in the *Methanobacterium* clade of which also *Methanobacterium formicicum* was often found (in 50% of the digesters) [Leclerc *et al.* 2004]. The nature of the influent (type of wastewater treated) did not have a clear effect on the archaeal diversity, although paper-mill wastewater treating digesters showed an increased number of clone vadinDC06 and *Methanobacterium formicicum* sequences [Leclerc *et al.* 2004]. In contrast, the digester process type had some effect, with the stirred-tank digesters harbouring a considerable archaeal diversity, which may mean that other processes impose more selection pressure, like adhesion and granulation [Leclerc *et al.* 2004]. The studies of

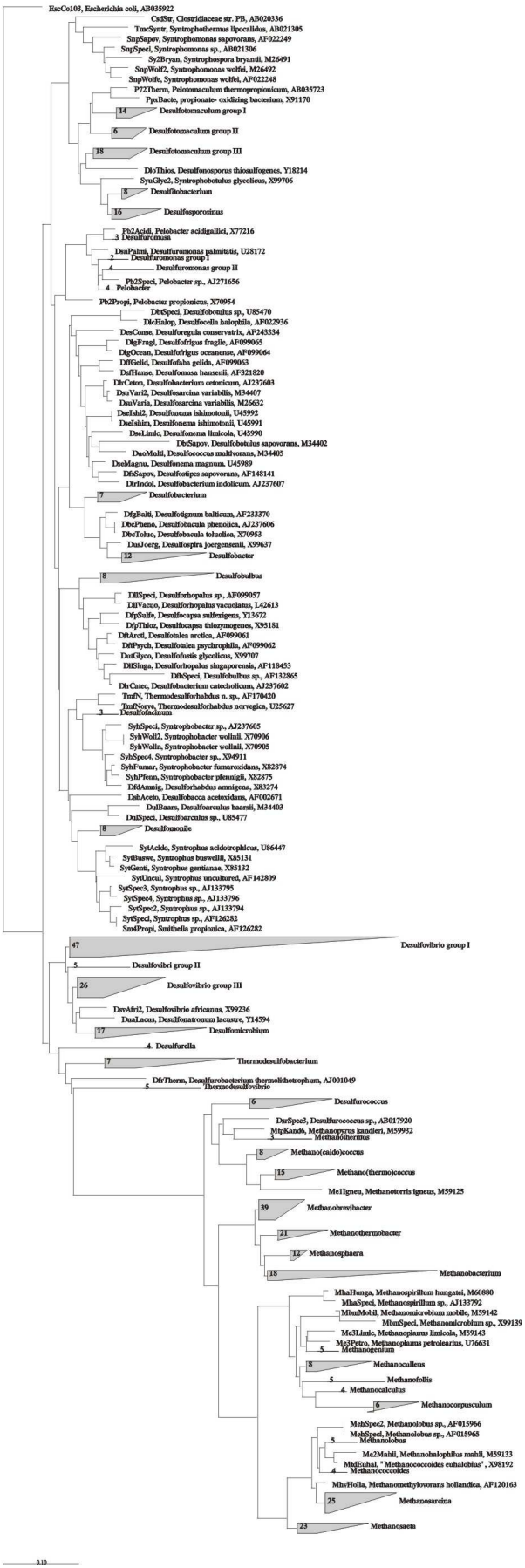
McHugh *et al.* (2003c) and Leclerc *et al.* (2004) strongly suggest that the source of metacommunity for *Euryarchaeota* in anaerobic digesters is low when assessed using rRNA gene sequences, although larger reactors have in general more diversity [Curtis and Sloan 2004, Leclerc *et al.* 2004, McHugh *et al.* 2003c].
Recently a comprehensive molecular inventory of a municipal anaerobic mesophilic wastewater treatment system was published [Chouari *et al.* 2005]. In total 246 archaeal and 579 bacterial nearly full-length 16S rRNA gene sequences were analysed, and with a 97% similarity threshold for OTU assignment, 20 archaeal OTU's and 206 bacterial OTU's were found. The archaeal OTU's grouped as follows: 2 (10%) *Methanosarcinales*, 7 (35%) *Methanomicrobiales*, 5 (25%) Arc I, a new *Euryarchaeota* phylum, and 6 (30%) *Crenarchaeota* [Chouari *et al.* 2005]. After specific probe development and enrichment culturing it was found that members of the novel predominant Arc I group used formate or $H_2/CO_2$ [Chouari *et al.* 2005]. OTU distribution within the bacterial library was as follows: 80 (38.8%) *Bacteroidetes*, 54 (26.2%) *Firmicutes*, 3 (1.5%) *Actinobacteria*, 44 (21.3%) *Proteobacteria* (mainly delta (19), but also beta (12) and alpha (10) subgroups), 7 (3.4%) *Chloroflexi*, 9 (4.4%) *Synergistes*, 5 (2.4%) *Thermotogales*, 1 (0.5%) OP8 and 3 (1.5%) OP9 [Chouari *et al.* 2005]. However, dot blot hybridisation showed that the *Chloroflexi* phylum accounted for 20.2% of the bacterial community in the municipal anaerobic mesophilic wastewater treatment system [Chouari *et al.* 2005]. The discrepancy between both analyses might be explained by skewed representations of the relative abundance of organisms in PCR-clone libraries [Hugenholtz & Goebel 2001].

Figure 3: Phylogenetic tree of organisms commonly found in anaerobic wastewater treatment systems, constructed with archaeal and bacterial 16S rRNA gene sequences retrieved from the ARB database, using the neighbour joining method and Felsenstein correction (*E. coli* positions 566 to 1091) as implemented in the ARB software package [Ludwig *et al.* 2004, Saitou & Nei 1987].

Table 4: Overview of results from clone library-based surveys of sludges from different anaerobic wastewater treatment reactors.

a. Percentages OTU's (> 96% sequence similarity within an OTU)
b. Clone percentages
c. 56% *Methanosaeta*-like clones and > 5% *Methanosarsina*-like (particular *Methanomethylovorans*) clones
d. Percentages of reactors
e. Closest related to clone vadinDC06 [Godon *et al.* 1997] with *Methanobacterium formicicum* as closest cultured relative
f. 84% *Methanosaeta concilli*-related
g. Percentages OTU's (> 97% sequence similarity within an OTU)
h. Percentages of clones with a unique electrophoretic position on DGGE
i. Clone percentages screened with ARDRA

ND. Not detected


EacCo103, Escherichia coli, AB035922
CndStr, Clostridiaceae str. PB, AB020336
TmcSyntr, Syntrophothermus lipocalidus, AB021305
SnpSapov, Syntrophomonas sapovorans, AF022249
SnpSpeci, Syntrophomonas sp., AB021306
SyzBryan, Syntrophospora bryantii, M26491
SnpWolf2, Syntrophomonas wolfei, M26492
SnpWolfe, Syntrophomonas wolfei, AF022248
P7ZTherm, Pelotomaculum thermopropionicum, AB035723
PpxBacte, propionate- oxidizing bacterium, X91170
14 Desulfotomaculum group I
6 Desulfotomaculum group II
18 Desulfotomaculum group III
DloThiox, Desulfonosporus thiosulfogenes, Y18214
SyuGlyc2, Syntrophobotulus glycolicus, X99706
8 Desulfitobacterium
16 Desulfosporosinus
Pb2Acidi, Pelobacter acidigallici, X77216
3 Desulfuromusa
DsnPalmi, Desulfuromonas palmitatis, U28172
2 Desulfuromonas group I
4 Desulfuromonas group II
Pb2Speci, Pelobacter sp., AJ271656
4 Pelobacter
Pb2Propi, Pelobacter propionicus, X70954
DbtSpeci, Desulfobotulus sp., U85470
DlcHalop, Desulfocella halophila, AF022936
DesConse, Desulforegula conservatrix, AF243334
DfgFragi, Desulfofrigus fragile, AF099065
DfgOcean, Desulfofrigus oceanense, AF099064
DftGelid, Desulfofaba gelida, AF099063
DsfHanse, Desulfomusa hansenii, AF321820
DlrCeton, Desulfobacterium cetonicum, AJ237603
DsuVari2, Desulfosarcina variabilis, M34407
DsuVaria, Desulfosarcina variabilis, M26632
DseIshi2, Desulfonema ishimotonii, U45992
DseIshim, Desulfonema ishimotonii, U45991
DseLimic, Desulfonema limicola, U45990
DbtSapov, Desulfobotulus sapovorans, M34402
DuoMulti, Desulfococcus multivorans, M34405
DseMagnu, Desulfonema magnum, U45989
DfsSapov, Desulfotignum balticum, AF148141
DlrIndol, Desulfobacterium indolicum, AJ237607
7 Desulfobacterium
DfgBalti, Desulfotignum balticum, AF233370
DbcPheno, Desulfobacula phenolica, AJ237606
DbcToluo, Desulfobacula toluolica, X70953
DsuJoerg, Desulfospira joergensenii, X99637
12 Desulfobacter
8 Desulfobulbus
DflSpeci, Desulforhopalus sp., AF099057
DflVacuo, Desulforhopalus vacuolatus, L42613
DfpSulfe, Desulfocapsa sulfexigens, Y13672
DfpThioz, Desulfocapsa thiozymogenes, X95181
DftArcti, Desulfotalea arctica, AF099061
DftPsych, Desulfotalea psychrophila, AF099062
DstGlyco, Desulfofustis glycolicus, X99707
DflSinga, Desulforhopalus singaporensis, AF118453
DfhSpeci, Desulfobulbus sp., AF132865
DlrCatec, Desulfobacterium catecholicum, AJ237602
TmfN, Thermodesulforhabdus n. sp., AF170420
TmfNorve, Thermodesulforhabdus norvegica, U25627
3 Desulfofaclinum
SyhSpeci, Syntrophobacter sp., AJ237605
SyhWoll2, Syntrophobacter wolinii, X70906
SyhWolin, Syntrophobacter wolinii, X70905
SyhSpec4, Syntrophobacter sp., X94911
SyhFumar, Syntrophobacter fumaroxidans, X82874
SyhPfenn, Syntrophobacter pfennigii, X82875
DfdAmnig, Desulforhabdus amnigena, X83274
DsbAceto, Desulfobacca acetoxidans, AF002671
DulBaars, Desulfoarculus baarsii, M34403
DulSpeci, Desulfoarculus sp., U85477
8 Desulfomonile
SytAcido, Syntrophus acidotrophicus, U86447
SyiBuswe, Syntrophus buswellii, X85131
SytGenti, Syntrophus gentianae, X85132
SyuUncul, Syntrophus uncultured, AF142809
SytSpec3, Syntrophus sp., AJ133795
SytSpec4, Syntrophus sp., AJ133796
SytSpec2, Syntrophus sp., AJ133794
SytSpeci, Syntrophus sp., AF126282
Sm4Propi, Smithella propionica, AF126282
47 Desulfovibrio group I
5 Desulfovibri group II
26 Desulfovibrio group III
DavAfri2, Desulfovibrio africanus, X99236
DunLacus, Desulfonatronum lacustre, Y14594
17 Desulfomicrobium
4 Desulfurella
7 Thermodesulfobacterium
DfrTherm, Desulfurobacterium thermolithotrophum, AJ001049
5 Thermodesulfovibrio
6 Desulfurococcus
DurSpec3, Desulfurococcus sp., AB017920
MtpKand6, Methanopyrus kandleri, M59932
3 Methanothermus
8 Methano(caldo)coccus
15 Methano(thermo)coccus
Me1Igneu, Methanotorris igneus, M59125
39 Methanobrevibacter
21 Methanothermobacter
12 Methanosphaera
18 Methanobacterium
MhaHunga, Methanospirillum hungatei, M60880
MhaSpeci, Methanospirillum sp., AJ133792
MbmMobil, Methanomicrobium mobile, M59142
MbmSpeci, Methanomicrobium sp., X99139
Me3Limic, Methanoplanus limicola, M59143
Me3Petro, Methanoplanus petrolearius, U76631
5 Methanogenium
8 Methanoculleus
5 Methanofollis
4 Methanocalculus
6 Methanocorpusculum
MehSpec2, Methanolobus sp., AF015966
MehSpeci, Methanolobus sp., AF015965
5 Methanolobus
Me2Mahii, Methanohalophilus mahii, M59133
MtuEuhal, "Methanococcoides euhalobius", X98192
4 Methanococcoides
MhvHolla, Methanomethylovorans hollandica, AF120163
25 Methanosarcina
23 Methanosaeta
0.10

| | *Bacteria* | | | | | | | | | | | | | | | | *Archaea* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reactor | *AlphaProteobacteria* | *BetaProteobacteria* | *GammaProteobacteri* | *DeltaProteobacteria* | *Cytophaga-Flexibacter-Bacteroidetes* | *Firmicutes* | *Actinobacteria* | *Planctomyces-Chlamydia* | GNS | *Spirochaetes* | *Synergistes* | *Chloroflexi* | *Thermotogales* | *Thermodesulfovibrio* | Green sulphur bacteria | *Methanobacteriales* | *Methanococcales* | *Methanomicrobiales* | *Methanosarcinales* | *Crenarchaea* | *Thermoplasma* | Arc I | Reference |
| Fluidised-bed fed by vinasses[a] | 17% | | | | 16% | 46% | 4% | 3% | 5% | 5% | 3% | ND | ND | ND | ND | 17% | ND | ND | 33% | 17% | 33% | ND | [Godon *et al.* 1997] |
| Mesophilic sucrose/ propionate/acetate fed[b] | ND | ND | ND | 27% | 8% | 7% | 4% | 10% | 14% | 3% | 1% | ND | ND | 5% | 2% | 19% | | | | | | | [Sekiguchi *et al.* 1998] |
| Thermophilic fed with sucrose/ propionate/acetate[b] | ND | ND | ND | ND | ND | 16% | ND | 13% | 18% | ND | 5% | ND | ND | 19% | 7% | 22% | | | | | | | [Sekiguchi *et al.* 1998] |
| Mesophilic terephthalate[h] | ND | ND | ND | 78.5% | ND | ND | ND | ND | 7.5% | ND | 0.9% | ND | ND | ND | ND | ND | ND | 18.3% | 81.7% | ND | ND | ND | [Wu *et al.* 2001] |
| Mesophilic citric acid[i] | ND | 7% | ND | 24% | 12% | 57% | | ND | ND | ND | ND | ND | ND | ND | ND | 44% | | | | 56% | ND | ND | [Collins *et al.* 2003] |
| Six anaerobic reactors[b] | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | 24% | 15% | ND | 61%[c] | ND | ND | ND | [McHugh *et al.* 2003c] |
| 44 anaerobic reactors[d] | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | 73%[e] | ND | ND | 98%[f] | ND | ND | ND | [Leclerc *et al.* 2004] |
| Municipal[g] | 4.8% | 5.8% | 1% | 9.2% | 38.8% | 26.2% | 1.5% | ND | 2% | ND | 4.4% | 3.4% | 2.4% | ND | ND | ND | ND | 35% | 10% | ND | 30% | 25% | [Chouari *et al.* 2005] |

Functional and compositional stability

In order to investigate the relation between functional and compositional stability in enegineered ecosystems Fernández *et al.* (1999) followed anaerobic methanogenic bioreactors over a period of almost two years. Microbial diversity was estimated by ARDRA, defining different profiles as OTU. Screening in total 252 clones from each domain in seven sampling points over a period of 605 days, a total of 75 bacterial OTU's and only 21 archaeal OTU's were found, of which the three most dominant ones accounted for 80% of the clones. Although the anaerobic reactor was functionally stable over time regarding COD removal and pH, the microbial community showed a dynamic composition. While *Methanobacterium formicicum* was detected initially at very high levels (between 72-94% of the clones), this OTU was quickly replaced by *Methanosarcina mazei* and a *Methanobacterium bryantii*-related organism [Fernández *et al.* 1999]. The bacterial community was more variable than the archaeal community and the total number of OTU's at each sampling point ranged from 8 to 24 with a mean of 13 [Fernández *et al.* 1999]. Since only 12 bacterial clones were sequenced, only a few phylogenetic groups were identified of which the *Spirochaetes* phylum was dominating the first period. In the second period, *Eubacterium* and *Propionibacterium* related organisms dominated [Fernández *et al.* 1999]. Phylogenetic analysis revealed that the genetic instability detected by ARDRA suggested metabolic variations in the community. If, however, similar physiology is assumed for phylogenetically related taxa, the metabolic variation was less dramatic than were the genetic changes observed [Fernández *et al.* 1999]. Multiple genera and species that are known or expected to have similar physiological properties can coexist in the same ecosystem, providing functional redundancy, and maybe the observed community instability was an early warning for the failure of the reactor that occurred at the end of the study. Interestingly, an increase of ribotype and morphotype diversity after glucose perturbations demonstrated that fluctuating environmental conditions can indeed increase the diversity of microbial communities and more stable communities appeared functionally less stable, suggesting that an inflexible community structure may be associated with larger functional instability [Fernandez *et al.* 2000]. It was suggested that subdominant populations participate in the reactive capacity of the digester ecosystem and may help it to face perturbations and foster functional stability [Delbès *et al.* 2000]. Microbial communities metabolizing substrates via several parallel pathways, which means via a network of multiple routes for substrate flow, exhibit rapid recovery from perturbations compared to communities that are organized along linear microbial food chains [Fernandez *et al.* 2000, Hashsham *et al.* 2000]. It is becoming clear that equilibrium-based concepts of stability apply mainly to aggregate communities or ecosystem properties, whereas individual populations are generally in a state of flux even under stable conditions [Briones & Raskin 2003]. It has been demonstrated that stability is better correlated to functional redundancy and not to population diversity *per se*, so processes and interactions that promote functional stability usually result from larger functional redundancy and functional niche complementation. Therefore, although functional stability is highly dependent on the role of functionally important populations, it is also essential to characterize so-called minor community components (i.e. their functional groupings and interactions) in order to better understand stability over long time periods and after a wide range of perturbations [Briones & Raskin 2003].

In another study an acetate crisis in a glucose-fed anaerobic reactor was investigated. A shift in the methanogens from *Methanobacterium formicicum*-related to *Methanosaeta concilii*-related Archaea was detected at the 16S rRNA level, but not at the 16S rRNA gene level [Delbès *et al.* 2001]. This indicates a major shift in metabolically active populations, rather than their contribution to biomass. Not more than 15 different populations were identified, but it was clear that *Spirochaetes*-related organisms were very active in the reactor, especially during the acetate crisis together with *Clostridium indolis*-related organisms and to a lesser extent other low-GC Gram-positives and GNS bacteria [Delbès *et al.* 2001]. On the contrary *Synergistes* showed a decreased activity during the acetate crisis [Delbès *et al.* 2001]. Unfortunately, only little is known about the ecological role of these and previously mentioned microbial groups. However, after starvation *Synergistes*-related organisms showed an upsurge when lactate was added and addition of starch caused an increase in

*Bacteroides* and *Clostridium*-related species [Delbès *et al.* 2000]. *Clostridia*-related populations, which are presumed versatile with respect to their fermentation spectrum, also increased after a shock at pH 6, while under normal operation conditions at neutral pH a *Spirochaetes*-related bacterium was most prominent and was suggested to retrieve energy from the fermentation of glucose [Delbès *et al.* 2000].
Leclerc *et al.* (2001) monitored the methanogenic community during the start-up of an anaerobic digester fed with glucose and found that the initial dominance of *Methanosaeta concilii* was changed within 21 days to *Methanobacterium formicicum*, which was not detected at day 1, but remained the dominating methanogen until the end of the experiment (107 days). *Methanosarcina* was not detected and together with the declining numbers of *M. concilii*, two instability periods of the reactor, characterized by high acetate concentrations, might be explained [Leclerc *et al.* 2001].
Pender *et al.* (2004) investigated the long-term effects of operating temperature and sulphate addition on the methanogenic community structure of anaerobic hybrid lab-scale reactors and found only 18 different archaeal OTU's (defined by different ARDRA profiles) over the course of the whole experiment (1081 days). The seed sludge, from a full-scale potato-processing wastewater digester, and the two mesophilic lab-scale reactors were dominated by close relatives of *Methanosaeta concilii*. While *Methanobacteriales* and *Methanocorpusculum parvum* related sequences were also detected at this stage, *M. parvum* related organisms were the only detected methanogens by the end of the experiment at elevated temperatures. Sulphate addition and consecutive temperature increase showed an even more profound effect on the methanogenic community. Hydrogen-utilising methanogens, related to *Methanobacterium thermoautotrophicum* dominated at the conclusion of the trial. This supports the idea that hydrogenotrophic methanogens show a tendency to dominate biomass from stressed bioreactors.

**Integrated approach**

Although microbial cells can now be retrieved and identified from almost any environmental sample without isolation and cultivation, quantification, activity measurements and clear understanding of microbial interactions cannot be achieved with only culture-independent techniques. Since stability and performance in anaerobic bioreactors are strongly dependent on complex microbial interactions, integration of cultivation-dependent and molecular microbial approaches provides an opportunity to couple the microbial structure and the functional characteristics of the system [Pereira *et al.* 2002]. From an ecological point of view it is also very important to know how the different organisms are operating and interacting with each other to establish and maintain a sustainable well-functioning ecosystem. The ultimate goal of environmental microbiology is to understand the mechanistic relationships between habitat characteristics, evolutionary pressures, microbial diversity, and biochemical processes and their genetic controls [Madsen 2005]. Molecular phylogenetic surveys have revealed a vast number of new microbial groups and a remaining challenge for microbiologists is to better characterize the biological properties of these newly described microbial taxa [DeLong and Pace 2001]. Although shortcomings of traditional culture techniques in capturing a large portion of the micro-organisms abundant in natural ecosystems are recognized, the availability of cultured isolates is certainly needed for a better understanding of their physiology and role in biochemical cycles [Connon & Giovannoni 2002]. However, to deal with not yet cultured organisms, some technological progress has been made in analysing the genetic potential within ecosystems and *in situ* activity of bacteria [Wagner 2004a]. High trough-put genome sequencing and DNA micro-array technologies make it possible to obtain a better insight into the genetic potential of organisms and gene expression at the genomic scale when exposed to a specific condition [Zoetendal *et al.* 2004a]. Also metagenomic libraries, which can contain > 100 kb of genomic DNA fragments, provide a tool to study the phylogenetic, physical and functional properties of communities without the need for cultivation [Handelsman 2004]. Subtractive hybridisation, like Representational Difference Analysis (RDA)

[Lisitsyn *et al.* 1993, Felske 2002], is an interesting PCR-based technique to obtain unique gene sequences, but is probably too sensitive to be applied for complex microbial communities. It also should be realised that genetic information alone is very hypothetical and has to be verified via expression experiments and mutagenesis schemes to confirm proposed functions. Although genome analysis has provided unforeseen insights into the physiology of prokaryotes, the function of almost half of the sequenced genes still remains enigmatic, indicating a clear need for better knowledge of functions [Cypionka 2005]. Such considerations reinforce the need for cultured isolates, but unfortunately there is still a lack of knowledge and pure cultures do not necessarily reflect the *in situ* situation. It is easy to be seduced by the elegance of the technology now available to microbial ecologists, but ingenious technology alone does not increase understanding, since only the application of these immensely powerful tools to address real ecological questions will show their full potential and increase our knowledge [Gray & Head 2001]. As new techniques are developed, our level of understanding increases and our knowledge expands [Kirk *et al.* 2004]. Some recent developments with isotope-labelled substrates, which integrate culture-dependent with culture-independent approaches, address the aforementioned problems and will be discussed below. Table 5 gives an overview of approaches used in microbial ecology.

Table 5: Overview of approaches used in microbial ecology.

| Approach | Description | Remarks |
|---|---|---|
| Cultivation | Study micro-organisms in defined circumstances. | Only a minor fraction of the micro-organisms can be cultivated. |
| PCR | Specific and sensitive amplification of genetic material (DNA/RNA). | Primers developed from known sequences and can cause bias. |
| Real-time PCR | Sensitive and sensitive quantitative amplification suitable for high-throughput over a wide dynamic range. | Primers developed from known sequences and can be biased. |
| Fingerprinting (DGGE/SSCP/T-RFLP etc.) | Rapid overview of diversity. Ideal for comparisons of ecosystems in time or between different samples. | Bias in nucleic acids extraction and PCR. Only dominant populations can be visualised. |
| Sequencing | Gold standard for sequence retrieval. | Nucleic acids extraction, PCR and cloning can be biased. |
| FISH | Enumeration of micro-organisms *in situ*. Allows localisation and quantification. | Laborious without automatisation and requires sequence information for probe development. Cell permeabilisation and fixation can cause bias. |
| FACS | Rapid enumeration and separation of micro-organisms. | Requires sequence information for probe development and is compromised with cell clusters. |
| Dot-blot hybridisation | Quantification of specific sequences. | Requires sequence information for probe development. |
| Identification arrays | Parallel detection and identification of many micro-organisms | Complicated, especially due to the amount of data and (statistical) result analysis. |
| Expression arrays | Allows rapid comparison of the transcriptome of different micro-organisms or of the same micro-organisms at a different condition. | Function of many expressed genes is not yet known. |
| Subtractive hybridisation (e.g. RDA) | Retrieval of unique sequences. | Delicate technique and complicated for mixed communities. |
| *In situ* isotope tracking (e.g. SIP, MAR-FISH, isotope array) | Combination of cultivation and molecular techniques allowing the functional identification of active micro-organisms. | Not suitable for all environments and cross-feeding might prove difficult to interpretate. |

Stable Isotope Probing

Stable Isotope Probing (SIP) makes use of a stable isotope-labelled substrate submitted to the environment of interest and then assessing the assimilation of the isotope into microbial biomarkers. Typical biomarkers that have been used for stable isotope studies include nucleic acids [Manefield *et al.* 2002, Radajewski *et al.* 2000], phospholipid fatty acids [Treonis *et al.* 2004], and ergosterol [Malosso *et al.* 2004]. Of these biomarkers, nucleic acids may be the most useful because they contain the most taxonomic information and have greatest turnover rates [Griffiths *et al.* 2004]. After the addition and incubation of a $^{13}$C-labelled substrate, total nucleic acids are extracted and the $^{13}$C-labelled portion separated using density gradient centrifugation for subsequent molecular analysis. This approach was first applied to DNA to identify functionally active methanotrophic populations [Radajewski *et al.* 2000]. Following this study, it was demonstrated in a phenol degrading bioreactor that RNA may be a more suitable biomarker because of significantly higher turnover rates of rRNA, not requiring cellular replication for isotope assimilation [Manefield *et al.* 2002]. With this technique even anaerobic syntrophic propionate oxidising micro-organisms can be studied [Lueders *et al.* 2004a]. RNA-SIP has the advantage of greater sensitivity than DNA-SIP, and extension of RNA-SIP techniques to examine mRNA could in theory access the entire transcriptomes of Bacteria involved in a specific metabolic process in the environment [Dumond & Murrell 2005]. The potential applications of SIP are immense, and this powerful tool offers the opportunity to elucidate the functional properties of the plethora of uncultivated micro-organisms that are now known to dominate most natural environments by linking the occurrence of particular organisms with specific processes in natural communities [Gray & Head 2001]. Labelling of the chromosomal DNA by stable-isotope-labelled substrates offers the advantage of isolating an entire copy of the genome of metabolically active micro-organisms, which allows not only the phylogenetic identification by rRNA analysis, but also the detection and cloning of functional genes [Spring *et al.* 2000]. SIP-based time-course experiments with environmental samples will probably yield valuable information on microbial interactions and microbial food webs in the environment [Dumond & Murrell 2005]. This was shown in a study combining RNA-SIP and DNA-SIP for the simultaneous incorporation of stable isotope-labelled methanol in microcosms containing oxic rice-field cover soil [Lueders *et al.* 2004b]. The future holds great promise for SIP, particularly when combined with other emerging technologies such as micro-arrays and metagenomics or in combination with MAR-FISH, which was done to study the denitrification of a methanol-fed microbial community in activated sludge [Dumond & Murrell 2005, Ginige *et al.* 2004, Madsen 2005]. While it remains difficult to understand the utilization of labelled complex substrates by multiple micro-organisms and extended pathways, combinations of advanced techniques will be useful and improve insights.

MAR-FISH

Micro-Auto-Radiography (MAR) combined with Fluorescence *In Situ* Hybridisation (FISH) is a strong technique to couple functional analysis with identification of micro-organisms *in situ* [Andreasen & Nielsen 1997, Nielsen & Nielsen 2005, Lee *et al.* 1999]. Comparable to SIP, an isotope-labelled substrate is added to an environment of interest, but in this case the isotope is radio-active. The uptake of this radio-active-labelled substrate can be detected and the micro-organisms can be identified by FISH [Andreasen & Nielsen 1997, Nielsen & Nielsen 2005, Lee *et al.* 1999]. The combination of *in situ* rRNA hybridisation techniques, MAR, and confocal laser scanning microscopy provides a unique opportunity for obtaining cultivation-independent insights into the structure and function of microbial communities, since the *in vivo* uptake of substrates by micro-organisms under different environmental conditions can be directly detected and the involved organisms can be identified *in situ*, which should facilitate the design of appropriate isolation techniques for many hitherto uncultured bacteria that are only identified by molecular techniques [Lee *et al.* 1999]. Disadvantages of MAR-FISH are the laborious procedure, specific equipment (for radioactive labelled material) and the relatively limited number of probes that can be used for

identification. To date, MAR-FISH has been applied in a small number of studies of natural and engineered environments and data should be interpreted with caution, since complex metabolism cannot simply be deduced from the uptake of a single radio-active labelled substrate [Gray & Head 2001].

Isotope-array

Identification of active micro-organisms can be done with an isotope array. It combines the advantages of identification with a micro-array and the detection of active organisms that utilize a certain labelled substrate, like with SIP or MAR-FISH, but with high-throughput abilities. In the functional diversity array, diversity screening is combined with detection of populations responsible for specific transformations in the community [Polz *et al.* 2003]. RNA, extracted from samples incubated with radio-active labelled substrates, is fluorescently labelled and is hybridised to an identification micro-array. The major advantages of rRNA detection are the linearity of the labelling process and the possible limitation to a few cell divisions, which ensure that community structure will be only minimally biased [Polz *et al.* 2003]. Compared to MAR-FISH, the isotope array allows to apply many probes in parallel, a feature which will be of major importance if the ecophysiology of complex microbial communities is of interest, but in ecosystems with less active and dense prokaryotic communities, the applicability of present isotope arrays will be limited to numerically more abundant micro-organisms [Adamczyk *et al.* 2003, Wagner *et al.* 2006]. The number of approaches which allow inference of the activity and specific function of a micro-organism in its environment is still very limited, but an isotope array can be used in a PCR-independent manner to exploit the high parallelism and discriminatory power of micro-arrays for the direct identification of micro-organisms which consume a specific substrate in the environment [Adamczyk *et al.* 2003]. If combined with time series experiments, isotope array analyses have the potential to indicate metabolic links between different community members and thus to reveal building blocks of natural metabolic community networks [Wagner 2005]. Nonetheless, elucidation of structure-function relationships or niche differentiation of populations within microbial communities remains one of the big challenges in microbial ecology [Polz *et al.* 2003].

## Concluding remarks and future perspectives

Anaerobic wastewater treatment systems have been applied for several years already and major improvements have been made. Also microbiological techniques and functional application of microbiological methods have seen impressive leaps forward. However, the fundamental microbiological background of anaerobic wastewater treatment is still not fully understood. Structure-function questions are still challenging and need integrated research efforts for resolution. Molecular ecology and physiological data can be obtained together with newly developed technologies and the combination of classical and modern microbiological methods will definitely improve ecophysiological knowledge of wastewater treatment and many other environmental systems. Compilation and analysis of data should not only be limited to microbiological disciplines, but multidisciplinary research teams should work hand-in-hand to tackle the fundamental and applied questions that still remain unsolved until now. Processes like sludge granulation or disintegration are often observed, but not very well understood. The ratio behind successful wastewater treatment systems or unwanted process failures is often lacking. Continued application of innovative culture-dependent and culture-independent techniques, and the use of novel strategies will be pivotal to a better understanding and predictability of anaerobic wastewater treatment processes and reactor performance.

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# Chapter 2

## Effect of temperature and sulphate on the anaerobic microbial community degrading short-chain fatty acids

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**Abstract**

The microbial community of anaerobic biomass that was incubated at different temperatures with a mixture of short-chain fatty acid in the absence and presence of sulphate was investigated. Biochemical activities of the incubations were compared with the microbial community shifts using DGGE fingerprinting and subsequent cloning and sequencing analysis. A total of 324 bacterial clones retrieved from the incubations belonged mainly to the *Firmicutes* (42% of the clones) and *Deltaproteobacteria* (40% of the clones). Microbiota profiling by DGGE and analysis of clone libraries revealed that at a fixed temperature the bacterial community was strongly affected by the presence of sulphate, even though the same inoculum was used. Whereas the sulphidogenic bacterial populations did not change significantly during the incubation at the different temperatures, community shifts were observed in the absence of sulphate during the incubation at 37°C. Integration of molecular characterisation of the communities and biochemical results suggested that *Syntrophomonas* sp. and *Desulfobulbus propionicus* were involved in the syntrophic degradation of butyrate and propionate, respectively. This study indicated that biochemical results compared with the microbial community shifts observed by DGGE profiles and cloning and sequencing could be used efficiently to link identity to function of the mixed cultures, especially for the communities predominantly degrading the substrates sequentially.

**Introduction**

Anaerobic treatment is an accepted sustainable technology for a wide range of wastewater and waste types, and its applicability is growing each year. In the anaerobic degradation process, short-chain fatty acids are important intermediates which are further degraded by proton-reducing acetogens and hydrogen- and acetate-utilizing methanogens to methane and carbon dioxide [Gujer & Zehnder 1983]. The major intermediates acetate, propionate and butyrate may account for up to 80% of methanogenesis in anaerobic bioreactors [Mackie & Bryant 1981, Fang *et al.* 1995, Zellner *et al.* 1996]. In the presence of sulphate, sulphate reducing bacteria (SRB) degrade organic acids and hydrogen with sulphate. Complete conversion of organic compounds in anaerobic digestion processes requires complex microbial communities [Stams *et al.* 2005, Schink & Stams 2006]. The degradation of butyrate and propionate through methane depends on the syntrophic interaction with hydrogen utilizing organisms. Several fatty acid oxidizing bacteria have been isolated in co-culture with a hydrogen-utilizing partner [Stams 1994]. In the presence of sulphate, sulphidogens and methanogens compete for substrate such as formate and hydrogen, and with acetogens resulting in a decrease of methane production. Due to kinetic parameters and threshold hydrogen concentrations, hydrogen utilizing sulphidogens can outcompete the methanogens [Lovley *et al.* 1982, Stams *et al.* 2005].

Anaerobic digestion processes can be operated at temperatures ranging from 10 to 60°C [Speece 1996]. Temperature has a determining effect on the activity and community structure [McHugh *et al.* 2004]. Some studies have focused on the impact of temperature on the microbial communities; many of these studies dealt with archaeal communities [McHugh *et al.* 2003, Pender *et al.* 2004, Leven *et al.* 2007] and mainly carbohydrate-based substrate mixtures were used in these studies. Several mesophilic and thermophilic methanogens and acetogens have been isolated from short-chain fatty acids degrading communities. However, the interactions of mixed populations, which reflect the *in situ* conditions, are not clear. A better understanding of the consortium involved in the degradation of short-chain fatty acids may lead to effective management and improvements in the anaerobic treatment of wastewaters.

Previous studies have focused on structure and function of anaerobic communities enriched in the presence of either acetate, propionate or butyrate using cultivation-independent 16S ribosomal RNA

(rRNA) and/or functional gene analysis [Roest *et al.* 2005a, Shigematsu *et al.* 2004, Shigematsu *et al.* 2006]. However, by feeding single carbon sources a bias is introduced as members that are specialized in one particular substrate are enriched. Rather, the analysis of the communities degrading fatty acids mixtures is necessary to more realistically mimic the situation in bioreactors, and to obtain improved insight in the *in situ* microbial interactions and processes. The effect of the feeding pattern, in the presence of two substrates, on the active microbial community was reported before [Kong *et al.* 2004]. Although the degradation of short chain fatty acids mixtures has been well characterized in terms of physiological traits [Gorris *et al.* 1988, Collins *et al.* 2003, Han *et al.* 2005], the corresponding microbial communities existing in these anaerobic processes have not been characterised.

The objective of this study was to determine the microbial community structure of anaerobic biomass as a function of temperature as well as feed composition by denaturing gradient gel electrophoresis (DGGE) together with cloning and sequencing analysis, and to link this to substrate consumption patterns. To this end, 6 different temperatures (10, 25, 37, 45, 55 and 65°C) and 2 feed mixtures were selected. The feed mixtures were composed of butyrate, propionate and acetate without sulphate (Feed mixture-A) and with sulphate (Feed mixture-B). The consumption of each C-source in the mixtures was monitored during the entire course of the incubation, and compared with the microbial communities present.

## Materials and methods

### Source of inoculum

The inoculum was collected from a paper mill wastewater treatment plant (Industriewater Eerbeek BV, Eerbeek, The Netherlands) exhibiting approximately 70-80% COD removal, in which both sulphate reduction and methanogenesis were observed. In the gas produced, $CH_4$ (80%), $CO_2$ (19%), and $H_2S$ (1%) are present. The COD/$SO_4^{2-}$ of the wastewater was between 9.5 and 10; the total COD 1.7 g.$l^{-1}$ and pH 6.9. The main constituents of the wastewater were as follows (mg COD/L): carbohydrate, mainly starch (850); acetate (500); propionate (300); butyrate (45); formate (20) and sulphate (180 mg/L) [Oude Elferink *et al.* 1998].

### Incubations

Basic medium was prepared as described previously [Plugge 2005]. 10 ml of washed granular inoculum with bicarbonate buffered medium was disintegrated by a Potter homogenizer (Tamson, Zoetermeer, The Netherlands). Sterile anaerobic incubation conditions were applied. Addition of yeast extract was omitted to eliminate the growth of primary fermenting microorganisms. Substrates were added to the basal medium from sterile anoxic concentrated stock solutions (1 M) by sterile syringes to the final concentrations. Two different feed mixtures were prepared. Feed mixture-A was composed of 10 mM acetate (640 mg COD.$l^{-1}$), 5 mM propionate (516), and 5 mM butyrate (832). Feed mixture-B had the same composition as Feed mixture-A, however included 31.25 mM sulphate.

Duplicate enrichments were incubated at 10, 25, 37, 45, 55, and 65°C in 580 ml serum bottles, containing 250 ml of medium. Since the duplicate experiments showed less than 5% of difference, in terms of biochemical performance, the average values of duplicates were used in the graphs and tables presented. The utilization of the substrate mixtures was followed by monitoring the decrease of each substrate, in addition to production of sulphide, methane and hydrogen. Autoclaved bottles including cell suspensions and substrates were incubated as abiotic controls. In addition, bottles including suspended biomass without substrates were incubated as negative controls at each temperature. Methane production values were corrected with the negative controls for all enrichments. All incubations were performed under static conditions and in the dark.

10% of active enrichments (vol:vol) were repeatedly transferred into fresh medium. After 3 consecutive transfers, incubations were used for experiments to determine the population dynamics together with biochemical performance. The growth rate measured after the first transfer to new media was the same growth rate measured after the third transfer of the enrichment culture. After the termination of each experiment, the exact amount of cell dry mass and pH were determined. The mass of the medium components was taken into account for correction of the biomass values. Optical density was monitored in 1 cm light path cuvettes at 600 nm. Substrate utilized by the cultures was calculated based on the equations presented in Oude Elferink *et al.* (1998). Percentage of carbon degradation by methanogens and sulphidogens was determined by methane and sulphide production, respectively.

Analytical techniques

Samples taken from the reactors and other incubations were centrifuged at 14,000 rpm for 10 minutes. Supernatant was analyzed for the volatile fatty acid (VFA) components by HPLC as described previously [Stams *et al.* 1993]. 0.2 ml of gas samples was removed from the incubations with a 1 ml sterile syringe to determine the amount of $CH_4$ and $H_2$ [De Bok *et al.* 2002]. Sulphide was determined as described by Trüper and Schlegel [Truper and Schlegel 1964].

Molecular characterization of enrichments

*i) Sampling.* Sampling for molecular and biochemical analyses was performed simultaneously. Well suspended samples were withdrawn from the batches with syringes under sterile conditions and were transferred into 10 ml pre-autoclaved screw-cap vials immediately. The aliquots were centrifuged at 10,000 rpm, 4$^{o}$C, for 15 min. Supernatants were discarded and the pellets were washed with phosphate buffered medium.

*ii) DNA extraction.* Biomass samples, as well as the inoculum samples from the Eerbeek paper mill anaerobic wastewater treatment plant, were directly subjected to DNA extraction, during which mechanical disruption by bead beating and phenol/chloroform/iso-amyl-alcohol extraction was performed as outlined elsewhere [Oude Elferink *et al.* 1997].

*iii) PCR amplification.* Aliquots (1 µl) of the DNA, isolated from the biomass samples, were used for subsequent PCR amplification of bacterial and archaeal 16S rRNA gene fragments. For bacterial PCR U968-GC (5′-AAC GCG AAG AAC CTT AC-3′) forward and L1401 (5′-GCG TGT GTA CAA GAC CC-3′) reverse primers [Nubel *et al.* 1996] were used to amplify the region between the V6 and the V8 regions of the bacterial 16S rRNA gene. For archaeal PCR, the V2-V3 region was amplified using A109 (5′-ACT GCT CAG TAA CAC GT-3′) [Roest *et al.* 2005a] forward and 515-GC (5′-ATC GTA TTA CCG CGG CTG CTG GCA-3′) [Lane 1991] reverse primers. The GC-clamp sequence was CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G. All primers were purchased from MWG-Biotech (Ebersberg, Germany). The reaction mixture was subjected to PCR under the following conditions: denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing for 1 min (annealing temperatures for *Bacteria* and *Archaea* were 56 and 52°C, respectively), and DNA extension at 68°C for 1 min. After the last amplification cycle, samples were kept for a final extension at 68°C for 7 min and were immediately cooled to 4°C. The size and the yield of the amplicons (5 µl) were checked by electrophoresis on a 1% (wt/vol) agarose gel (Invitrogen Breda, The Netherlands) in the presence of ethidium bromide.

*iv) DGGE.* GC-clamped PCR amplicons were subjected to electrophoresis in 8% polyacrylamide (37.5:1acrylamide-bisacrylamide) gels containing urea and formamide using DCode TM System apparatus (BioRad). DGGE was performed using a denaturing gradient ranging from 55% to 38% (bottom to top) and from 50% to 30% for bacterial and archaeal amplicons, respectively [Roest *et al.* 2005b]. DGGE gels were analyzed with the BioNumerics, v. 4.0 software package (Applied Maths, Sint-Martens-Latem, Belgium) to calculate similarity indices between the DGGE profiles using the Pearson product-moment correlation [Hane *et al.* 1993]. Unweighted Pair Group Method Arithmetic (UPGMA) averages were calculated and corresponding dendrograms showing the

relationships between the DGGE profiles were constructed. For each temperature and feed mixture DGGE was performed in duplicate.

*v) Cloning and Sequencing.* Representative samples for each incubation condition (given in italic and bold in the figures of DGGE profiles) were selected for further cloning and sequencing. The amplified V6-V8 regions (*Bacteria*) and V2-V3 regions (*Archaea*) of the 16S rRNA gene (~400 bp) were purified with the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The quality and the amount of the purified PCR products were determined by electrophoresis on a 1% (wt/vol) agarose gel with a DNA marker (GeneRuler™ 100bp DNA Ladder Plus, MBI Fermentas). These amplicons were cloned into XL1-Blue competent cells (Stratagene, La Jolla, CA) using the pGEM®-T Easy Vector System I (Promega, Madison, WI) following the manufacturer's instructions. Randomly picked clones were transferred into 20 µl TE buffer and incubated at 95°C for 10 min to lyse the cells. The resulting crude lysate was used as template for PCR with the pGEM®-T specific PG1 (5′-TGG CGG CCG CGG GAA-3′) and the PG2 (5′-GGC CGC GAA TTC ACT AGT G-3′) primers. Amplicons were subsequently digested with a mixture of AluI, CfoI, and MspI (Promega) restriction enzymes at 37°C for 90 min. To differentiate these digested clones, restriction fragment length polymorphism (RFLP) analysis was conducted by using the Elchrom Submerged Gel Electrophoresis System (Cham, Switzerland). The digested PCR products were run in 12% (wt/vol) pre-cast agarose gels (Elchrom, Cham, Switzerland) with a DNA marker (GeneRuler™ 50 bp DNA Ladder Plus, MBI Fermentas) at 100 V, 55 °C, for 45 min and were visualized with ethidium bromide staining. Representative clones with distinct RFLP profiles were selected for further DGGE and sequencing analyses. Sequence analysis was carried out with the Sequenase sequencing kit (Amersham, Slough, United Kingdom) and vector-targeted primer T7 ( 5′-TAA TAC GAC TCA CTA TAG GG-3′ ) (Promega). The sequences were automatically analysed on a LI-COR (Lincoln, NE) DNA sequencer 4000 L, and were checked and edited manually. Alignments were performed using the Clustal X program [Chenna *et al.* 2003]. The alignments were manually checked using GeneDoc Multiple Sequence Alignment Editor & Shading Utility Version 2.6.002 (http://www.psc.edu/biomed/genedoc). A phylogenetic tree was constructed using the ARB program package [Ludwig *et al.* 2004]. All sequences were checked for chimeric artefacts using the CHIMERA_CHECK program version 2.7 in the Ribosomal Database Project II (RDP II) [Maidak *et al.* 2001]. Homology searches of the databases were conducted by using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) [Altschul *et al.* 1997].

The nucleotide sequences obtained in this study have been deposited in the GenBank database under accession numbers EU156183 to EU156241.

## Results and discussion

### Biochemical performance of incubations

Substrate degradation and product formation data of the batch incubations are given in Table 1. For feed mixture-A, with butyrate (642 mg COD/L), propionate (516 mg COD/L), and acetate (832 mg COD/L), growth was observed only at 25, 37, and 45ºC. All constituents of the feed were consumed at 37ºC, whereas acetate was not degraded at 45ºC. It was produced from propionate and butyrate in stoichiometries of 1 mol acetate/mol propionate and 2 mol acetate/mol butyrate consumed. The accumulation of acetate, in combination with the observed production of methane, indicated that acetogenic bacteria and hydrogenotrophic archaea were still active. Hence, acetate-utilizing methanogens seemed more sensitive to high temperature than hydrogen-utilizing methanogens. Similar trends have been observed previously, such as reported for methanogenesis in rice field soils at different temperatures [Frey & Conrad 2000]. For feed mixture-B, competition between sulphate reducers and methanogens was influenced by temperature. A comparison of methanogenesis and sulphate reduction showed that ~50% of the COD of the acetate, propionate and butyrate mixture was degraded completely by sulphidogens at 25 and 37ºC. At 45ºC, all propionate and butyrate was degraded by sulphidogens without the production of acetate

demonstrating complete oxidation of butyrate and propionate to $CO_2$, whereas the initially present acetate in the feed mixture was not degraded at all. It has been shown previously that at higher temperatures acetotrophic methanogens were not developed [Colleran & Pender 2002]. Although a slight decrease was observed for butyrate at other temperatures (10, 55, and 65ºC), such a decrease was not substantial for propionate and acetate.

Comparison of the DGGE profiles of Eerbeek biomass cultivated at different temperatures and with different feed mixtures

DGGE analysis of PCR-amplified fragments of bacterial and archaeal 16S rRNA gene fragments was used to compare the respective community fingerprints of the Eerbeek biomass enriched at 25, 37, and 45°C with different feed compositions A and B (Fig. 1). Duplicate DGGE profiles of all incubations were run independently in parallel, and no differences were detected in the duplicate experiments (data not shown).

Table 1: Experimental and calculated data for the mass balance of the anaerobic degradation in batch cultures enriched at different temperatures (10, 25, 37, 45, 55 and 65ºC) with feed mixture of acetate, propionate and butyrate, in the absence (A) and presence (B) of excess concentrations of sulphate

| Incubation temperature (°C) | Feed mixture | Substrate concentrations at the beginning and the end of incubations (mM) | | | | | | | | Product (mM) | % of carbon removed as COD | | Electron recovery (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Acetate | | Propionate | | Butyrate | | $SO_4$ | | | | | |
| | | Beg. | End | Beg. | End | Beg. | End | Beg. | End | $CH_4$ | MPA | SRB | |
| 10 | A | 9.9 | 9.4 | 5.1 | 4.9 | 4.9 | 4.9 | - | - | N.D. | - | - | - |
| | B | 9.9 | 9.1 | 5.1 | 4.4 | 4.8 | 4.1 | 29.6 | 26.0 | N.D. | - | - | - |
| 25 | A | 10.1 | N.D. | 4.9 | 4.2 | 4.9 | N.D. | - | - | 21.1 | 100 | - | 89.5 |
| | B | 10.2 | N.D. | 5.1 | N.D. | 5.0 | N.D. | 31.0 | 18.0 | 13.7 | 51.4 | 48.6 | 84.6 |
| 37 | A | 10.0 | N.D. | 4.9 | N.D. | 4.9 | N.D. | - | - | 30.1 | 100 | - | 97.2 |
| | B | 10.3 | N.D. | 5.1 | N.D. | 4.9 | N.D. | 29.7 | 15.42 | 14.6 | 50.6 | 49.4 | 91.6 |
| 45 | A | 10.0 | 22.4 | 5.1 | N.D. | 4.9 | N.D. | - | - | 7.9 | 100 | - | 91.4 |
| | B | 10.3 | 9.9 | 5.0 | N.D. | 4.9 | N.D. | 30.9 | 12.4 | 0.2 | 1.1 | 98.9 | 87.6 |
| 55 | A | 10.0 | 8.1 | 5.1 | 4.4 | 4.9 | 4.9 | - | - | 0.1 | - | - | - |
| | B | 10.1 | 9.0 | 4.9 | 3.9 | 4.9 | 4.8 | 29.0 | 27.4 | 2.3 | - | - | - |
| 65 | A | 9.9 | 9.3 | 4.9 | 4.8 | 5.0 | 5.1 | - | - | N.D. | - | - | - |
| | B | 9.7 | 8.8 | 5.0 | 4.7 | 5.0 | 4.8 | 29.4 | 28.2 | N.D. | - | - | - |

N.D.: Not Detected, MPA: Methane Producing Archaea, SRB: Sulphate Reducing Bacteria, Average of two independent experiments were presented. The duplicate experiments showed less than 5% of difference.

Overall, bacterial community profiles clustered separately for the two different feed mixtures A and B, indicating a pronounced effect of the addition of an excess amount of sulphate (Fig. 1, upper panel). DGGE profiles of feed mixture-A clustered together for the enrichments incubated at 25, 37 and 45°C with a similarity of more than 75%, which is comparable to a similarity of more than 70% for profiles generated for incubations with feed mixture-B. The 25 and 37°C incubations of feed mixtures A and B, respectively, were clustered to each other with more than 85% similarity for both feed mixtures. The inoculum formed an outgroup with a similarity of less than 50% with bacterial profiles of the other incubations. Incubations at 10, 55, and 65°C did not show noticeable degradation of the feed components, and were not taken into account for further analysis.

The archaeal profiles showed a high similarity with each other of in general more than 70% (Fig. 1, lower panel). There was a significant relatedness between community profiles of A and B incubations, depending more on temperature then on the feed mixture used. Only the 45°C incubation of feed mixture-B with sulphate showed some archaeal community differences compared to the other archaeal DGGE profiles. All archaeal DGGE patterns showed more than 50% similarity to the inoculum.

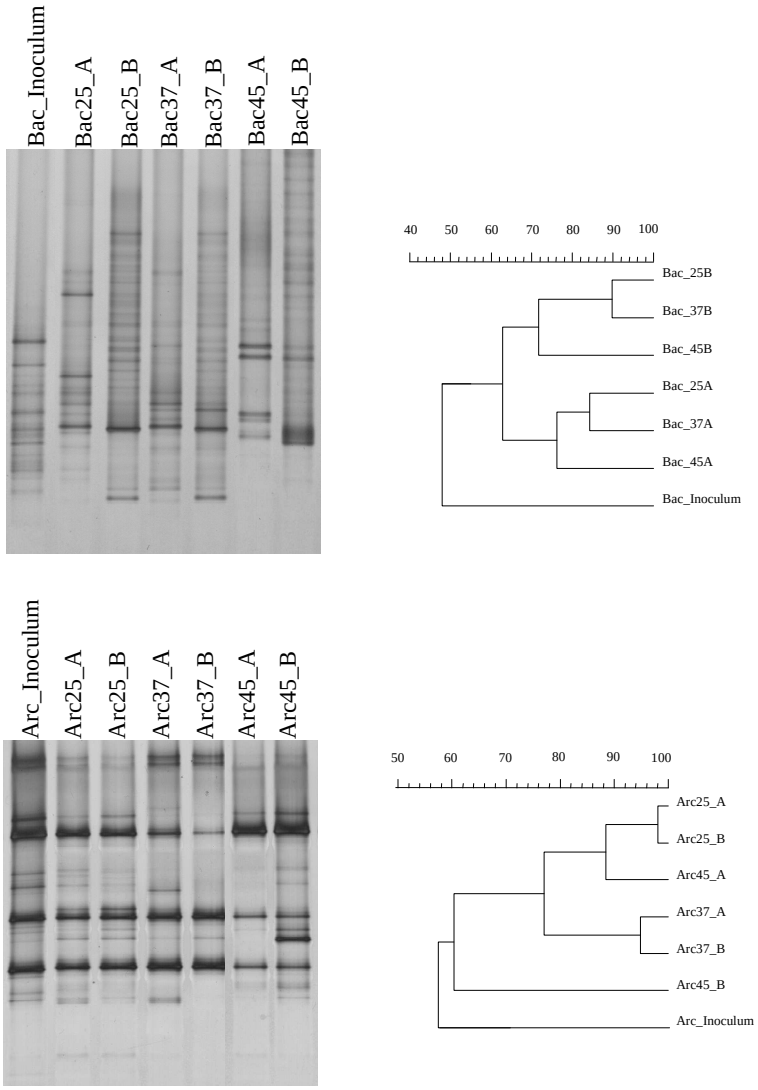


Figure 1: Bacterial (upper panel) and archaeal (lower panel) DGGE community fingerprints, and corresponding similarity dendrograms of the Eerbeek biomass enriched at 25, 37 and 45°C with the feed mixtures A and B. Primer couples used in PCR-DGGE were F968-GC, R1401 and F109(T), R515-GC for bacteria and archaea, respectively. Dendrograms of the DGGE gels illustrate the correlation between different profiles. The clustering of patterns was calculated using the unweighted-pair group method using average linkages (UPGMA). The first letters Bac and Arc refer to bacteria and archaea, respectively. Numbers indicate the temperatures. Second letters, A or B, correspond to the two different feed mixtures.

## Bacterial 16S rRNA gene clone libraries

*Feed mixture-A at 25°C.* The most pre-dominant (34%) sequence represented in this clone library was most closely related to clone IIIA-8 derived from polluted river sediment, actively dechlorinating polychlorinated dibenzo-*p*-dioxins/ dibenzofurans [Yoshida *et al.* 2005]. The closely affiliated cultured *Geospirillium* sp. (95% sequence similarity) [Heising *et al.* 1999] ferments fumarate to acetate, succinate, and carbon dioxide (Table 2). *Eubacterium aggregans* related clones made up 20% of the clone library. This homoacetogenic bacterium mediates the reaction of hydrogen with carbon dioxide to form acetate [Mechichi *et al.* 1998]. 18% of the clone library corresponded to butyrate oxidizers of the genus *Syntrophomonas*. Even though no sulphate was present, populations related to *Desulfovibrio alcoholovorans*, *Desulfovibrio aminophilus* and *Desulfobulbus elongatus* were detected with an abundance of 7% of the total clone library. Whereas *Desulfovibrio*-related populations probably functioned as proton reducers in syntrophic association with hydrogen and formate consuming methanogens, *Desulfobulbus elongatus* most likely contributed to the partial degradation of propionate in syntrophy with hydrogen utilizing

methanogens or SRBs. *Acetobacterium* related populations were detected with an abundance of 14% and can ferment $H_2/CO_2$, formate and several sugars to acetate. Hence, they might grow autotrophically on $H_2/CO_2$, formate and CO in the absence of carbohydrates. Although observed sequence similarities were only moderate (97 and 89%) preventing unamibiguous conclusions on ecophysiology, the most closely related *Acetobacterium* species are psychrophilic acetogens, and *Acetobacterium fimetarium* can grow up to a temperature of 35°C, suggesting a similar lifestyle for the detected populations [Kotsyurbenko *et al.* 1995].

*Feed mixture-B at 25°C.* The clone library mainly represented *Deltaproteobacteria*, occupying 78% of the total clones (Table 2). This phylum was composed of sulphidogenic bacteria, *Desulfovibrionaceae* (55%), *Desulfobulbaceae* (22%), and *Desulfobacteraceae* (2%). These abundant SRB were also related to the rate of carbon source used by sulphidogens which was 49% of feed mixture-B. Beside these SRB-related clones, clones related to *Clostridia* were detected at 7% of the total clone library. The following microorganisms are the closest cultured relatives of less dominant *Clostridia* species in the clone library. *Acetobacterium malicum* ferments $H_2/CO_2$, formate and several sugars to acetate [Kotsyurbenko *et al.* 1995]. Therefore, this organism could act as homoacetogenic bacterium in this incubation, converting $H_2/CO_2$ and/or formate to acetate. *Eubacterium infirmum* is known as a saccharolytic organism [Cheeseman *et al.* 1996]. Although homeacetogenic properties were not known for this species, based on other species of the *Eubacterium* genus found in the clone library of feed mixture-A, it could be that *Eubacterium infirmum* acts as a homoacetogen in this sulphate reducing consortia. Less abundant clones related to saccharolytic anaerobic species *Clostridium cylindrosporum* [Stackebrandt *et al.* 1999], haloalkaliphilic *Spirochaeta asiatica* [Zhilina *et al.* 1996], and *Treponema* sp. isolated from human periodontal samples, were also observed in this clone library. The low abundance (2%) of the *Syntrophomonas flectens* related clone strongly suggested that butyrate was mainly degraded by sulphidogens.

*Feed mixture-A at 37°C.* The consumption of feed mixture-A was monitored via the supernatants sampled during the course of the entire incubation. Metabolic data were compared with the microbial community shifts determined for the biomass samples harvested at the corresponding time points (Figure 2). The main community shift occurred between day 7 and 10 of incubation (Fig. 2, Table 2). At the $7^{th}$ day of incubation, butyrate, beside acetate, was degraded, while propionate remained almost stable.
However, on day 42, propionate was consumed. Therefore, the clones from the libraries of the $7^{th}$ and the $42^{nd}$ days were expected to be dominated by clones related to organisms degrading butyrate and propionate, respectively. Correspondingly, band X, dominantly present in the DGGE profile of the $7^{th}$ day, corresponded to the butyrate degrader *Syntrophomonas* sp. MGB-C1 (Figure 2). DGGE band Y of the $42^{nd}$ day corresponded to *Desulfobulbus propionicus*, known as propionate degrader, which was also coherent with the biochemical results.
Sequences most closely related to syntrophic short chain fatty acid oxidisers and sulphate reducers were found on day 7. In contrast to the DGGE patterns, where the prominent band corresponded to *Syntrophomonas* sp. and the amplicon related to *Desulfobulbus propionicus* appeared less intense, *Desulfobulbus propionicus* was found as the most abundant (44%) member of the clone library, whereas the *Syntrophomonas* sp. related clones slightly less abundant (33%) at the moment mainly butyrate degradation was observed in the culture. This discrepancy might be originating from selective amplification during PCR. Based on the known species properties, it could be attained that the *Syntrophomonas* sp. is responsible for the syntrophic butyrate degradation.

Table 2: Phylogenetic assignment of the bacterial clones derived from Eerbeek biomass enriched at 25, 37 and 45°C with feed mixtures A and B.

| Clone | Length (bp) | Closest phylotype | % Seq. sim. | Closest cultured species | % Seq. sim. | Taxonomic position (class, family, or genus) | # Clones | % Freq. |
|---|---|---|---|---|---|---|---|---|
| **25°C-Feed Mixture-A** | | | | | | | | |
| 25C_059 | 437 | clone IIIA-8 | 99 | *Geospirillium* sp. KoFum | 95 | Epsilonproteobacteria Campylobacteraceae | 15 | 34.1 |
| 25C_067 | 359 | clone E21 | 99 | *Desulfovibrio alcoholovorans* | 99 | Deltaproteobacteria; Desulfovibrionaceae | 1 | 2.3 |
| 25C_019 | 437 | clone R1p15 | 94 | *Desulfobulbus elongatus* | 94 | Deltaproteobacteria; Desulfobulbaceae | 1 | 2.3 |
| 25C_018 | 437 | eubacterium OCG4' | 96 | *Desulfovibrio aminophilus* | 96 | Deltaproteobacteria; Desulfovibrionaceae | 1 | 2.3 |
| 25C_026 | 433 | *Trichlorobacter thiogenes* | 98 | | | Deltaproteobacteria; Geobacteraceae | 1 | 2.3 |
| 25C_051 | 434 | clone E12 | 99 | *Syntrophomonas* sp. MGB-C1 | 96 | Firmicutes; Syntrophomonadaceae | 8 | 18.2 |
| 25C_074 | 434 | *Eubacterium aggregans* | 99 | | | Firmicutes; Clostridia | 9 | 20.4 |
| 25C_083 | 379 | *Acetobacterium fimetarium* | 97 | | | Firmicutes; Clostridia | 2 | 4.6 |
| 25C_058 | 440 | *Acetobacterium wieringae* | 89 | | | Firmicutes; Clostridia | 4 | 9.1 |
| 25C_050 | 431 | bacterium HB31 | 96 | *Papillibacter cinnaminovorans* | 95 | Firmicutes; Clostridia; Papillibacter | 1 | 2.3 |
| 25C_066 | 432 | clone LJ8 | 98 | *Clostridium aminobutyricum* | 90 | Firmicutes; Clostridia | 1 | 2.3 |
| **25°C-Feed Mixture-B** | | | | | | | | |
| 25D-012 | 440 | *Desulfovibrio putealis* | 93 | | | Deltaproteobacteria; Desulfovibrionaceae | 24 | 52.2 |
| 25D-006 | 307 | *Desulfomicrobium apsheronum* | 93 | | | Deltaproteobacteria; Desulfovibrionales | 1 | 2.2 |
| 25D-028 | 441 | bacterium LTCE-T2A 4A | 99 | *Desulfobulbus propionicus* | 98 | Deltaproteobacteria; Desulfobulbaceae | 8 | 17.4 |
| 25D-085 | 441 | clone TANB52a | 93 | *Desulfobulbus propionicus* | 86 | Deltaproteobacteria; Desulfobulbaceae | 1 | 2.2 |
| 25D-044 | 438 | clone R1p15 | 91 | *Desulfobulbus elongatus* | 91 | Deltaproteobacteria; Desulfobulbaceae; | 1 | 2.2 |
| 25D-004 | 439 | clone TANB52a | 96 | *Desulfococcus multivorans* | 86 | Deltaproteobacteria; Desulfobacteraceae | 1 | 2.2 |
| 25D-021 | 429 | clone PL-37B6 | 87 | *Eubacterium infirmum* | 84 | Firmicutes; Clostridia | 1 | 2.2 |
| 25D-068 | 433 | *Acetobacterium malicum* | 91 | | | Firmicutes; Clostridia | 1 | 2.2 |
| 25D-036 | 436 | clone HKT160 | 98 | *Clostridium cylindrosporum* | 91 | Firmicutes; Clostridia | 1 | 2.2 |
| 25D-045 | 434 | eubacterium RFLP11 | 95 | *Syntrophomonas flectens* | 94 | Firmicutes; Syntrophomonadaceae | 1 | 2.2 |
| 25D-014 | 436 | bacterium SHA-7 | 96 | *Bacteroides* sp. SA-11 | 90 | Bacteroidetes; Bacteroidaceae | 1 | 2.2 |
| 25D-077 | 440 | clone TANB52a | 98 | *Spirochaeta asiatica* | 87 | Spirochaetes; Spirochaetaceae | 3 | 6.5 |
| 25D-084 | 436 | clone SSE9 | 99 | *Treponema* sp. 10:A:C25 | 92 | Spirochaetes; Spirochaetaceae | 1 | 2.2 |
| 25D-069 | 440 | clone IIIA-8 | 93 | *Sulfurospirillum* sp. Am-N | 91 | Epsilonproteobacteria; Campylobacteraceae | 1 | 2.2 |
| **37°C-Feed Mixture-A (Day 7)** | | | | | | | | |
| 37C1_091 | 442 | bacterium LTCE-T2A 4A | 99 | *Desulfobulbus propionicus* | 99 | Deltaproteobacteria; Desulfobulbaceae | 21 | 43.8 |
| 37C1_027 | 435 | Proteobact. isolate WB-25 | 95 | *Desulforhabdus amnigena* | 94 | Deltaproteobacteria; Syntrophobacteraceae | 1 | 2.1 |
| 37C1_059 | 433 | Uncultured *Geobacter* sp. | 89 | *Geobacter metallireducens* | 88 | Deltaproteobacteria; Desulfuromonadales | 2 | 4.2 |
| 37C1_004 | 439 | *Desulfovibrio* sp. PA35E4 | 99 | *Desulfovibrio alcoholovorans* | 99 | Deltaproteobacteria; Desulfovibrionaceae | 2 | 4.2 |
| 37C1_036 | 436 | clone R1p15 | 92 | *Desulfobulbus elongatus* | 92 | Deltaproteobacteria; Desulfobulbaceae | 1 | 2.1 |
| 37C1_034 | 439 | clone E27 | 98 | *Syntrophobacter wolinii* | 92 | Deltaproteobacteria; Syntrophobacteraceae | 2 | 4.2 |
| 37C1_066 | 434 | *Syntrophomonas* sp. MGB-C1 | 99 | | | Firmicutes; Syntrophomonadaceae | 16 | 33.3 |
| 37C1_028 | 431 | clone W4A-A56 | 96 | *Slackia heliotrinreducens* | 90 | Actinobacteria; Coriobacterineae | 3 | 6.3 |
| **37°C-Feed Mixture-A (Day 42)** | | | | | | | | |
| 37C2_037 | 441 | bacterium LTCE-T2A 4A | 99 | *Desulfobulbus propionicus* | 99 | Deltaproteobacteria; Desulfobulbaceae | 21 | 44.7 |
| 37C2_085 | 438 | *Syntrophobacter sulfatereducens* | 94 | | | Deltaproteobacteria; Syntrophobacteraceae | 1 | 2.1 |
| 37C2_005 | 439 | clone E27 | 98 | *Syntrophobacter wolinii* | 92 | Deltaproteobacteria; Syntrophobacteraceae | 2 | 4.3 |
| 37C2_068 | 436 | *Syntrophobacter fumaroxidans* | 98 | | | Deltaproteobacteria; Syntrophobacteraceae | 4 | 8.5 |
| 37C2_084 | 431 | clone W4A-A56 | 97 | *Slackia heliotrinreducens* | 91 | Actinobacteria; Coriobacterineae | 14 | 29.8 |
| 37C2_045 | 434 | *Syntrophomonas* sp. MGB-C1 | 95 | | | Firmicutes; Syntrophomonadaceae | 3 | 6.4 |
| 37C2_077 | 437 | clone SSE9 | 99 | *Treponema* sp. 10:A:C25 | 92 | Spirochaetes; Spirochaetaceae | 2 | 4.3 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **37°C-Feed Mixture-B** | | | | | | | | |
| 37D_007 | 432 | isolate WB-25 | 98 | *Desulforhabdus amnigena* | 98 | Deltaproteobacteria; Syntrophobacteraceae | 19 | 43.2 |
| 37D_006 | 332 | *Desulforhabdus amnigena* | 99 | | | Deltaproteobacteria; Syntrophobacteraceae | 14 | 31.8 |
| 37D_054 | 441 | bacterium LTCE-T2A 4A | 99 | *Desulfobulbus propionicus* | 98 | Deltaproteobacteria; Desulfobulbaceae; | 1 | 2.3 |
| 37D_015 | 448 | clone:ALU43 | 88 | *Desulfuromonas palmitatis* | 84 | Deltaproteobacteria; Desulfuromonadaceae | 1 | 2.3 |
| 37D_038 | 430 | Eubact. oral P2PC_29 P2 | 93 | *Eubacterium brachy* | 92 | Firmicutes; Clostridia | 1 | 2.3 |
| 37D_078 | 446 | bacterium TA19 | 98 | *Aminobacterium colombiense* | 89 | Firmicutes; Clostridia | 1 | 2.3 |
| 37D_070 | 436 | clone E3 | 99 | *Treponema* sp. 10:A:C25 | 92 | Spirochaetes; Spirochaetaceae | 1 | 2.3 |
| 37D_046 | 439 | bacterium MTCE-T2 1G | 98 | *Clostridium* sp. (BN II) | 94 | Firmicutes; Clostridia; Clostridiaceae | 4 | 9.1 |
| 37D_094 | 435 | anaerobic bact. clone B-1C | 91 | *Petrimonas sulfuriphila* | 91 | Bacteroidetes; Bacteroidales | 1 | 2.3 |
| 37D_014 | 435 | clone EUB53-2 | 98 | *Bacteroidales* str. WB4 | 91 | Bacteroidetes; Bacteroidales | 1 | 2.3 |
| **45°C-Feed Mixture-A** | | | | | | | | |
| 45C_042 | 435 | clone 008D10_B_SD_P15 | 94 | *Syntrophomonas flectens* | 93 | Firmicutes; Syntrophomonadaceae | 10 | 20.8 |
| 45C_090 | 418 | *Syntrophomonas flectens* | 95 | | | Firmicutes; Syntrophomonadaceae | 34 | 70.8 |
| 45C_074 | 435 | *Desulfacinum subterraneum* | 90 | | | Deltaproteobacteria; Syntrophobacteraceae | 1 | 2.1 |
| 45C_019 | 438 | isolate WB-25 | 98 | *Desulforhabdus amnigena* | 97 | Deltaproteobacteria; Syntrophobacteraceae | 2 | 4.4 |
| 45C_058 | 437 | *Syntrophobacter sulfatereducens* | 99 | | | Deltaproteobacteria; Syntrophobacteraceae | 1 | 2.1 |
| **45°C-Feed Mixture-B** | | | | | | | | |
| 45D_043 | 411 | *Desulfotomaculum thermobenzoicum* | 97 | | | Firmicutes; Clostridia; Peptococcaceae | 43 | 89.6 |
| 45D_091 | 434 | clone: SmB60fl | 97 | Clostridium sp. ANP2 | 90 | Firmicutes; Clostridia; Clostridium | 3 | 6.3 |
| 45D_020 | 429 | clone Lgja-11 | 95 | *Eggerthella sinensis* | 92 | Actinobacteria; Coriobacteriaceae | 1 | 2.1 |
| 45D_059 | 438 | clone M79 | 96 | *Thermosipho ferriphilus* | 84 | Thermotogae; Thermotogaceae | 1 | 2.1 |

Although *Desulfobulbus propionicus* (44%), *Desulforhabdus amnigena* (2%), *Desulfobulbus elongatus* (2%), and *Syntrophobacter wolinii* (4%) degrade propionate under sulphidogenic conditions, only *Syntrophobacter wolinii* can grow in co-culture with hydrogen utilizing methanogens in the absence of sulphate [Boone and Bryant 1980].

The clone library of the sample taken on day 42 was dominated by sequences closely related to the propionate and sulphate reducing bacterium, *Desulfobulbus propionicus* (45%). Although the similarity of the related clone to these species is as high as 99%, the presence of this culture in the absence of sulphate is not clear. Its involvement in syntrophic interaction was not reported so far. Predicted syntrophic propionate degraders were affiliated with *Syntrophobacter sulfatereducens* (2%), *Syntrophobacter wolinii* (4%) and *Syntrophobacter fumaroxidans* (9%). The second most abundant clone (30%) was remotely related (91% sequence similarity) to *Slackia heliotrinreducens*. Although sequence similarity is too low to predict the physiology of the population detected in this incubation, the fact that *Slackia heliotrinreducens* has been described to reduce fumarate and does not grow on carbohydrates [Lanigan 1976] might indicate that this abundant population could be attained to the functioning of the propionate degradation pathway. Clone SSE9, affiliated with the *Spirochaetes*, was also detected in an anaerobic bioreactor and from a trial to determine the effect of feed composition on the performance and microbial community dynamics.

*Feed mixture-B at 37°C. Desulforhabdus amnigena* and *Desulfobulbus propionicus* covered 77% of the total clone library (Table 2). Due to their functional properties of propionate degradation and also the presence of sulphate in this incubation, it could be attained that these species were degrading propionate and concomitantly reducing sulphate. Other populations were detected at much lower abundance and could be linked to mainly carbohydrate fermenting organisms.

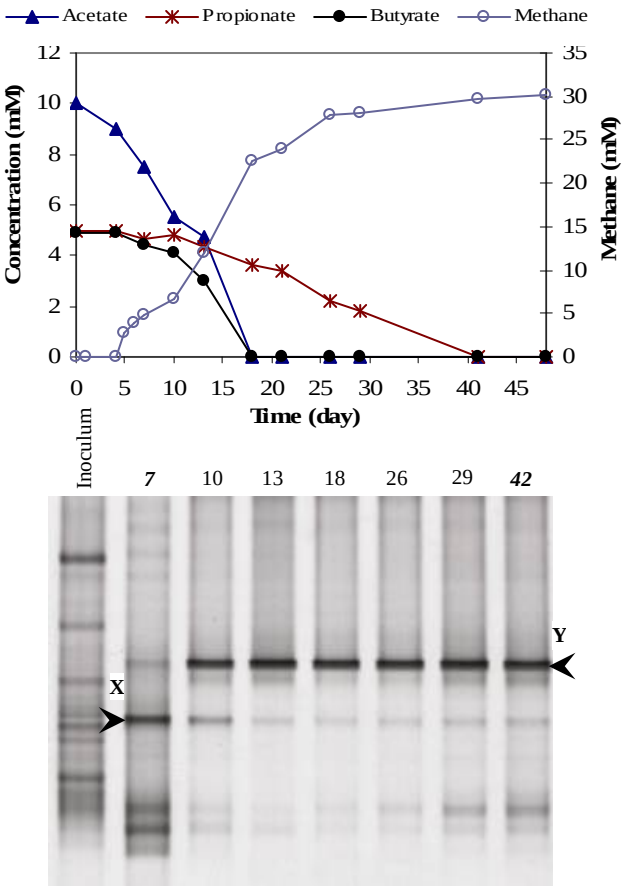


Figure 2: The biochemical performance (upper panel) in relation to the bacterial population dynamics (lower panel): Eerbeek biomass enriched at 37°C with Feed Mixture-A. Bands X and Y show the amplicons related to *Syntrophomonas* sp. and *Desulfobulbus* sp., respectively. Inoculum: Original Eerbeek biomass as Marker. Numbers indicate sampling days.

*Feed mixture-A at 45°C.* Sequences that were most closely affiliated with the syntrophic butyrate degrading *Syntrophomonas flectens*, albeit at similarities of only 93-95%, dominated this clone library with an abundance of 91% (Table 2). It is noteworthy that both butyrate and propionate were consumed in this enrichment, suggesting that the corresponding populations might be involved in the degradation of obht fatty acids. Clones most closely related to *Desulfacinum subterraneum*, a thermophilic sulphate-reducing bacterium isolated from a high temperature oil field, *Desulforhabdus amnigena* and *Syntrophobacter sulfatereducens* covered only 9% of this syntrophic enrichment. While *Syntrophobacter sulfatereducens* can grow syntrophically in the presence of hydrogenotrophic methanogens [Chen *et al.* 2005], *Desulforhabdus amnigena* can grow only in the presence of sulphate [Oude Elferink *et al.* 1995]. *Syntrophomonas flectens* has been reported to be able to grow up to a temperature of 42°C [Zhang *et al.* 2004], which was very close to the enrichment temperature of 45°C. The other mesophilic species *Desulforhabdus amnigena and Syntrophobacter sulfatereducens* can grow to a temperature of up to 45°C [Oude Elferink *et al.* 1995, Chen *et al.* 2005].

*Feed mixture-B at 45°C. Desulfotomaculum thermobenzoicum* affiliated clones were an important group under sulphidogenic conditions at 45°C, comprising 90% of the total community (Table 2). *Desulfotomaculum thermobenzoicum* is a thermophilic, syntrophic, propionate-oxidizing bacterium [Plugge *et al.* 2002]. Propionate and butyrate were completely degraded by sulphidogens, and methane production was not observed. *Desulfotomaculum thermobenzoicum* could degrade propionate completely to $CO_2$. However, at lower temperatures of the same feed mixture, incomplete oxidation was observed.

Comparative evalutation of the bacterial clone libraries.
Members of the anaerobic consortia incubated at different temperatures ranging from 25 to 45°C and with short chain fatty acids based feed mixtures were classified into 10 bacterial divisions, indicating considerable microbial diversity as observed for other wastewater treatment plants [LaPara *et al.* 2000, Wagner & Loy 2002, Roest *et al.* 2005a]. However, a total of 324 bacterial clones retrieved from all incubations belonged mainly to only two different phyla, namely *Firmicutes* (42% of the clones) and *Deltaproteobacteria* (40%) (Figure 3). While *Firmicutes* appeared in all incubations, *Deltaproteobacteria* were not detected in incubations at 10, 55 and 65°C (results not shown), for which growth was not observed on VFA's.

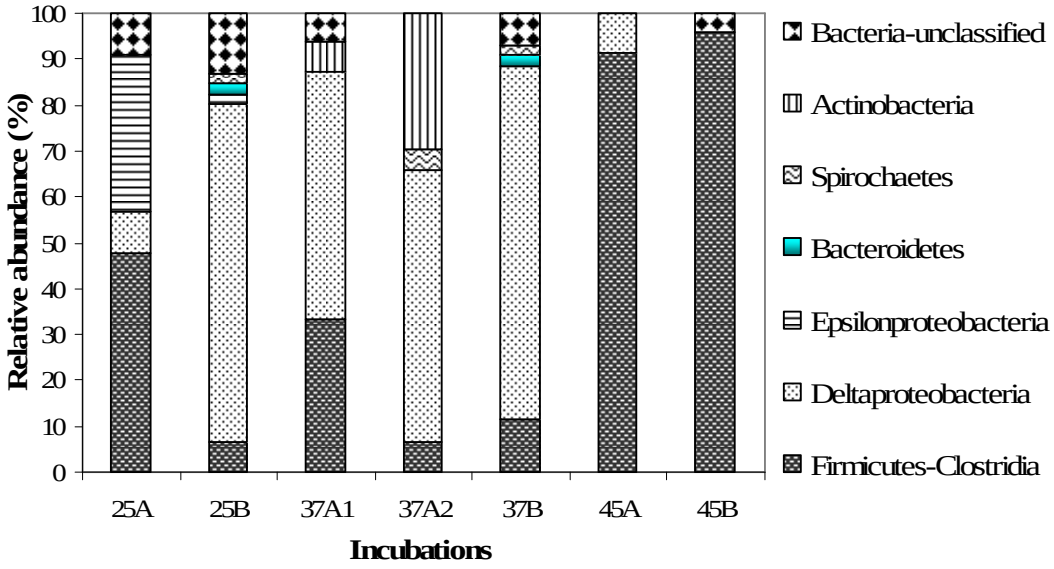


Figure 3: 16S rRNA based diversity of the incubations evaluated on the phylum level. Each incubation represented approximately 48 clones.

The archaeal clone library
In the batch culture incubated with feed mixture-A at 25°C, the archaeal community was composed of clones representing the *Methanosarcinaceae* and *Methanobacteriaceae* lineages of methanogenic *Euryarchaeota* (Table 3). *Methanobacterium beijingense*, and *Methanobacterium formicicum* relatives were the dominant populations, which utilise hydrogen and formate. They made up 77.3% of the total clone library. The remaining 23% of the clone library was represented by an acetate utilizing methanogenic relative, *Methanosaeta concilii*.

Table 3: Phylogenetic assignment of archaeal clones isolated from Eerbeek biomass enriched at 25°C with Feed Mixture-A.

| Clone | Length (bp) | Closest phylotype | % Seq. sim. | Closest cultured species | % Seq. sim. | Taxonomic position (class, family, or genus) | # Clones | % Freq |
|---|---|---|---|---|---|---|---|---|
| **25°C-Feed Mixture A** | | | | | | | | |
| ArcM_009 | 383 | archaeon clone 5LOC9 | 99 | *Methanosaeta concilii* | 98 | Euryarchaeota; Methanosaetaceae | 10 | 22.7 |
| ArcM_072 | 385 | archaeon clone CM17 | 98 | *Methanobacterium beijingense* | 98 | Euryarchaeota; Methanobacteriaceae | 26 | 59.1 |
| ArcM_017 | 384 | archaeon clone 1G1 | 99 | *Methanobacterium formicicum* | 95 | Euryarchaeota; Methanobacteriaceae | 8 | 18.2 |

The archaeal community was rather similar in all incubations, although some changes were detected in the thermophilic incubations (Fig. 1, lower panel). In these thermophilic incubations nearly 50% of the screened archaeal clones were related to *Bacteria*, eventhough archaeal-specific primers were used. This has been observed before and could be due to low numbers of *Archaea* compared to

*Bacteria* [Roest *et al.* 2005b]. The main methanogen detected in the thermophilic incubations was closely related to *Methanothermobacter thermautotrophicus* strain GC-1 (98%).

Integrated discussion of results

Assuming that each band in a given DGGE profile represents of a specific population, the number of distinctive bands should be proportional to the observed diversity in the clone libraries. Although these correlations between clone libraries and corresponding DGGE profiles were observed for some of the incubations, most of them were not correlated well with each other. This might be due to PCR amplification biases [Reysenbach *et al.* 1992, von Wintzingerode *et al.* 1997, Polz & Cavanaugh 1998] as well as the 16S rRNA gene copy numbers [Wilkinson & Young 1995, Klappenbach *et al.* 2001] of species, which can result in detection of multiple bands corresponding to one species in the DGGE profiles whereas the different amplicons might still yield identical RFLP profiles.

Temperature dependent changes of microbial communities were observed between incubations at 37 and 45°C for starch-based feed mixtures incubated at the same conditions as in this study [Altinbas 2007]. Thermophilic saccharolytic species were observed starting from an incubation temperature of 45°C and through higher incubation temperatures. For feed mixture-A, this situation was not the same; e.g. the mesophilic syntrophic species also survived at 45°C. However at higher temperatures no growth was observed, suggesting the sensitivity of the syntrophic players to temperatures of more than 45°C. For feed mixture-B, sulphidogens were present in all different incubations (25-45°C), indicating that the presence of unlimited concentrations of sulphate affected the microbial comunity structure. While butyrate and propionate were degraded incompletely in the 25 and 37°C incubations, complete oxidation was observed for both substrates at 45°C. Clones from this study matched with sequences derived from a variety of environments such as anaerobic bioreactors, soils, faecal samples, rumen and periodontal samples. Altogether a diverse community has been detected in the different incubations.

Monitoring and evaluation of the bacterial population shifts using DGGE patterns demonstrated that the microbial community shifts were not completely in agreement with the biochemical results (data not shown), except for the incubation at 37°C with feed mixture-A. Whereas the degradation pattern changed, the corresponding DGGE profiles remained relatively unchanged for most of the incubations. However, it can not be ruled out that the stable community profiles might be caused by the even contribution of each member of the community to the degradation of each compound present in the incubations. On the other hand, the nucleic acids subjected to community profiling might not be sensitive enough to reflect the population changes during the incubation. It is known that nucleic acids that correspond to certain specific microorganisms can stay longer in the enrichments; even if the microorganisms lost their metabolic activity [Wagner *et al.* 1995]. The DNA of small subunit rRNA-encoding genes reflects all existing organisms in the community and does not enable the distinction between active and resting cells. Therefore, it might not have been possible to detect shifts of the bacterial community with different types of C-sources along the incubation periods. However, a community shift was clearly observed for the enrichment incubated at 37°C with feed mixture-A. While butyrate consumption was observed at the beginning of the incubation, propionate concentrations stayed stable. After consumption of butyrate, propionate was degraded. This degradation profile was in agreement with the shift in community structure, suggesting that the populations related to *Syntrophomonas* sp. and *Desulfobulbus propionicus* were involved in the syntrophic degradation of butyrate and propionate pathways respectively. Although *Syntrophomonas* sp. is known as a butyrate degrading organism, *Desulfobulbus propionicus* can degrade propionate only in the presence of sulphate. Therefore, it could be suggested that this species might play an important role in the pathway of propionate degradation; also in the full scale bioreactor.

It was reported that anaerobic inocula can be used for a wide range of incubation temperatures [Leven *et al.* 2007, Wu and Conrad 2001, Sekiguchi *et al.* 2001]. However, this study demonstrated that it is not feasible to use mesophilic anaerobic sludge if temperature shifts to either psychrophilic or thermophilic conditions are applied with a mixture of acetate, propionate, and butyrate and in the presence of sulphate.

Metabolic interactions between different trophic groups should be elucidated in order to optimize the anaerobic treatment. Understanding of the microbial aspects and expanding our knowledge of the extent to which microbial community composition is related to physiological properties were possible by monitoring substrate utilisation and metabolite formation compared with the microbial community shifts using DGGE fingerprinting, especially for the communities predominantly degrading the substrates sequentially. This study revealed also the effect of different temperatures on anaerobic biomass for different feed mixtures, which provided knowledge about members of the communities as well as the physiological behaviour of them under these various conditions.

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# Chapter 3

## Enrichment and detection of micro-organisms involved in direct and indirect methanogenesis from methanol in an anaerobic thermophilic bioreactor

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**Abstract**

To get insight into the micro-organisms involved in direct and indirect methane formation from methanol in a lab-scale thermophilic (55°C) methanogenic bioreactor, reactor sludge was disrupted and serial dilutions were incubated in specific growth media containing methanol and possible intermediates of methanol degradation as substrates. With methanol growth was found till a dilution of $10^8$. However, when *Methanothermobacter thermoautotrophicus* strain Z245 was added for $H_2$ removal, growth was observed up to a $10^{10}$-fold dilution. With $H_2/CO_2$ and acetate, growth was observed till dilutions of $10^9$ and $10^4$, respectively. Dominant micro-organisms in the different dilutions were identified by 16S rRNA-gene diversity and sequence analysis. Furthermore, dilution PCR revealed a similar relative abundance of Archaea and Bacteria in all investigated samples, except the enrichment with acetate, which contained 100 times less archaeal DNA than bacterial DNA. The most abundant bacteria in the culture with methanol and strain Z245 were closest related to *Moorella glycerini*. *Thermodesulfovibrio* relatives were found with high sequence similarity in the $H_2/CO_2$ enrichment, but also in the original lab-scale bioreactor sludge. In the $H_2/CO_2$ enrichment *Methanothermobacter thermoautotrophicus* strains were the most abundant hydrogenotrophic archaea. The dominant methanol-utilising methanogen, which was present in the $10^8$-dilution, was most closely related to *Methanomethylovorans hollandica*. Compared to direct methanogenesis, results of this study indicate that syntrophic, interspecies hydrogen transfer-dependent methanol conversion is equally important in the thermophilic bioreactor, confirming previous findings with labelled substrates and specific inhibitors.

**Introduction**

Methanol is a main pollutant in some specific wastewaters, like the evaporator condensate of pulp and paper industries, coal-gasification plants, potato-starch producing factories, and landfill leachates. Such wastewaters can be treated anaerobically [Bérubé & Hall 2000, Minami *et al.* 1991, Paulo *et al.* 2001, Yamaguchi *et al.* 2001]. Under mesophilic (30-40°C) conditions stable high-rate methanol conversion to methane is well possible in properly designed and operated bioreactors [Weijma & Stams 2001]. Thermophilic treatment, however, is an attractive alternative, particularly when the wastewater is discharged at high temperatures [Van Lier 1996], e.g. in the evaporator condensate of pulp and paper industries [Bérubé & Hall 2000, Minami *et al.* 1991]. Whereas the anaerobic treatment of methanol-containing wastewaters under mesophilic conditions has been investigated by several researchers [Florencio *et al.* 1994, Fukuzaki & Nishio 1997, Lettinga *et al.* 1979], treatment under thermophilic conditions received less attention.

Under anaerobic conditions methanol can be degraded in different ways [Weijma & Stams 2001]. Methylotrophic methanogens (e.g. *Methanosarcina* species) are able to use methanol directly to produce methane and $CO_2$. In the presence of hydrogen, these organisms even degrade methanol solely to methane. Indirect methanogenesis is possible as well. Homoacetogens can ferment methanol ($+CO_2$) to acetate, which can be cleaved to methane and $CO_2$ by acetoclastic methanogens. In addition, homoacetogens and methylotrophic methanogens can also convert methanol to $H_2$ and $CO_2$, but only when the hydrogen partial pressure is kept low by hydrogenotrophic methanogens or sulphate reducers [Balk *et al.* 2002, Weijma & Stams 2001].

Understanding of anaerobic methanol conversion in high-rate reactors at any environmental condition requires the elucidation of the degradation routes of methanol by methanogens, sulphate reducers and homoacetogens [Weijma & Stams 2001]. In mesophilic methanogenic bioreactors, methanol was mainly consumed by *Methanosarcina* species [Florencio *et al.* 1994, Gonzalez-Gil *et al.*, Weijma & Stams 2001]. However, recent research with a thermophilic methanogenic bioreactor suggested that a major part of the methanol was degraded by syntrophic consortia [Paulo *et al.* 2002, Paulo *et al.* 2003, Weijma & Stams 2001]. By using $^{13}C$-labelled substrates and specific inhibitors it was shown that in an anaerobic methanol-fed thermophilic bioreactor, about 50% of the

methanol was directly converted to methane by methylotrophic methanogens, and about 50% via the intermediates $H_2/CO_2$ and acetate [Paulo *et al.* 2001, Paulo *et al.* 2003]. The aim of the study presented here was to get insight into the microbial consortia involved in direct and indirect methanogenesis from methanol in that anaerobic thermophilic bioreactor. Sludge of this bioreactor was used for enrichment of micro-organisms with methanol and possible intermediates of methanol metabolism, and the dominant micro-organisms were identified using 16S rRNA-based molecular approaches.

## Methods

### Enrichment cultures

Starting sludge for dilution series was taken from a continuous methanol-fed thermophilic (55°C) anaerobic lab-scale bioreactor [Paulo *et al.* 2001]. This sludge was crushed under anaerobic conditions and ten-fold dilution series were prepared in basal mineral bicarbonate-buffered medium with 0.1 g/l yeast extract [Stams *et al.* 1993]. The different dilutions were inoculated in media containing methanol (20 mM), acetate (20 mM) or 170 kPa $H_2/CO_2$ (80:20) as substrates. One of the dilution series with methanol contained *Methanothermobacter thermoautotrophicus* strain Z245 (DSM 3720) for removal of $H_2$ to promote syntrophic growth. The methanogen was pre-grown with $H_2/CO_2$, and after growth the gas phase was changed to $N_2/CO_2$ and methanol and different dilutions of the sludge were added. All incubations were done in 120-ml vials containing 50 ml medium. The vials were incubated under non-shaking conditions at 55°C in the dark.

### Molecular characterisation

DNA was isolated from cells harvested by centrifugation via mechanical disruption by bead beating, and phenol/chloroform/iso-amyl-alcohol extraction, and ethanol precipitation [Oude Elferink *et al.* 1997]. Amplification of 16S rRNA genes, Denaturing Gradient Gel Electrophoresis (DGGE), cloning, screening of clones with Amplified Ribosomal DNA Restriction Analysis (ARDRA) and sequencing of clones with a unique ARDRA-profile was done as described previously [Roest *et al.* 2005]. Sequenced clones were subsequently subjected to DGGE analysis, and the resulting bands were compared with the fingerprints from the highest enrichment dilutions with growth.

The ratio of Archaea and Bacteria was estimated by dilution PCR. To determine PCR detection thresholds for different groups of micro-organisms, PCR reactions with the Archaea specific primer A109-f [Groβkopf *et al.* 1998], Bacteria specific primer 7-f and universal primer 1492-r [Lane 1991] were first performed with a ten-times-dilution series of known DNA concentrations from *E. coli* JM109 (Invitrogen, Breda, The Netherlands), *Lactobacillus rhamnosus* (ATCC 53103), *Methanospirillum hungatei* strain JF1 (DSM 864) and *Methanobacterium formicicum* (DSM 1535). Second, ten-times-dilution series of DNA extracted from sludge and enrichment cultures were amplified with the same primers under identical conditions [Roest *et al.* 2005].

### Phylogenetic analysis

Similarity searches of 16S rRNA sequences derived from clones against sequences deposited in public databases were performed using the NCBI Blast search tool at http://www.ncbi.nlm.nih.gov/BLAST/, January 2004.

Alignment with FastAligner, followed by manual correction according to secondary structures, and phylogenetic analyses were performed using the ARB program package [Ludwig *et al.* 2004]. A phylogenetic tree was constructed with Felsenstein correction in ARB, using the neighbour joining method (*E. coli* positions 539 to 1449) [Saitou & Nei 1987]. Nucleotide sequences obtained in this study have been deposited in GenBank (accession no. AY526498 to AY526520).

## Results

Enrichment cultures

Microbial populations were enriched from sludge samples with methanol in the presence or absence of *Methanothermobacter thermoautotrophicus* strain Z245, acetate or $H_2/CO_2$. Growth and substrate conversion was obtained to the following dilutions: $10^8$ with methanol alone, $10^{10}$ with methanol in the presence of *Methanothermobacter thermoautotrophicus* strain Z245, $10^4$ with acetate, and $10^9$ with $H_2/CO_2$.

Table 1: Cloning and sequencing results of bacterial amplicons (> 1450 bp) obtained from thermophilic enrichment cultures. Numbers of clones with identical ARDRA profiles out of the total number of picked and screened clones are given to indicate relative abundance. The letters of the Band ID correspond to DGGE bands in Figure 1. Subscript numbers indicate different sequences in Figure 2 that showed the same mobility in DGGE. If the first closest database hit was an uncultured micro-organism, phylum assignment as well as the first hit with a cultured micro-organism is given.

| ARDRA | Band ID | Accession number | Closest database hit | Similarity |
|---|---|---|---|---|
| | | | **MeOH / (Z245) $10^{10}$** | |
| 17 of 35 | A | AY526500 | *Moorella glycerini* | 98.0% |
| 7 of 35 | $B_1$ | AY526501 | Uncultured low G+C Gram+ clone OPB54 (*Firmicutes*) | 89.7% |
| | | | *Clostridium fervidus* (ATCC 43204) | 90.0% |
| 6 of 35 | $B_2$ | AY526502 | Thermophilic eubacterium ST12 (*Firmicutes*) | 94.9% |
| | | | *Thermanaeromonas toyohensis* | 90.4% |
| | | | **Acetate $10^4$** | |
| 12 of 36 | C | AY526503 | *Flexistipes* sp. vp180 (*Deferribacteres*) | 95.3% |
| | | | Deferribacter desulfuricans | 88.1% |
| 7 of 36 | D | AY526504 | Petrobacter succinatimandens *BON4* | 99.7% |
| 4 of 36 | E | AY526505 | Uncultured eubacterium SJA-102 (*Spirochaetes*) | 95.0% |
| | | | Spirochaeta caldaria | 89.4% |
| | | | **$CO_2$ / $H_2$ $10^9$** | |
| 25 of 37 | $G_1$ | AY526507 | *Thermodesulfovibrio* sp. TGL-LS1 (*Nitrospirae*) | 98.8% |
| | | | Thermodesulfovibrio islandicus | 99.1% |
| 4 of 37 | F | AY526506 | Uncultured bacterium clone TA55_Ba_141 (*Thermotogae*) | 95.5% |
| | | | *Thermotoga lettingae* | 94.8% |
| 4 of 37 | $G_2$ | AY526508 | *Thermodesulfovibrio sp.* TGL-LS1 (*Nitrospirae*) | 99.1% |
| | | | Thermodesulfovibrio islandicus | 99.4% |
| | | | **MeOH $10^8$** | |
| 9 of 15 | H | AY526509 | Uncultured bacterium clone TA55_Ba_74 (*Bacteroidetes*) | 96.3% |
| | | | Brumimicrobium glaciale | 90.0% |
| 4 of 15 | I | AY526510 | Uncultured bacterium clone TA55_Ba_74 (*Bacteroidetes*) | 96.2% |
| | | | Brumimicrobium glaciale | 90.0% |

Relative abundance of Archaea and Bacteria

To gain insight into the relative contribution of Archaea and Bacteria to the microbial communities, a dilution PCR approach was chosen. Initially, PCR with Archaea- and Bacteria-specific primers on dilutions of genomic DNA isolated from two archaeal and two bacterial strains was used to assess the detection threshold of the respective reactions. As the same detection limit was observed for the archaeal and bacterial specific PCR reactions, the relative ratio of Archaea and Bacteria can be revealed from dilution PCR with the DNA samples of the enrichments. This approach indicated that in the highest dilutions with growth on methanol, with and without *Methanothermobacter thermoautotrophicus* strain Z245, and $H_2/CO_2$, Archaea and Bacteria were present at a comparable order of magnitude. The enrichment with acetate, however, contained a 100-fold excess of bacterial DNA. In a separate experiment the relative contribution of Bacteria and Archaea in the original

reactor sludge was analysed. Relative abundance of archaeal and bacterial DNA isolated from the sludge was found to be in a similar order of magnitude.

Table 2: Cloning and sequencing results of archaeal amplicons (app. 1300 bp) retrieved from thermophilic enrichment cultures. Numbers of clones with identical ARDRA profiles out of the total number of picked and screened clones are given to indicate relative abundance. The letters of the Band ID correspond to DGGE bands in Figure 1. Subscript numbers indicate different sequences in Figure 2 that showed the same mobility in DGGE. If the first closest database hit was an uncultured micro-organism, phylum assignment as well as the first hit with a cultured micro-organism is given.

| ARDRA | Band ID | Accession number | Database search result | Similarity |
|---|---|---|---|---|
| | | | **MeOH / (Z245) $10^{10}$** | |
| 2 of 21 | $J_1$ | AY526511 | *Methanothermobacter thermoautotrophicus* strain ΔH | 99% |
| 18 of 21 | $J_2$ | AY526512 | *Methanothermobacter thermoautotrophicus* strain ΔH | 99% |
| 1 of 21 | $J_3$ | AY526513 | *Methanothermobacter thermoautotrophicus* strain ΔH | 99% |
| | | | **Acetate $10^4$** | |
| 18 of 22 | $K_1$ | AY526514 | *Methanomethylovorans hollandica* strain ZB | 97% |
| 1 of 22 | $K_2$ | AY526515 | Uncultured bacterium SJA-102 (*Spirochaetes*) | 96% |
| | | | *Spirochaeta caldaria* | 88% |
| 2 of 22 | L | AY526517 | *Thermodesulfovibrio* sp. TGL-LS1 | 99% |
| | | | (*Nitrospirae*) | 99% |
| | | | *Thermodesulfovibrio islandicus* | |
| 1 of 22 | $K_3$ | AY526516 | *Methanomethylovorans hollandica* strain ZB | 96% |
| | | | **$CO_2$ / $H_2$ $10^9$** | |
| 28 of 28 | M | AY526518 | *Methanothermobacter thermoautotrophicus* strain ZH3 | 99% |
| | | | **MeOH $10^8$** | |
| 30 of 31 | $N_1$ | AY526519 | *Methanomethylovorans hollandica* strain ZB | 97% |
| 1 of 31 | $N_2$ | AY526520 | *Methanomethylovorans hollandica* strain ZB | 97% |

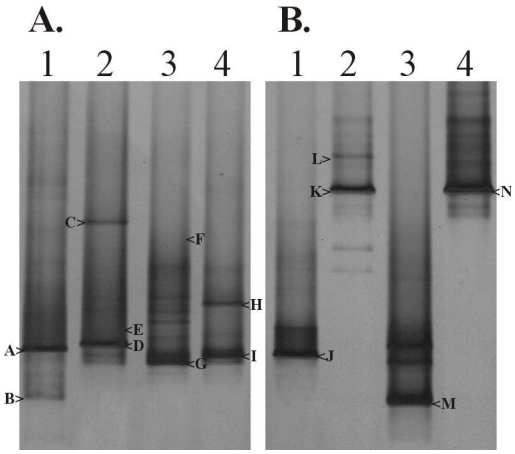


Figure 1: Bacterial (A) and archaeal (B) DGGE patterns of the highest dilutions with growth from the sludge dilution enrichment series from the anaerobic thermophilic lab-scale bioreactor.
1, methanol + Z245; 2, acetate; 3, $H_2$ with $CO_2$; 4, methanol.
See Tables 1 and 2 for band IDs.

## Molecular characterisation and phylogenetic analysis

The highest dilutions with growth from the four different enrichment series were used for DNA isolation and subsequent 16S rRNA-gene-targeted PCR-DGGE analysis. Bacterial and archaeal 16S rRNA sequences of dominant micro-organisms are listed in Tables 1 and 2, were also the number of picked and screened clones can be found. Dominant clones (based on ARDRA screening) in the clone libraries were also found as dominant bands in the DGGE profiles (Figure 1). From each ARDRA profile one clone was sequenced, and the obtained information was then used for

phylogenetic analysis (Figure 2). Micro-organisms from several microbial divisions were found, although the dominant bacterial sequences from the enrichments clustered mainly in *Firmicutes* and the *Thermodesulfovibrio* (*Nitrospirae*) groups. Archaea found in this study could be divided in two groups, *Methanobacteriales* and *Methanosarcinales*.
The most abundant methylotrophic methanogens in the methanol enrichment was related to *Methanomethylovorans hollandica* strain ZB. The most closely related cultivated relative of the dominant bacterial sequences from this methanol enrichment was *Brumimicrobium glaciale*. This organism was isolated from sea ice, but the sequence similarity was only 90%. The sequences best match was with an uncultured bacterium clone derived from a thermophilic terephthalate-degrading anaerobic sludge community.

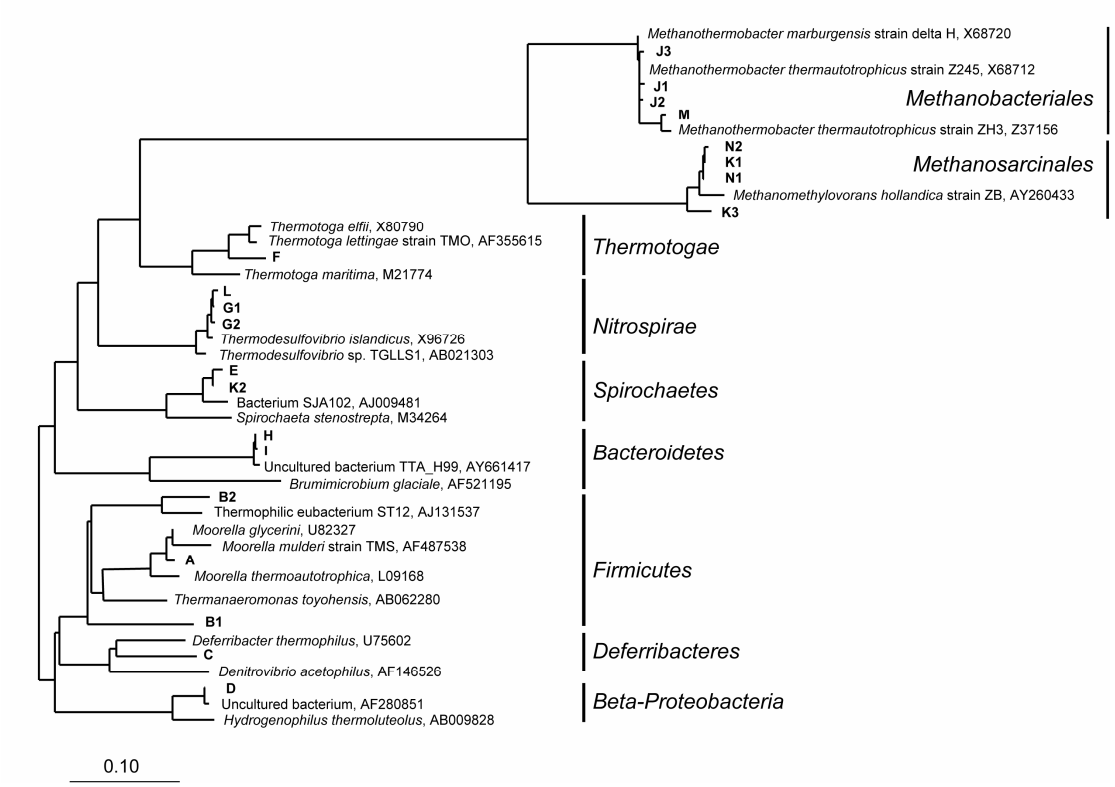

Figure 2: Phylogenetic tree constructed with 16S rRNA sequences of clones from anaerobic thermophilic enrichments and closely related sequences. Genbank accession numbers are given for reference sequences.

In the enrichment with methanol and *Methanothermobacter thermoautotrophicus* strain Z245 a bacterium closely related to *Moorella glycerini* was most dominant. In the same enrichment also some clones of bacteria related to *Clostridium fervidus* and *Thermanaeromonas toyohensis* were detected. Furthermore, *Methanothermobacter thermoautotrophicus*-related sequences were found in this enrichment. Relatives of *M. thermoautotrophicus* ZH3 were also found in high numbers in the $H_2/CO_2$ enrichment.
*Thermodesulfovibrio*-relatives were found in high numbers in the $H_2/CO_2$ enrichment. This enrichment also contained *Thermotoga lettingae* related sequences.
Acetate did not give rise to growth to a high dilution. In the highest dilution with growth ($10^4$), Archaea were only detected as a minor component of the enriched consortium. Besides *Methanomethylovorans hollandica* strain ZB relatives, some bacterial clones were detected in the archaeal clone library. In the bacterial clone library, most abundant sequences showed relatively low sequence similarity (88.1%) with *Deferribacter desulfuricans*. Sequences related to *Petrobacter succinatimandens* BON4, a moderate thermophilic, nitrate-reducing bacterium isolated from an Australian oil well [Bonilla Salinas *et al.* 2004], were found with high sequence similarity (99.7%).

## Discussion

Enrichment cultures

Selective enrichment cultivation indicated that acetate is not an important intermediate in thermophilic methanol degradation in the lab scale bioreactor. On the other hand high numbers of hydrogen-utilizing micro-organisms were present in the sludge, suggesting that hydrogen is an important intermediate in methanol conversion. In addition, the presence of the hydrogenotrophic methanogen *Methanothermobacter thermoautotrophicus* strain Z245 resulted in growth in higher dilutions with methanol as substrate. These findings are in agreement with results of a previous study with the sludge from the lab-scale bioreactor [Paulo *et al.* 2003].

Relative abundance of Archaea and Bacteria

With a dilution PCR, insight can be gained into the relative abundance of Archaea and Bacteria in the microbial communities. Archaea and Bacteria were found to be present at similar orders of magnitude in the original reactor sludge and all enrichments, except in the enrichment with acetate, were a 100-fold excess of bacterial DNA was detected. Since initially the same detection limit was observed for the archaeal and bacterial specific PCR reactions, and all samples showed comparable amplification results, the bacterial excess in the acetate enrichment is interesting. The lack of an expected $H_2$-sink can not be directly explained, but maybe the visible growth was due to bacterial growth on the added yeast extract and the small amount of substrate that still might be present in these rather low dilutions.

Molecular characterisation and phylogenetic analysis

Previously, it was reported that the homoacetogen *Moorella glycerini* cannot utilise methanol [Slobodkin *et al.* 1997]. However, the closely related *M. mulderi* [Balk *et al.* 2003], *M. thermoacetica* [Wiegel *et al.* 1981] and *M. thermoautotrophica* [Fontaine *et al.* 1942] can utilize methanol. *M. mulderi*, which was isolated from a thermophilic sulphate-reducing bioreactor, can oxidize methanol completely to $CO_2$ when grown in the presence of a hydrogenotrophic methanogen or hydrogenotrophic sulphate reducer [Balk *et al.* 2003]. Therefore, it seems evident that the detected dominant *Moorella*-relative plays an important role in the degradation of methanol to $H_2/CO_2$ in the thermophilic methanogenic bioreactor. The detected relatives of *Clostridium fervidus* and *Thermanaeromonas toyohensis* also seem to have the ability to grow on methanol in syntrophic association with methanogens. The *Methanothermobacter thermoautotrophicus*-related sequence in the enrichment with methanol including *M. thermoautotrophicus* strain Z245 is most probably the added strain Z245, although it cannot be excluded from sequence data that other, endogenous strains e.g. closely related to strain ΔH were also maintained in this dilution with growth [Touzel *et al.* 1992]. Relatives to strain ZH3 were also found in the $H_2/CO_2$ enrichment. *M. thermoautotrophicus* strains are hydrogenotrophic methanogens and cannot grow on methanol. Thus, their presence and abundance in the sludge is an extra indication that interspecies $H_2$–transfer is pivotal in thermophilic methanol degradation.

The type strain of *Methanomethylovorans hollandica* is mesophilic. Lomans *et al.* reported that this methylotrophic methanogen does not grow above 40ºC [Lomans *et al.* 1999]. Recently, another strain of *M. hollandica* (strain ZB) was isolated from cold lake sediment [Simankova *et al.* 2003]. This organism had a temperature growth range from 1-38ºC, with an optimum of 30ºC. The *M. hollandica*-relative from this study was related to *M. hollandica* strain ZB, but it was derived from a thermophilic environment and was enriched at 55°C. From our observations it is clear that the *Methanomethylovorans* genus comprise psychro- to mesophilic and moderately thermophilic species. Such different ecophysiologies among strains that are closely related at the 16S rRNA gene level, are found more often, and have recently caught significant attention in microbial ecology [Jaspers & Overmann 2004, Schloter *et al.* 2000]. Our results indicate that methylotrophic methanogens are present in lower numbers ($10^8$) in the thermophilic sludge than methylotrophic homoacetogens ($10^{10}$) growing in syntrophic association with hydrogenotrophic methanogens.

Presently, it is not clear yet which environmental conditions determine the occurrence of direct and indirect methanogenesis from methanol. However, the amount of available cobalt is one factor that seems to be important (Bo Jiang, unpublished results). The first micro-organism described with the ability of indirect methane formation from methanol, *Methanobacillus kuzneceovii*, was reported to degrade methanol via acetate to methane [Pantskhava & Pchelkina 1969a/b]. Mah *et al.* (1977) discussed that this methanogen might be a syntrophic culture of an acetogen and a methanogen. Indeed Ilarionov (1985) isolated a *Clostridium thermoautotrophicum* strain from the *Methanobacillus kuzneceovii* culture and Collins *et al.* (1994) reclassified *Clostridium thermoautotrophicum* into *Moorella thermoautotrophica*.
*Thermodesulfovibrio*-relatives, with high sequence similarity, were found in high numbers in the highest $H_2/CO_2$-dilution with growth. This was very surprising as *Thermodesulfovibrio* spp. are not known to utilise methanol. They can use $H_2$, but only in the presence of sulphate or other electron acceptors. In the media that were used, no sulphate or other electron acceptors were present and methane was the only end product. In an additional experiment, however, *Thermodesulfovibrio*-related sequences were also directly detected in the original sludge of the lab-scale bioreactor [results not shown]. Gram-positive sulphate reducers belonging to the *Desulfotomaculum* genus can use methanol and $H_2/CO_2$ in the absence of sulphate to some extent [Goorissen *et al.* 2004, Min & Zinder 1990]. While it might be that the detected *Thermodesulfovibrio* species have similar physiological properties, there is currently no experimental evidence supporting this hypothesis. In the $H_2/CO_2$ enrichment also bacteria related to *Thermotoga lettingae* were present. *T. lettingae* has been shown to degrade methanol to $H_2$ and $CO_2$ in syntrophic culture with *Methanothermobacter thermoautotrophicus* ΔH or *Thermodesulfovibrio yellowstonii* and has the ability to grow homoacetogenically with $H_2/CO_2$ [Balk *et al.* 2002].
Some bacterial sequences were detected with archaeal specific primers in the acetate enrichment (3 out of 22). It is not uncommon to have some non-specific amplification with archaeal specific primers (e.g. Leclerc *et al.* (2004)). This might be especially the case in samples with an excess of bacterial DNA.
In conclusion, the integrated application of enrichment cultivation- and molecular approaches presented here provided novel insights into the microbial consortia involved in the conversion of methanol in thermophilic anaerobic bioreactors. Besides direct methanogenesis, results indicate that syntrophic, interspecies hydrogen transfer-dependent methanol conversion is important in the thermophilic bioreactor, confirming previous findings with labelled substrates and specific inhibitors. In addition, indications were obtained that certain phylogenetic groups of micro-organisms have yet-unknown physiological properties.

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# Chapter 4

## Molecular Monitoring of Microbial Diversity in Expanded Granular Sludge Bed (EGSB) reactors Treating Oleic Acid

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**Abstract**

A molecular approach was used to evaluate the microbial diversity of bacteria and archaea in two expanded granular sludge bed (EGSB) reactors fed with increasing oleic acid loading rates up to 8 kg of chemical oxygen demand (COD) $m^{-3}$ $day^{-1}$ as the sole carbon source. One of the reactors was inoculated with granular sludge (RI) and the other with suspended sludge (RII). During operation, the sludge in both reactors was segregated in two layers: a bottom settled one and a top floating one. The composition of the bacterial community, based on 16S rDNA sequence diversity, was affected most during the oleate loading process in the two reactors. The archaeal consortium remained rather stable over operation in RI, whereas in RII the relative abundance of *Methanosaeta*-like organisms became gradually weaker, starting in the bottom layer. In the range of oleate loads evaluated, 6 kg of COD $m^{-3}$ $day^{-1}$ was found as the maximum value that could be applied to the system. A further increase to 8 kg of oleate-COD $m^{-3}$ $day^{-1}$ induced a maximal shift on the microbial structure of the sludges. At this time point, methanogenic acetoclastic activity was not detected and only very low methanogenic activity on $H_2/CO_2$ was exhibited by the sludges.

**Introduction**

Oleic acid (C18:1) is, in general, the most abundant LCFA (long chain fatty acid) present in industrial and domestic wastewater as well as one of the more toxic ones [Galbraith *et al.* 1971, Komatsu *et al.* 1991]. LCFAs result from hydrolysis of lipids and are especially problematic for anaerobic wastewater treatment. Besides their direct toxicity to the two main trophic groups involved in LCFAs degradation, methanogens and acetogens, they can adsorb onto biomass particles causing biomass flotation and washout [Hwu *et al.* 1998, Koster & Cramer 1987, Rinzema *et al.* 1994]]. The study of these phenomena, as well as reactor operation and alternative configurations in the application of the anaerobic digestion technology to effluents with high lipid/LCFA content have been subject of research work [Alves *et al.* 2001a/b, Hwu *et al.* 1997a/b, Pereira *et al.* 2001, Rinzema *et al.* 1988, Sam-Soon *et al.* 1991]. However, there is still a lack of knowledge regarding the microbiological aspects of the complex consortia involved in degradation.
Recent developments in molecular ecology have provided new molecular techniques that make it feasible to investigate complex microbial communities, overcoming the problems associated with the traditional cultivation-dependent methods [Amann *et al.* 1995]. Especially in anaerobic bioreactors, where stability and performance is strongly dependent on complex microbial interactions, this development can provide an opportunity to establish the connection between the microbial structure and the functional characteristics of the system. The use of 16S rDNA-based methods employing denaturing gradient gel electrophoresis (DGGE) [Muyzer & Smalla 1998], molecular cloning and sequencing [Godon *et al.* 1997, Wu *et al.* 2001] and fluorescent in situ hybridization [Harmsen *et al.* 1996, Raskin *et al.* 1994b, Sekiguchi *et al.* 1999] can provide an accurate estimate of the microbial composition and diversity in a complex community. Furthermore, when combined with other techniques, chemical, biochemical and/or physiological assays, they can provide considerable information and improve our understanding about the role and dynamics of microorganisms [Oude Elferink *et al.* 1998, Raskin *et al.* 1994a, Verstraete *et al.* 1996].
In previous work, it was found that after feeding a reactor with oleate as the sole carbon source, the biomass became encapsulated by a whitish matter. When this biomass was washed to remove the residual substrate, and incubated in batch vials, it was able to produce methane by degradation of the adsorbed substrate [Alves *et al.* 2001, Pereira *et al.* 2001]. The maximum potential methane production due to the degradation of the adsorbed substrate exhibited by the sludge of two expanded granular sludge bed (EGSB) reactors fed with increasing loads of oleic acid was studied in batch assays [Pereira *et al.* 2002]. The behaviour of granular and suspended sludge was compared since, although being more resistant to LCFAs toxicity than the suspended or flocculent sludge, granular stability is critical for lipid/LCFAs containing wastewaters [Amaral *et al.* 2001,

Hwu *et al.* 1998]. The aim of the present work was to evaluate the microbial diversity of bacteria and archaea in the granular and suspended sludge collected during the operation of these EGSB reactors, using a molecular approach.

## Materials and methods

Sludge sources

Sludge samples were obtained from two 10 l EGSB reactors operated at mesophilic conditions (37ºC) as described elsewhere [Pereira *et al.* 2002]. One reactor (RI) was inoculated with granular sludge whereas another reactor (RII) was inoculated with suspended sludge. Both reactors were operated in parallel with increasing oleate concentrations from 2 to 8 g chemical oxygen demand (COD) $l^{-1}$, in a total of four periods. The hydraulic retention time was set at 1 day. Table 1 summarizes the operating conditions and performance of RI and RII. During the operation a significant amount of sludge accumulated as a floating top layer in both digesters. At the end of each period, samples from the bottom and top layers were collected from each reactor, washed and centrifuged (1681 g, 10 min) twice with the same anaerobic basal medium used in the batch experiments.

Table 1: Operating conditions and performance of RI-granular sludge and RII-suspended sludge (Mean ± 95% confidence intervals) (adapted from Pereira *et al.*, 2002).

| Time (days) [period] | Influent COD ($g\ l^{-1}$) | Influent oleate-COD ($g\ l^{-1}$) | COD Removal efficiency (%) | | Effluent VSS ($g\ l^{-1}$) | | Methane production ($l\ CH_4\ day^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | | | **RI** | **RII** | **RI** | **RII** | **RI** | **RII** |
| 0-70 [1] | 3.8 (± 0.3) | 1.9 (± 0.2) | 96.5 (± 0.6) | 85.8 (± 3.2) | 0.38 (± 0.07) | 0.65 (± 0.04) | 10.6 (± 1.0) | 7.9 (± 1.2) |
| 70-119 [2] | 3.8 (± 0.3) | 3.8 (± 0.3) | 83.4 (± 4.8) | 74.4 (± 5.5) | 0.85 (± 0.22) | 0.72 (± 0.15) | 2.3 (± 0.5) | 2.6 (± 0.5) |
| 119-162 [3] | 6.2 (± 0.7) | 6.2 (± 0.7) | 74.2 (± 3.8) | 74.6 (± 2.9) | 1.96 (± 0.43) | 1.57 (± 0.17) | 1.6 (± 0.2) | 2.0 (± 0.8) |
| 162-219 [4] | 8.2 (± 0.5) | 8.2 (± 0.5) | 68.8 (± 3.4) | 69.4 (± 5.5) | 2.71 (± 0.57) | 2.50 (± 0.58) | 1.5 (± 0.2) | 2.2 (± 0.6) |

Batch experiments

Methanogenic activity tests were performed using the pressure transducer technique [Colleran *et al.* 1992]. The pressure increase in sealed vials fed with non-gaseous substrates (acetate, propionate, butyrate and ethanol) or the pressure decrease in vials previously pressurized with gaseous substrates ($H_2/CO_2$) was monitored. The hand held pressure transducer was capable of measuring a pressure increase or decrease of two bar (0 - ± 202.6 kPa) over a device range of -200 to +200 mV, with a minimum detectable variation of 0.005 bar, corresponding to 0.05 ml biogas in 10 ml headspace. The basal medium used in the batch experiments, made up with demineralized water, was composed of cysteine-HCl (0.5 $g\ l^{-1}$) and sodium bicarbonate (3 $g\ l^{-1}$), the pH was adjusted to 7.0-7.2 with 8 N NaOH and was prepared under strict anaerobic conditions. No calcium or trace-nutrients were added.

Methanogenic toxicity tests were also performed using the pressure transducer technique, for oleate concentration ranging from 100 - 900mg $l^{-1}$. Acetate was added as co-substrate to characterize the toxicity towards acetoclastic methanogens. The oleate concentration that caused a 50% relative methanogenic acetoclastic activity loss was defined as inhibitory concentration at 50% ($IC_{50}$). All batch tests were performed in triplicate assays. Methane content of the biogas was measured by gas

chromatography using a Chrompack Haysep Q (80 to 100 mesh) column, with $N_2$ carrier gas at 30 ml $min^{-1}$ and a flame-ionization detector. Temperatures of the injection port, column, and flame-ionization detector were 120, 40, and 130ºC, respectively.

Microbial community analysis

*DNA extraction and amplification*. Total DNA was extracted from approximately 1ml of homogenized sludge sample as previously described by Harmsen *et al*. (1995). The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using a *Taq* DNA polymerase kit (Life Technologies, Gaithersburg, MD, USA). Complete bacterial 16S rDNA was selectively amplified for cloning and sequencing using 7-f (5'-AGAGTTTGAT(C/T)(A/C)TGGCTCAG-3') and 1510-r (5'-ACGG(C/T)TACCTTGTTACGACTT-3') primers [Lane 1991] with the following thermocycling 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. The reactions were subsequently cooled to 4ºC. For DGGE a specific region of eubacterial 16S rDNA (V6-V8 region) was amplified using 968-GC-f (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3') and 1401-r (5'-CGGTGTGTACAAGACCC-3') primers [Nübel *et al.* 1996] with the same thermocycle program, but increased number of cycles to 35 and an annealing temperature of 56ºC. For archaea, primers A109-f (5'-AC(G/T)GCTCAGTAACAGTAACACGT-3') [Grosskopf *et al.* 1998] and 1510-r were used for complete 16S rDNA amplification and A109(T)-f (original Grosskopf *et al*. (1998), third nucleotide changed into T only, Hans G.H.J. Heilig personal communication) and 515-GC-r (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGATCGTATTACCGCGGCTGCTGGCAC-3') [Lane 1991] for V2-V4 region amplification for DGGE use. Both reactions were performed with the following thermocycle program: 94ºC for 5 min; 24 (34 for DGGE use) cycles of 52ºC for 40 s, 68ºC for 1 min and 94ºC for 30 s; 52ºC for 40 s and 68ºC for 7 min. All primers were purchased from MWG-Biotech (Ebersberg, Germany). The size and amount of PCR products were estimated by 1% agarose gel (w/v) electrophoresis and ethidium bromide staining.

*DGGE analysis* of the amplicons was done as described by Zoetendal *et al.* (2001). Denaturant gradients from 35 to 50% for bacterial amplicon separation and from 30 to 45% for the archaeal ones, were used. DGGE gels were scanned at 400 dpi and the DGGE profiles compared using the Molecular Analyst 1.12 software package (BioRad, Hercules, CA, USA). Similarity indices of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product-moment correlation coefficient [Häne *et al.* 1993]. Community shifts were described as changes in the DGGE profiles of the partial 16S rDNA amplicons.

*Cloning and sequencing* with the Sp6 primer (5'-GATTTAGGTGACACTATAG-3') (MWG-Biotech, Ebersberg, Germany), was done as described by Heilig *et al.* (2002). Similarity search of the partial 16S rDNA sequences derived from the sludge clones was performed using the NCBI sequence search service available in the internet (http.//www.ncbi.nlm.nih.gov/blast/).

*Cell fixation and fluorescent in situ hybridization*. After being washed and resuspended in phosphate-buffered saline, sludge samples were fixed overnight according to Amann (1995). Fixed samples were submitted to sonication for 5 min at 150 W, spotted to wells on gelatine-coated slides, dried for 20 min at 45ºC and dehydrated (1995). Thereupon, *in situ* hybridization was performed with the MX825-CY3 probe (5'-TCGCACCGTGGCCGACACCTAGC-3'; target group: *Methanosaeta*; [Raskin *et al.* 1994b]) as detailed by Manz *et al.* [Manz *et al.* 1992]. For detection of all DNA, 4,6-diamidino-2-phenylindole (DAPI) was added to the wash buffer at a final concentration of 100 ng $ml^{-1}$. After rinsing the slides in water, they were immediately air-dried and mounted in Vectashield (Vector Labs, Burlingame, CA, USA). Digital images of the slides, viewed with a Leica (Wetzlar, Germany) DMR HC epifluorescence microscope, were taken with a Leica DC 250 digital camera. These images were analysed with Leica QFluoro image analysis software at a Leica Q550 FW computer. Phase contrast microscopy was performed with a Zeiss (Oberkochen, Germany) Axioscope microscope.

## Results and discussion

*Specific methanogenic activity*

Both granular and suspended sludge inocula were characterized in batch experiments in terms of specific methanogenic activity and oleic acid toxicity toward acetoclastic methanogens (Table 2). The granular sludge exhibited significantly higher activities with acetate, propionate, ethanol and $H_2/CO_2$. However, methanogenic activity with butyrate was not detected in this sludge whereas a value of 52 ml $CH_{4(STP)}$ ($g^{-1}$ volatile suspended solids (VSS) $day^{-1}$) was detected in the suspended sludge. The toxicity limit ($IC_{50}$) of oleic acid towards acetoclastic methanogens was higher for the granular than for the suspended sludge, indicating the higher resistance of the granular inoculum to the toxicant studied. This result is consistent with the higher resistance to LCFA toxicity of granular sludge when compared with suspended or flocculent sludge previously reported by Hwu *et al.* (1996).

Table 2: Methanogenic activity for both seed sludge and at the end of reactors operation. Oleic acid toxicity for granular and suspended seed sludge. (Mean ± 95% confidence interval)

| | Seed sludge | | End of operation | |
|---|---|---|---|---|
| | granular | suspended | RI-granular | RII-suspended |
| Methanogenic activity in presence of: | | ml $CH_{4(STP)}$ (gVSS day)$^{-1}$ | | |
| Acetate | 327±11 | 107±6 | (n.d.) | (n.d.) |
| Propionate | 160±10 | 48±14 | (n.d.) | (n.d.) |
| Butyrate | (n.d.) | 52±3 | (n.d.) | (n.d.) |
| Ethanol | 514±94 | 106±2 | (n.d.) | (n.d.) |
| $H_2/CO_2$ | 597±16 | 487±31 | 27.8±1.1 | 12.4±2.2 |
| Oleic acid toxicity limit (IC50) (mg $l^{-1}$) | 345±26 | 133±16 | | |

(n.d.) - Not detected.
STP - Standard temperature and pressure conditions.

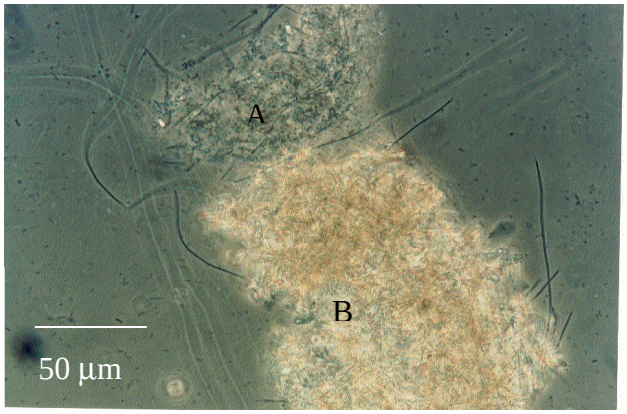



Figure 1: Microscopic aspect of the encapsulated sludge: A - biomass, B - whitish matter.

At the end of operation, the biomass from both reactors was also characterized in terms of methanogenic activity with acetate, propionate, butyrate, ethanol and $H_2/CO_2$. Both sludges exhibited low activity with $H_2/CO_2$ and no activity with the other substrates (Table 2). The granular sludge (from RI) exhibited higher methanogenic activity for hydrogenotrophic methanogens, an important group that acts syntrophically with proton reducing acetogenic bacteria such as LCFA-degraders. Furthermore, in this sludge methane production from $H_2/CO_2$ proceeded without delay, whereas in the suspended sludge (from RII) a lag-phase of 520 h preceding the initial methane production was found.

When considering digestors performance (Table 1), a clear methane production decrease of 20-30% of the initial production, could be observed during the operation. In the last operation period (organic loading rate at 8 kg oleate-COD $m^{-3}$ $day^{-1}$) the effectively to methane converted fraction of COD, was 27 l $CH_4$ (kg $COD_{removed}$)$^{-1}$ in RI and 39 l $CH_4$ (kg $COD_{removed}$)$^{-1}$ in RII. Only 7.7-11% of

the removed oleate-COD was used for methane production. Phenomena such as precipitation with divalent cations and adsorption onto the biomass can also be responsible for LCFAs removal [Hwu *et al.* 1998, Roy *et al.* 1985]. In Figure 1, the encapsulated sludge is shown and clear whitish zones, which represent the absorbed substrate, can be observed surrounding the biomass. This accumulation of non-degraded substrate onto the biomass due to adsorption can hinder the transfer of substrate and products, inducing a delay on initial methane production as well as a reduction of the methane production rate. The potential maximum methane production from the adsorbed substrate exhibited by both sludges during the four operation periods was also evaluated [Pereira *et al.* 2002]. It was found that the suspended sludge had a higher capacity of LCFAs adsorption, which can explain the lower methane production rates and higher delays on initial methane production found for this sludge in the activity tests.

*Population dynamics*

DNA extractions from the granular samples (I) and suspended sludge samples (II), collected from the bottom (b) and the floating top layer (t) of both reactors (RI and RII, respectively) at the end of each operation period (1, 2, 3 and 4), were used as template for amplification of the V6-V8 bacterial regions and the V2-V4 archaeal regions. These amplicons were separated by DGGE and the obtained band patterns of each lane, which corresponded to each sample, were compared. Figs. 2 and 3 present the obtained results.

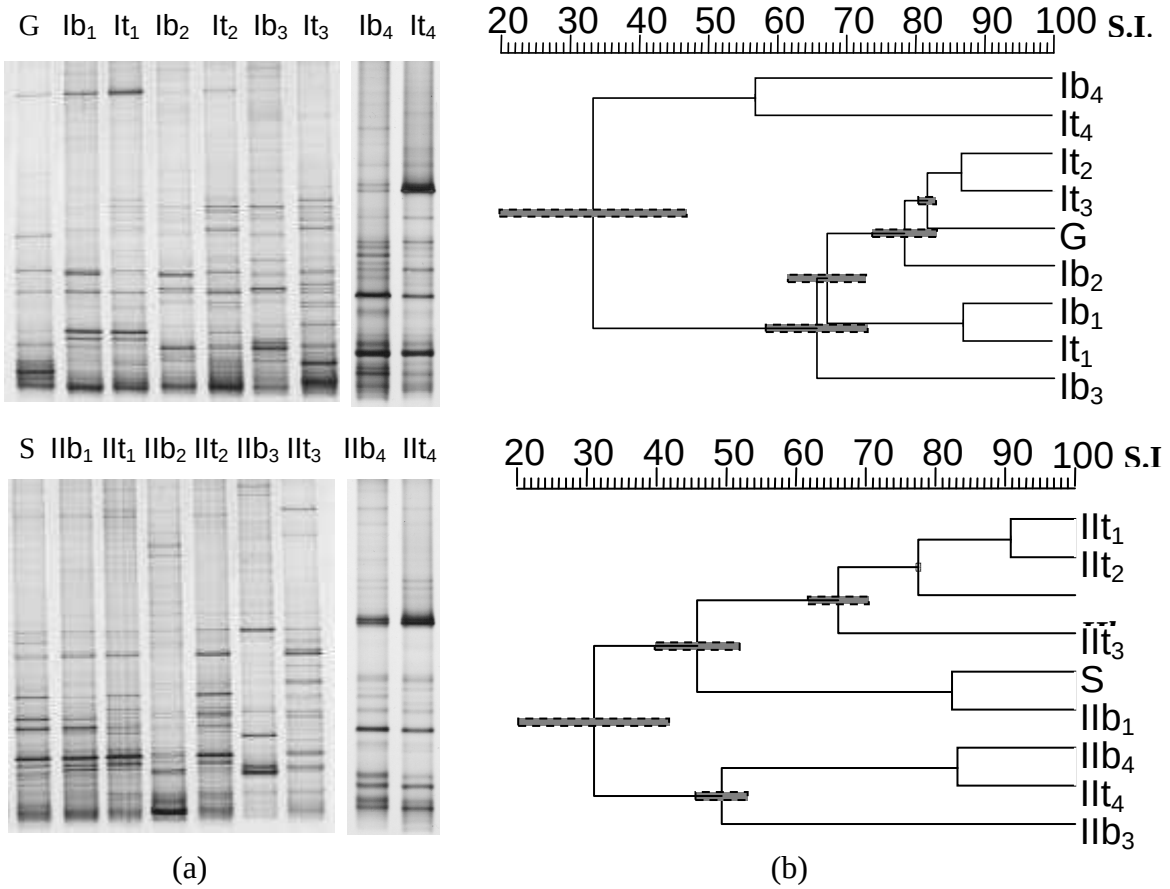
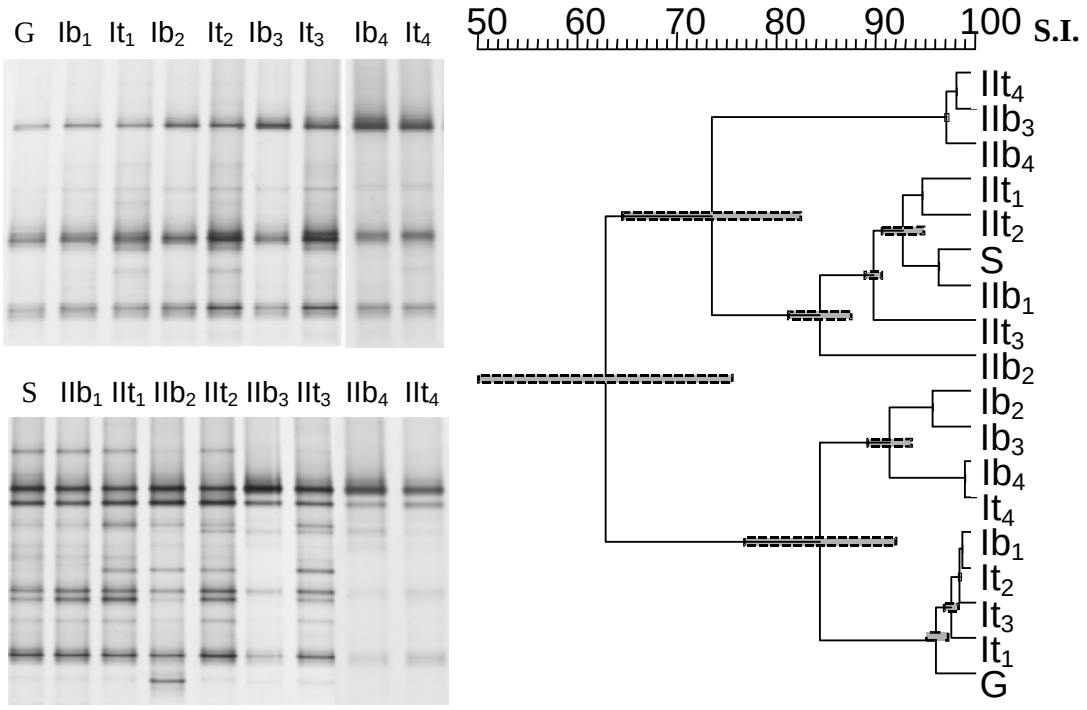



Figure 2: DGGE profiles of bacterial amplicons from sludge samples (a) and correspondent similarity index (S.I.) dendogram (UPGMA clustering) (b). I - granular sludge, II - suspended sludge, b - bottom layer, t - top layer, 1,2,3 and 4 - operation periods, G - granular inoculum, S - suspended inoculum.

For the bacterial domain (Fig. 2), comparison of DGGE band-patterns revealed a clear shift in the community structure with a decrease in the similarity indices between the bottom and top of RI-granular sludge from 86.8, in period 1, to 56.7 in period 4. At the end of the operation, the similarity index between bottom sludge and the inoculum was 42.8, and between top sludge and the inoculum

17.3, suggesting that a higher shift in the community structure occurred in the top than in the bottom sludge. This shift was maximal when the oleate loading rate was increased from 6 to 8 kg COD $m^{-3}$ $day^{-1}$ (the similarity index between the top sludge at the end of period 3 and 4 was of only 14.9). In RII-suspended sludge, the similarity index between bottom and top sludge attained a minimum value of 29.4 at the end of period 3 and increased to 83.1 at the end of the operation, indicating that at the end of period 3 the microbial structure from the top sludge was significantly different from the bottom sludge. This was not the case at the end of the operation, when both layers exhibit also low similarity indices to the inoculum (28.7 between bottom and inoculum and 15.2 between top and inoculum). Furthermore, the suspended top sludge characterized at the end of period 3 revealed the highest methane production capacity from the adsorbed substrate (1145 ± 307 ml $CH_{4(STP)}$ $g^{-1}$ VSS) [Pereira *et al.* 2002], suggesting that the microbial structure of this sludge includes groups of microorganisms particularly important for the degradation of LCFA to methane.




(a) (b)

Figure 3: DGGE profiles of archaeal amplicons from sludge samples (a) and correspondent similarity index (S.I.) dendogram (UPGMA clustering) (b). I - granular sludge, II - suspended sludge, b - bottom layer, t - top layer, 1,2,3 and 4 - operation periods, G - granular inoculum, S - suspended inoculum.

The lowest similarity index among the community patterns of the top layer sludge was found between period 3 and 4, i.e. 27.3. This maximal shift induced in the predominant bacterial composition may include the loss of microorganisms important for the degradation of LCFA to methane, and thus explain the sharp decrease on the methanization capacity of the adsorbed substrate exhibited by this sludge at the end of period 4 (111 ± 24 ml $CH_{4(STP)}$ $g^{-1}$ VSS) [Pereira *et al.* 2002].
In the archaeal consortium (Fig. 3) no significant shift in RI-granular sludge community patterns was detected indicating that the dominant microbial composition remained rather stable over operation, whereas in RII-suspended sludge the diversity decreased, starting in the bottom layer.

The differences observed between the two sludges can be understood based on their different morphological and physiological structure. In fact, contrary to the weak and random microorganisms aggregation of suspended and flocculent sludge, granules form dense aggregates with a layered microbial organization, in which the internal core consists mostly of acetoclastic

methanogens, surrounded by a second layer of acetogenic and hydrogenotrophic bacteria, with a peripheral layer comprising acidogenic, sulfate-reducing and hydrogenotrophic bacteria [Fang *et al.* 1994, Guiot *et al.* 1992, MacLeod *et al.* 1990, Quarmby & Forster 1995]. This layered structure was observed in granules fed with various substrates including brewery wastes, as was the case of the granular inoculum used in RI. Thus, being located in the inner core of the granules, methanogenic organisms (archaeal domain) can be more protected from the toxic effect and from the hydraulic shear stress, which can explain the insignificant shift in archaeal population observed in this sludge as opposed to the case of suspended sludge. The lower protection offered to the archaeal consortium in the case of the suspended sludge did not prevent the toxic effect, mainly on the community present at the feed inlet, i.e. the bottom layer.
Besides microbial organization, good settling properties are also pointed as an advantage of biomass aggregation [MacLeod *et al.* 1990]. However, for lipid/LCFA containing wastewaters, granule stability is very problematic and disintegration is often observed [Amaral *et al.* 2001, Hawkes *et al.* 1995, Sam-Soon *et al.* 1991]. In the thermodynamic respect, the disintegration of granules is predictable, when in contact with this compound, because at neutral pH, LCFAs act as surfactants, lowering the surface tension, compromising the aggregation of hydrophobic bacteria, like most acetogens (LCFA-degraders) [Daffonchio *et al.* 1995]. Thus, the decrease in the similarity indices between the bacterial consortium (located in the outer layer) from bottom and top layers on RI-granular sludge with the increase in the toxicant fed to the reactors can be a result of disintegration. The granules suffering from disintegration by contact with oleate would become lighter and accumulated, together with smaller fragments from disintegration, in a floating top layer. Results obtained in a previous work by applying image analysis to characterize the morphology of the granules during the operation of this reactor (RI) confirmed the occurrence of disintegration [Amaral *et al.* 2001].

*DGGE bands identification*
To identify the prominent bands in the DGGE patterns of sludge samples from the last operation period, bacterial and archaeal 16S rRNA genes from samples $Ib_4$, $It_4$, $IIb_4$ and $IIt_4$ were amplified, cloned and sequenced. The DGGE mobility of the obtained amplicons from the clones were compared to the DGGE profiles of the sludge samples in order to determine to which fragments they corresponded. Due to the considerable shift induced on the bacterial structure during the last operation period and, to retrieve more information, the same procedure was applied to sample $IIt_3$. This sample, taken from the top of RII-suspended sludge at the end of period 3, when oleate was fed at 6 g COD $l^{-1}$, was chosen since it exhibited the highest methane production capacity from the adsorbed substrate [Pereira *et al.* 2002], and thus, would include groups of microorganisms particularly important for the degradation of LCFAs to methane. Table 3 summarizes the sequencing results and Fig. 4 illustrates their corresponding position in each DGGE profile.
Sequencing and BLAST searching of the bacterial clones resulted mainly in matches with unknown and uncultured microorganisms assigned to the Gram-positive group (clones B1, B3, B6, B9), *Proteobacteria* (clone B2) and *Spirochaetales* (clone B7). Close relatives to *Desulfovibrio mexicoense* (clone B4) and *Trichlorobacter thiogenes* (clone B11) belonging to the delta subdivision of *Protebacteria* were also found, although corresponding to very diffuse fragments on the DGGE profiles. Clones B8 and B10 were closely related to the *Syntrophomonas* genus. The presence of saturated fatty acid-β-oxidizing syntrophic bacteria is desirable to maximize the consumption of butyrate and higher fatty acids resulting from LCFA conversion into acetate and hydrogen through β-oxidation mechanism by the proton reducing acetogenic bacteria [Weng & Jeris 1976]. *Syntrophomonas*-like organisms were found in sample $IIt_3$ but corresponded to very diffuse fragments in the DGGE profiles from the other samples (end of operation), suggesting that the presence of these microorganisms became weaker by increasing the oleate concentration from 6 to 8 g COD $l^{-1}$. *Pseudomonas*-like organisms (clone B5) corresponded to strong DGGE-bands in the suspended sludge at the end of digesters operation, whereas in the granular sludge they were found only in the top layer.

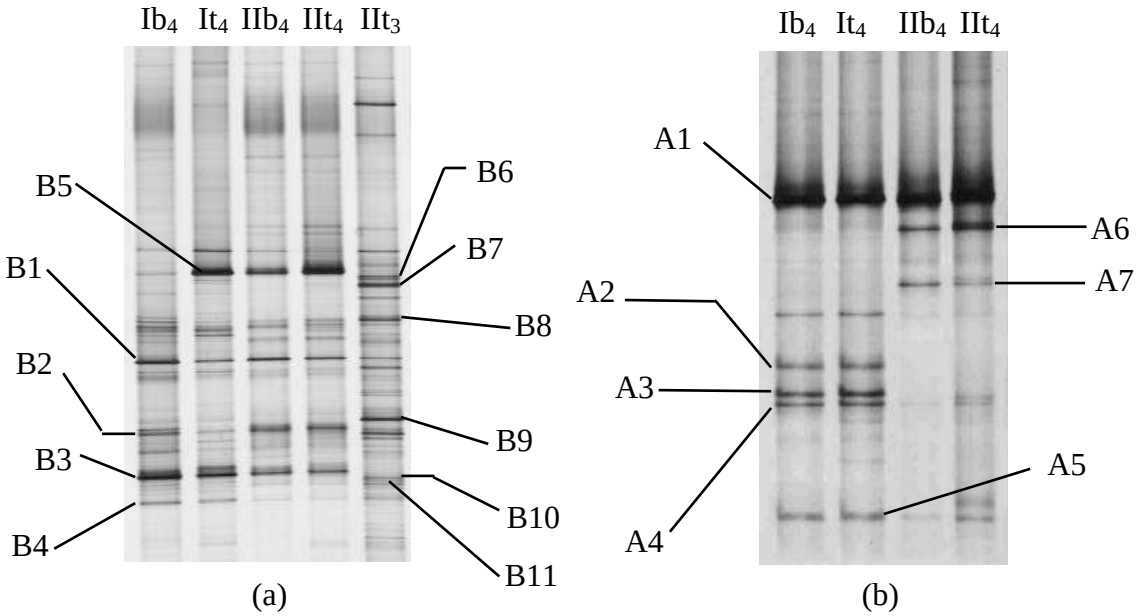



Figure 4: Correspondent position of each bacterial (a) and archaeal (b) sludge clone in the total DGGE profiles of the analyzed samples. I - granular sludge, II - suspended sludge, b - bottom layer, t - top layer, 3,4 - operation periods.

Table 3: Sequencing results of the sludge clones.

| Clone | GenBank Accession number | Sequence length (bp) | Closest relatives (% sequence similarity) |
|---|---|---|---|
| B1 | AF455055 | 811 | Uncultured eubacterium WCHB1-71 (94%), *Syntrophomonas sapovorans* (94%) |
| B2 | AF455056 | 813 | Uncultured bacterium clone C (97%), *Buchnera aphidicola* (91%) |
| B3 | AF455057 | 800 | Uncultured bacterium mle l-42 (97%), *Aminomonas paucivorans* (88%) |
| B4 | AF455058 | 903 | *Desulfovibrio mexicoense* (98%) |
| B5 | AF455059 | 736 | *Pseudomonas stutzeri* (96%) |
| B6 | AF455060 | 904 | Gram-positive bacterium MOL361 (87%), *Erysipelothrix rhusiopathiae* (88%) |
| B7 | AF455061 | 657 | Unidentified eubacterium cloneVadinBA43 (93%), *Spirochaeta africana* (88%) |
| B8 | AF455062 | 906 | *Syntrophomonas sapovorans* (97%) |
| B9 | AF455063 | 902 | Uncultured bacterium SJA-88 (90%), *Clostridium cellobioparum* (87%) |
| B10 | AF455064 | 804 | *Syntrophomonas* sp. MGB-C1 (97%) |
| B11 | AF455065 | 812 | *Trichlorobacter thiogenes* (97%) |
| A1 | AF455066 | 441 | *Methanobacterium formicicum* (97%) |
| A2 | AF455067 | 815 | *Methanosaeta concilli* (97%) |
| A3 | AF455068 | 775 | *Methanobacterium* sp. DSM 11106 (95%) |
| A4 | AF455069 | 884 | *Methanosaeta concilli* (98%) |
| A5 | AF455070 | 827 | Uncultured archaeon TA05 (98%), *Methanosaeta concilli* (98%) |
| A6 | AF455071 | 852 | *Methanobacterium formicicum* strain FCam (98%) |
| A7 | AF455072 | 774 | *Methanobacterium* sp. DSM 11106 (96%) |

For the archaeal domain the clone sequences were affiliated with the two main groups, the acetoclastic *Methanosaeta* and the hydrogenotrophic *Methanobacterium.* In the last operation period *Methanosaeta*-like organisms (clones A2, A4 and A5), known to be sensitive to LCFAs [Alves *et al.* 2001b, Hanaki *et al.* 1981] were clearly present in the granular biomass, but corresponded to very faint bands in the suspended sludge profiles (Fig. 4b). Along the operation, these DGGE bands exhibited stable relative intensities in the RI-granular sludge profiles, whereas in RII-suspended sludge they gradually faded, indicating that the relative abundance of this group became weaker in this sludge during the operation but remained quit constant in the granular one (Fig. 3a). This fact can be related to the layered granular organization referred to before, where M*ethanosaeta*-like organisms, being located in the internal granule core, take advantage of a more protected environment against the toxic effect and hydraulic shear stress. This protective concept is

also sustained by the higher toxicity limit ($IC_{50}$) of oleic acid towards acetoclastic bacteria exhibited by the granular inoculum when compared with the suspended sludge (Table 2). In a previous work, Zheng and Raskin (2000) used a genus-specific probe to evaluate the levels of *Methanosaeta* sp. in a number of bioreactor samples and found that they were more abundant in granular sludge than in flocculent sludge. In this work, the *Methanosaeta* genus specific probe MX825 was also used to evaluate the levels of this group of microorganisms in both reactors at the end of operation (samples $Ib_4$ and $IIb_4$). The results obtained by fluorescent in situ hybridization were consistent with previous data found by DGGE/sequencing. In the suspended sludge only a weak fluorescent signal was observed whereas in the granular sludge small chains of *Methanosaeta*-like organisms were still detected (Fig. 5). However, in both sludges no acetoclastic activity was detected at the end of operation (Table 2).

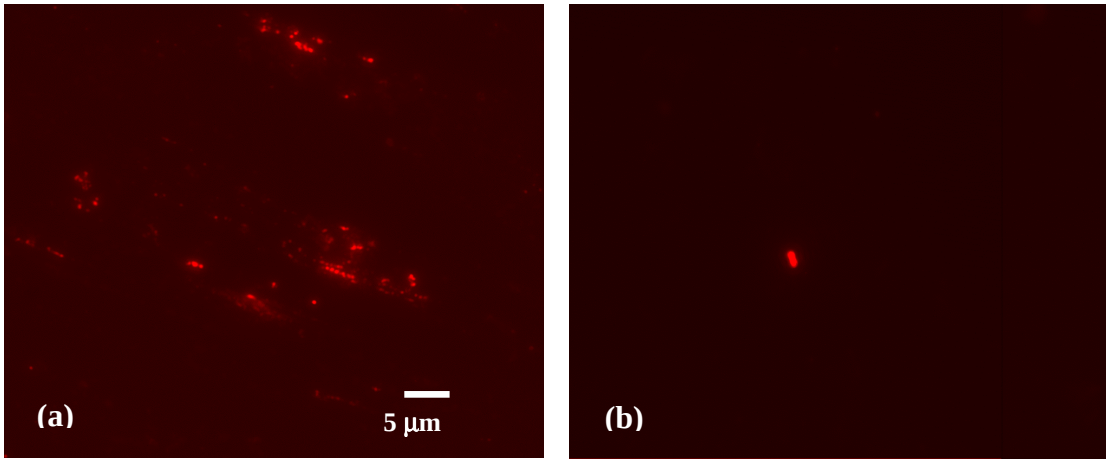



Fig. 5. Epifluorescence photographs showing in situ hydridization with probe MX825-CY3 of (a) granular and (b) suspended sludge present in the bottom layer of reactors, at the end of operation.

In this work, it is shown that the combination of physiological and molecular-based sludge characterization can be a powerful approach to study and optimize the anaerobic digestion process for treating lipids/LCFA-based effluents. Although in general, the methanogenic sludge population can be characterized relatively well, for the group of microorganisms involved in β-oxidation, the presence of many unidentified species in the sludge underlines the need for future research on classical isolation and characterization studies. This need is of utmost importance as it was observed that the bacterial domain was more affected by the contact with increasing oleic acid concentrations. Furthermore, the increase in the organic loading rate to 8 kg oleate-COD $m^{-3}$ $day^{-1}$ induced a maximal shift on the microbial community structure of the sludges. Also at this time point, methanogenic acetoclastic activity was not detected and only a very low methanogenic activity on $H_2/CO_2$ was still exhibited by the sludges. These results, together with the higher methane production capacity from the adsorbed substrate exhibited by the sludges in the previous organic loading rate evaluated, i.e. 6 kg oleate-COD $m^{-3}$ $day^{-1}$ [Pereira *et al.* 2002], suggest that this should be the maximum organic loading rate applied to these reactors when treating oleic acid-based effluents.

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# Chapter 5

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

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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.

**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 smelting plant. 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.

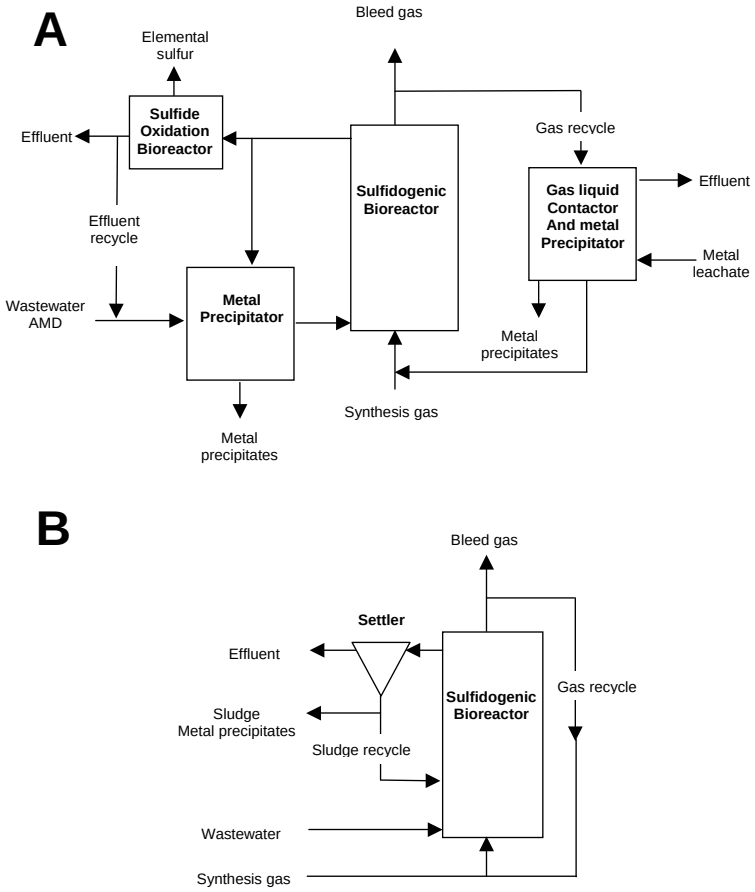


**Figure 1**: Simplified flow diagrams of the Kennecott (A) and Budel (B) reactors.

## Materials and 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 ºC | 30 - 35 ºC |
| 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 $^oC$ 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 by Oude Elferink *et al.* (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 ºC, reactor temperature was 1000 ºC. 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 ºC, and detector temperature was 200 ºC.

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 ºC. 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 *et al.* (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. 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. 2C). 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. 2B). With the increase of the biomass concentration (Fig. 2C) 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).

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.

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.

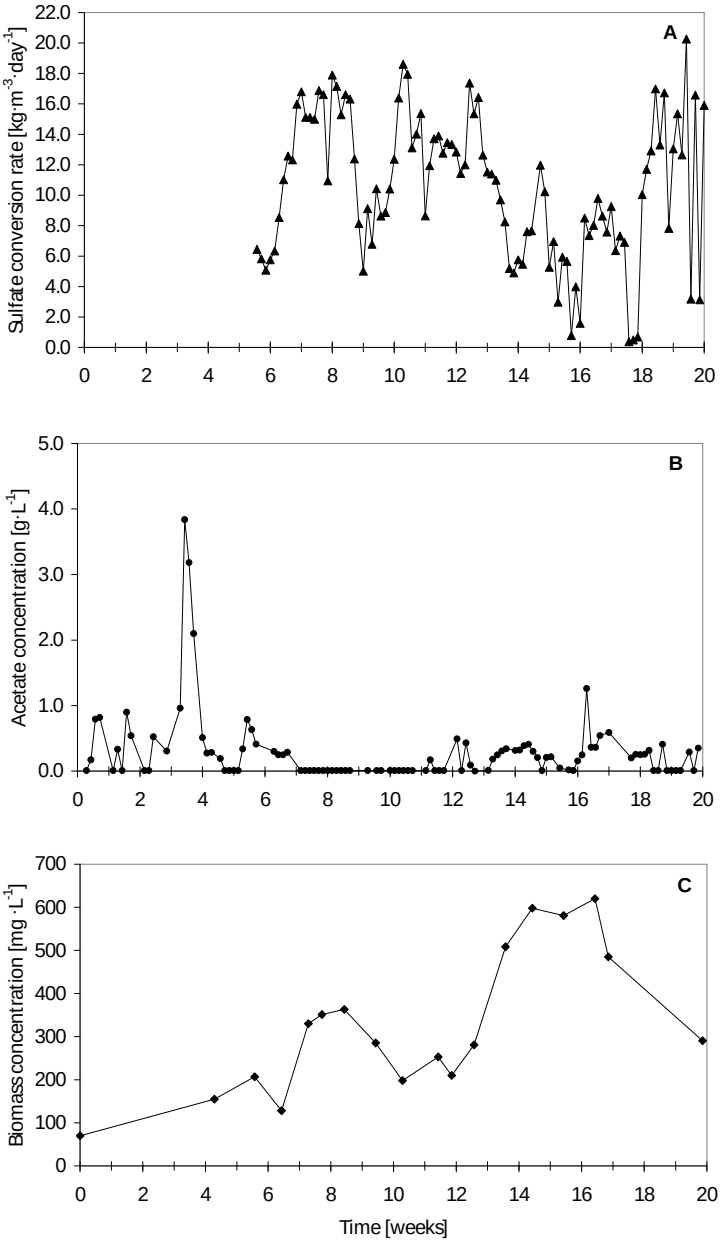


Figure 2: Sulfate conversion rate (A), acetate concentration (B), and biomass concentration (C) in the Budel reactor. The biomass concentration is based on the organic N.

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 | $1.5 \times 10^{11}$ | $4.4 \times 10^{12}$ |
| Methanogens | $6.8 \times 10^{8}$ | $4.5 \times 10^{9}$ |
| Homoacetogens | $3.6 \times 10^{5}$ | $6.2 \times 10^{6}$ |

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 relatives 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 3). 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 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$^{o}$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 ($\mu_{max}$) 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. 2B), 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]. 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.

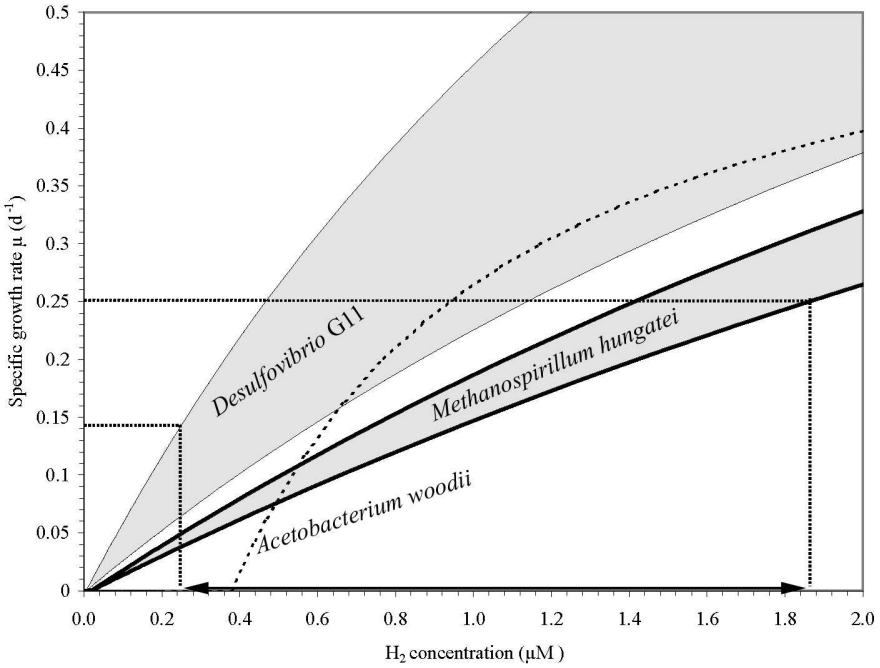


**Figure 3:** Hydrogen concentration range to sustain growth rates of 0.14 to 0.25 $d^{-1}$ based on Monod kinetics* for *Desulfovibrio* G11, *Methanospirillum hungatei* JF1 and *Acetobacterium woodii*.

*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 ºC. Monod parameters for *Acetobacterium woodii* were obtained from Peters *et al.* (1998) for growth at 30 ºC. Hydrogen thresholds measured at 28 – 34 ºC ,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.

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# Chapter 6

## Community analysis of a full-scale anaerobic bioreactor treating paper mill wastewater

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## Abstract

To get insight into the microbial community of an Upflow Anaerobic Sludge Blanket reactor treating paper mill wastewater, conventional microbiological methods were combined with 16S rRNA gene analyses. Particular attention was paid to micro-organisms able to degrade propionate or butyrate in the presence or absence of sulphate. Serial enrichment dilutions allowed estimating the number of micro-organisms per ml sludge that could use butyrate with or without sulphate ($10^5$), propionate without sulphate ($10^6$), or propionate and sulphate ($10^8$). Quantitative RNA dot-blot hybridisation indicated that Archaea were two-times more abundant in the microbial community of anaerobic sludge than Bacteria. The microbial community composition was further characterised by 16S rRNA-gene-targeted Denaturing Gradient Gel Electrophoresis (DGGE) fingerprinting, and via cloning and sequencing of dominant amplicons from the bacterial and archaeal patterns. Most of the nearly full length (approximately 1.45 kb) bacterial 16S rRNA gene sequences showed less than 97% similarity to sequences present in public databases, in contrast to the archaeal clones (approximately. 1.3 kb) that were highly similar to known sequences. While *Methanosaeta* was found as the most abundant genus, also *Crenarchaeote*-relatives were identified. The microbial community was relatively stable over a period of 3 years (samples taken in July 1999, May 2001, March 2002 and June 2002) as indicated by the high similarity index calculated from DGGE profiles (81.9 ± 2.7% for Bacteria and 75.1 ± 3.1% for Archaea). 16S rRNA gene sequence analysis indicated the presence of unknown and yet uncultured micro-organisms, but also showed that known sulphate-reducing bacteria and syntrophic fatty acid-oxidising micro-organisms dominated the enrichments.

## Introduction

Treatment of industrial wastewater in anaerobic bioreactors has grown in importance since the introduction of the Upflow Anaerobic Sludge Blanket (UASB) reactor about 25 years ago [Lettinga *et al.* 1980]. Although the general processes occurring in anaerobic biological wastewater treatment plants, such as hydrolysis, fermentation, acetogenesis, methanogenesis and sulphidogenesis, are well understood, the microbial community responsible for these conversions is often considered as a black box [Head *et al.* 1998]. This fact is not due to an underestimation of the biological component, but is caused by the limitations of methods available for the microbial identification and activity measurements.

Short chain fatty acids are important intermediates in the anaerobic degradation process. Propionate and butyrate oxidation to acetate, $CO_2$, $H_2$ and formate are energetically very unfavourable reactions and syntrophic interaction with methanogens or sulphate-reducing bacteria (SRB) is needed to make these oxidations feasible [Schink & Stams 2002, Schink 2002]. Micro-organisms involved in the degradation of propionate and butyrate play a crucial role in the anaerobic degradation process in methanogenic bioreactors. The degradation of these compounds in anaerobic bioreactors is strongly influenced by the presence or absence of sulphate. In the absence of sulphate, propionate or butyrate conversion is thermodynamically only possible at a low partial hydrogen pressure and formate concentration that characterise syntrophic consortia of propionate- and butyrate degraders with hydrogenotrophic methanogens [Boone *et al.* 1989, Harmsen *et al.* 1996, Schink 1997]. In the presence

of sulphate, propionate and butyrate can be converted by SRB without the participation of methanogens [Harmsen *et al.* 1996]. SRB are a polyphyletic group of physiologically versatile organisms that are not restricted to sulphate reduction. Because of the obligate syntrophy, examination of pure cultures, growing on these specific compounds, is not possible and substrate conversion and product formation data for mixed multi-species consortia can be difficult to interpret. However, the availability of cultured isolates is certainly needed for a better understanding of their physiology and role in the cycling of elements [Connon & Giovannoni 2002].

Cultivation-dependent methods give in general insufficient insight into the *in situ* structure and dynamics of microbial assemblages. Even when non-selective media are used, growth of particular microbial groups is favoured. Developments in molecular ecology, such as microbial community fingerprinting, cloning and sequence analysis, and Fluorescent *in situ* Hybridisation, have provided novel approaches that enable the investigation of complex microbial communities, overcoming the problems associated with the traditional cultivation dependent methods [Amann *et al.* 1995, Amann 1995, Kowalchuk *et al.* 2004, Vaughan *et al.* 2000]. Especially in anaerobic bioreactors, where stability and performance is strongly dependent on complex microbial interactions, this development provides an opportunity to couple the microbial structure and the functional characteristics of the system [Pereira *et al.* 2002]. Genetic fingerprinting approaches are particularly useful for the high-throughput comparison of microbial communities from different environments or to follow changes in community structure in time [Dijkshoorn *et al.* 2001, Muyzer 1999, Vaughan *et al.* 2000]. Molecular tools are also helpful in monitoring enrichment cultures and in facilitating the successful isolation of ecologically relevant bacterial populations. With this integrated approach metabolic functionality can be assigned to molecular data [Muyzer 1999].

The aim of this study was to establish a comprehensive description of the most dominant organisms present in an anaerobic paper-mill wastewater treatment system. This system is treating wastewater from three different paper-mills containing mainly starch and short chain fatty acids with a COD/sulphate ratio of 9.5 [Oude Elferink *et al.* 1998]. 16S rRNA gene-targeted molecular approaches, namely quantitative dot blot hybridisation of total community RNA, PCR-DGGE fingerprinting, and cloning and sequencing were used. In concert with selective enrichment of intermediate fatty-acid-degrading microbial consortia, the identity of key players involved in these processes was established.

## Materials and Methods

### Sludge samples

Granular mesophilic sludge samples of a stable full-scale anaerobic paper-mill wastewater treatment system in Eerbeek, The Netherlands, were taken from a sampling port at a fixed height and position of the reactor in July 1999, May 2001, March 2002 and June 2002. The major constituents of the paper-mill wastewater fed to the UASB reactor were carbohydrates (mainly starch) and short chain fatty acids [Oude Elferink *et al.* 1998]. More details of this treatment plant were given elsewhere [Oude Elferink *et al.* 1998].

Cultivation and DNA extraction

Five millilitres of the July 1999 sludge was homogenised with mortar and pestle and used as starting material for enrichment cultures. Dilution series were made in a basal bicarbonate-buffered medium containing 20 mM propionate or butyrate with and without 20 mM sulphate according to Stams *et al.* (1993). From a fresh *Methanospirillum hungatei* culture growing in exponential phase, 2.5 ml was added to the cultures with only propionate and butyrate to enhance growth of the syntrophic fatty acid-degrading organisms. Sludge samples and enrichments were used for total DNA isolation, based on mechanical disruption by bead beating and phenol/chloroform/iso-amyl-alcohol extraction according to the method of Oude Elferink *et al.* (1997).

RNA extraction and oligonucleotide probe hybridisation

RNA from the sludge samples was isolated with TRIZOL®, a commercially available ready-to-use, monophasic solution of phenol and guanidine isothiocyanate, (Invitrogen, Breda, The Netherlands) according to the manufacturers protocol, including the bead beating step. The resulting RNA pellet was further purified using the SV RNA isolation kit (Promega, Madison, WI, USA). After dissolving the pellet in the lysis buffer the protocol of the manufacturers was followed. RNA was blotted on Natran SuperCharge filters (Schleicher & Schuell, Dassel, Germany) and hybridised overnight at 42°C in QuickHyb™ (Stratagene, LaJolla, CA. USA) with the following [$\gamma$-$^{32}$P] dATP-labelled probes and conditions. All organisms, S-*-Univ-1390-a-A-18 (GAC GGG CGG TGT GTA CAA), washing at 44°C [Zheng *et al.* 1996]; Bacteria, S-D-Bact-0338-a-A-18 (GCT GCC TCC CGT AGG AGT), washing at 54°C [Amann *et al.* 1990]; and Archaea, S-D-Arch-0915-a-A-20 (GTG CTC CCC CGC CAA TTC CT), washing at 58°C [Stahl & Amann 1991]. A phosphor screen was exposed and signals were quantified with a STORM phosphor imager (Molecular Dynamics, Little Chalfont, Buckinghamshire, United Kingdom).

16S rRNA gene amplification

The 16S rRNA-genes were amplified from genomic DNA by PCR using *Taq* DNA polymerase (Life Technologies, Gaithersburg, MD, USA) with primers targeting conserved domains. Bacterial 16S rRNA genes were selectively amplified using 7-f and 1510-r primers [Lane 1991] with the following thermocycling program: pre-denaturation at 94ºC for 5 min; 25 cycles of denaturation at 94ºC for 30 s, annealing at 52ºC for 20 s, and elongation at 68ºC for 40 s; and post-elongation at 68ºC for 7 min. The reactions were subsequently cooled to 4ºC. For DGGE the V6-V8 region of bacterial 16S rRNA genes was amplified using 968-GC-f and 1401-r primers [Nübel *et al.* 1996]. Conditions were as above, but with 35 cycles and an annealing temperature of 56ºC. For Archaea, primers A109-f [Groβkopf *et al.* 1998] and 1510-r [Lane 1991] were used for nearly complete 16S rRNA gene amplification, whereas for DGGE, the V2-V3 region was amplified using A109(T)-f (original Groβkopf *et al.* (1998), but 3$^{rd}$ nucleotide position modified into T) and 515-GC-r [Lane 1991], with a GC-clamp. Both archaeal PCR reactions were performed with the following thermocycle program: pre-denaturation at 94ºC for 5 min; 25 (35 for DGGE use) cycles of denaturation at 94ºC for 30 s, annealing at 52ºC for 40 s, and elongation at 68ºC for 1 min; and post-elongation at 68ºC for 7 min. All primers were purchased from MWG-Biotech (Ebersberg, Germany). Size and yield of PCR products were estimated using a 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania) by 1% agarose gel (w/v) electrophoresis and ethidium bromide staining.

DGGE

DGGE analysis of the amplicons was performed on 8% (w/v) polyacrylamide gels containing denaturant gradients of 30 to 50%. A 100% denaturing solution contained 7M urea (Invitrogen, Breda, The Netherlands) and 40% (v/v) formamide (Merck, Whitehouse Station, NJ, USA). Gelbond film (Amersham Biosciences, Little Chalfont, England, UK) was used as physical support. Electrophoresis was performed in 0.5-X TAE buffer (20 mM Tris, 10 mM acetic acid and 0.5 mM EDTA pH 8) at 85V and 60ºC for 16 hours using a DCode™ System (BioRad, Hercules, CA, USA). During the first 5 min of electrophoresis, a voltage of 200 V was applied. Silver-staining and development of the gels was performed as described by Sanguinetti *et al.* (1994). Furthermore the effect of the number of PCR cycles on the bacterial DGGE profile of Eerbeek sludge was investigated in the range from 20 to 35 cycles, however significant differences were not observed (results not shown). Similarity indices of the compared profiles, based on the Pearson product-moment correlation coefficient, were calculated as described before [Häne *et al.* 1993, Pereira *et al.* 2002].

Cloning and sequencing

The amplified 16S rRNA gene products were purified with a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and cloned in *E. coli* JM109 (Invitrogen, Breda, The Netherlands) by using the pGEM®-T Easy vector system (Promega, Madison, WI, USA) with ampicillin selection and blue/white screening, according to the manufacturer's manual. Clones were screened by Amplified Ribosomal DNA Restriction Analysis (ARDRA), using the enzyme *MspI* (Invitrogen, Breda, The Netherlands). Fragments were separated by 2% (w/v) agarose (Boehringer Mannheim GmbH, Mannheim, Germany) gel electrophoresis and visualised by ethidium bromide staining. Clones with different ARDRA patterns were used for mobility comparison on DGGE. Plasmids of selected transformants were 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 pGEM®-T vector-targeted sequencing primers Sp6 (5'-GATTTAGGTGACACTATAG-3'), and T7 (5'-TAATACGACTCACTATAGGG-3'), and when necessary 16S rRNA-gene-targeted internal primers Uni-519-rev (5'-G(A/T)ATTACCGCGGC(G/T)GCTG-3') [Lane 1991], Uni-533-forw (5'-GTGCCA GC(A/C)GCCGCGGTAA-3') [Lane 1991], Arch-907-rev (5'-CCGTCAATTCCTTT GAGTTT-3') [Lane 1991] and Bact-1100-rev (5'-GGGTTGCGCTCGTTG-3') [Lane 1991]. All primers were labelled with IRD800 (MWG-Biotech, Ebersberg, Germany). The sequences were automatically analysed on a LI-COR (Lincoln, NE) DNA sequencer 4000L, checked and edited manually.

Phylogenetic analysis

Similarity searches of 16S rRNA gene sequences derived from clones against sequences deposited in publicly accessible databases were performed using the NCBI Blast search tool at http://www.ncbi.nlm.nih.gov/BLAST/.

Alignment with FastAligner, followed by manual aligning guided by secondary structures models, and phylogenetic analyses were performed using the ARB program package [Ludwig *et al.* 2004]. A phylogenetic tree was constructed in ARB, using the neighbour joining method [Saitou & Nei 1987] (*E. coli* positions 133 to 1086), with Felsenstein correction and a filter, excluding positions that are conserved <50% of sequences from phylogenetic lineages commonly found in anaerobic wastewater

treatment system communities. Nucleotide sequences obtained in this study have been deposited in GenBank (accession no. AY426437 to AY426485).

## Results and discussion

### Composition and stability of anaerobic bioreactor community

To get insight into the relative abundance of Archaea and Bacteria, freshly collected Eerbeek sludge was used for direct isolation of RNA. Dot blot hybridisation of total sludge RNA with radioactively labelled universal and domain-specific 16S-rRNA-targeted oligonucleotide probes revealed that archaeal rRNA (63%) was more abundant than bacterial rRNA (37%). This is in accordance with other studies of anaerobic wastewater treatment bioreactors, where Archaea were also twice as abundant as Bacteria [Gonzalez-Gil *et al.* 2001, Harmsen *et al.* 1996, Oude Elferink *et al.* 1998, Raskin *et al.* 1994].

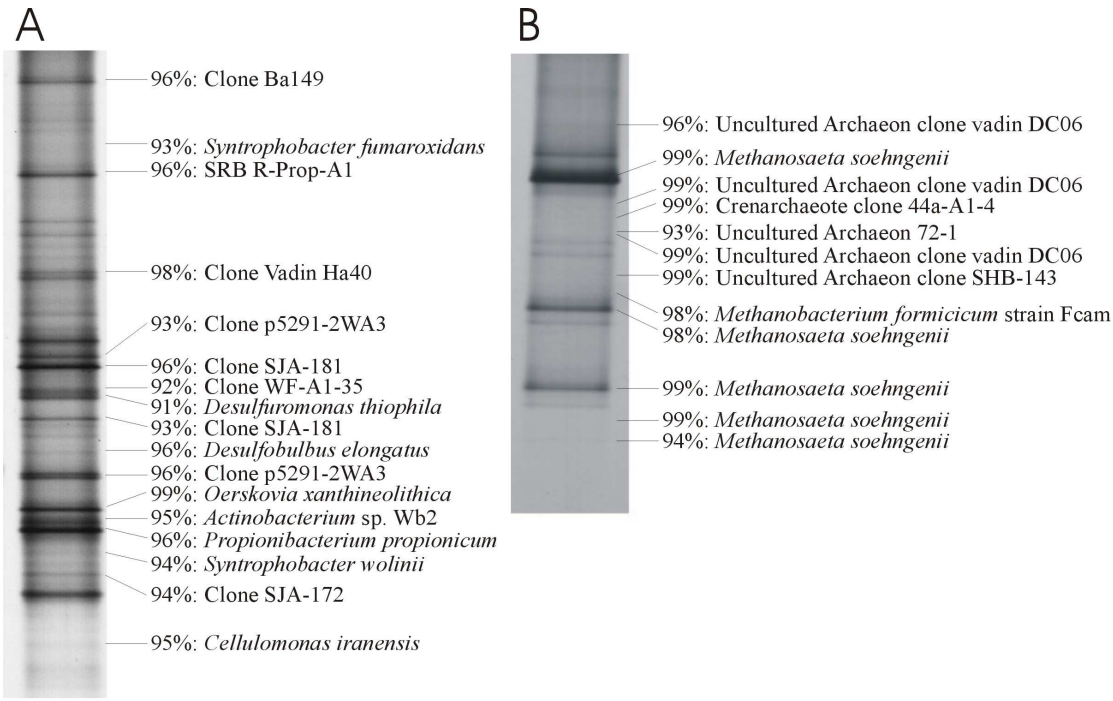


Figure 1: Identification of bands in the bacterial (A) and archaeal (B) DGGE pattern of sludge from the anaerobic wastewater treatment system in Eerbeek, the Netherlands. Percentage values indicate sequence similarity with closest relative present in the database.

Bacterial and archaeal 16S rRNA gene-targeted PCR amplification was performed to generate fragments suitable for DGGE fingerprinting analysis (Bacteria, V6-V8; Archaea, V2-V3). In addition, the bacterial DGGE-PCR amplicons were used for the construction of a clone library and for sequence analysis. Clones were subsequently subjected to DGGE analysis, and the resulting bands were compared with the bacterial fingerprint of Eerbeek sludge yielding the identification of bands observed in the pattern (Fig. 1). Furthermore, nearly full-length bacterial and archaeal 16S rRNA gene fragments were amplified, and used for the construction of clone libraries, and for sequence analysis (Table 1). Representative archaeal clones were subjected to DGGE analysis, and bands were compared with the archaeal DGGE fingerprint of Eerbeek sludge, resulting in the identification of bands observed in this pattern (Fig. 1). Most

archaeal sequences of the Eerbeek sludge were >97% similar to 16S rRNA gene sequences of cultured organisms.

Table 1: Identity of Bacterial (B-clones) (approximately 1.45 kb) and Archaeal (other clones) (approximately 1.3 kb) cloned 16S rRNA gene amplicons retrieved from sludge from the anaerobic wastewater treatment system in Eerbeek, the Netherlands (% = percentage of similarity between cloned 16S rRNA gene and the closest relative in the NCBI database).

| Clone | Closest relative in database | % | **Accession number** |
|---|---|---|---|
| | *Planctomycetes* | | |
| B4 | Uncultured soil bacterium PBS-III-20 | 90 | AY426449 |
| B31 | Uncultured soil bacterium PBS-II-13 | 88 | AY426447 |
| | Uncultured cluster | | |
| B13 | Uncultured eubacterium WCHB1-81 | 94 | AY426438 |
| | *Alpha-Proteobacteria* | | |
| B14 | Unidentified eubacterium clone DA122 | 95 | AY426439 |
| | *Delta-Proteobacteria* | | |
| B16 | Uncultured bacterium SJA-172 | 94 | AY426441 |
| | *Actinobacteria* | | |
| B2 | *Propionibacterium lymphophilum* DSM 4903 | 93 | AY426442 |
| B5 | Uncultured bacterium SJA-181 | 93 | AY426450 |
| B8 | Cellulomonas gelida | 96 | AY426452 |
| B20 | Uncultured thermal soil bacterium clone YNPFFP59 | 89 | AY426443 |
| | *Firmicutes* | | |
| B7 | Uncultured bacterium gene clone:BCf4-07 | 93 | AY426451 |
| B9 | Unidentified eubacterium clone vadinHB04 | 98 | AY426453 |
| B35 | *Desulfitobacterium sp.* Viet-1 | 90 | AY426448 |
| | *Chloroflexi* | | |
| B30 | Uncultured bacterium SJA-61 | 99 | AY426446 |
| B15 | Uncultured bacterium C1-28 | 89 | AY426440 |
| B21 | Uncultured bacterium SHA-105 | 91 | AY426444 |
| B3 | Uncultured bacterium a2b042 | 92 | AY426445 |
| | *Flavobacteria* | | |
| B1 | Benzene mineralising consortium clone SB-5 | 91 | AY426437 |
| | *Crenarchaeota* | | |
| 1A8 | Uncultured archaeon 72-1 | 93 | AY426476 |
| 2B5 | Uncultured crenarchaeote clone 44a-A1-4 | 99 | AY426482 |
| | *Methanobacterales* | | |
| 1C11 | Unidentified archaeon clone vadinDC06 | 99 | AY426478 |
| 1G1 | *Methanobacterium formicicum* strain FCam | 98 | AY426480 |
| 2C2 | Unidentified archaeon clone vadinDC06 | 96 | AY426483 |
| 2C4 | Unidentified archaeon clone vadinDC06 | 99 | AY426484 |
| | *Methanomicrobiaceae* | | |
| 1A3 | *Methanosaeta soehngenii* | 99 | AY426474 |
| 1A7 | *Methanosaeta soehngenii* | 98 | AY426475 |
| 1B7 | *Methanosaeta soehngenii* | 99 | AY426477 |
| 1E4 | *Methanosaeta soehngenii* | 99 | AY426479 |
| 1H10 | *Methanosaeta soehngenii* | 94 | AY426481 |
| 2H1 | Uncultured archaeon clone SHB-143 | 99 | AY426485 |

The majority of prominent bands from the archaeal DGGE profile as well as a major proportion (>40%) of the archaeal clone library matched with sequences from *Methanosaeta* spp. In contrast, the degree of similarity of bacterial clones to known sequences was relatively low (<97%). While the majority (approximately 80%) of the

retrieved bacterial 16S rRNA gene sequences were most similar to those of yet uncultured micro-organisms, some members of the family *Cellulomonadaceae* (*Cellulomonas*, *Oerskovia*) were represented as thick bands in DGGE (Fig. 1). These organisms are capable of hydrolysing cellulose and carbohydrates [Barlows *et al.* 1992]. Other prominent phylotypes belonged to the genus *Propionibacterium*, members of which produce propionate [Barlows *et al.* 1992]. Furthermore, several sequences most closely related to syntrophic short chain fatty acid oxidisers and sulphate reducers were found. Together with the prominent methanogenic Archaea, phylotypes that can be affiliated with all steps along the anaerobic degradation pathway of organic matter to methane have been detected. As rRNA gene sequence databases continue to expand, especially with sequences from cultivated and well-characterized micro-organisms, it will be easier to link the community structure in anaerobic sludge granules more precisely to function [Sekiguchi *et al.* 1998].

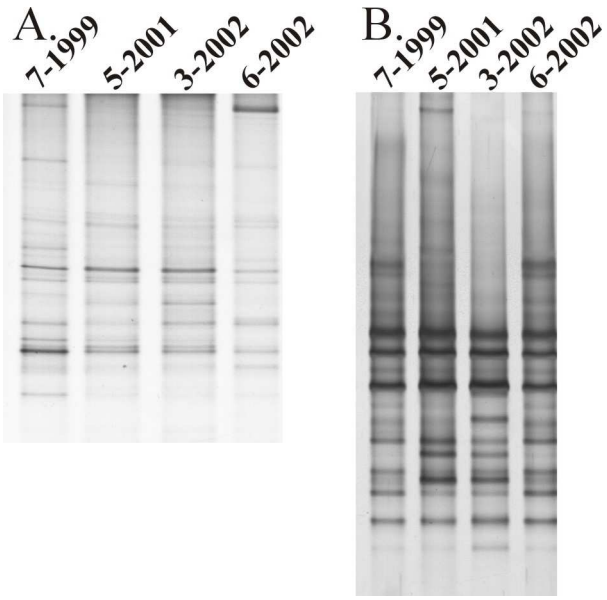


Figure 2: Bacterial (A) and Archaeal (B) DGGE patterns of sludge taken at different dates from the anaerobic wastewater treatment system in Eerbeek, the Netherlands.
The calculated similarity index was 81.9 ± 2.7% for Bacteria and 75.1 ± 3.1% for Archaea, indicating a small temporal microbial variation.

Sludge from the bioreactor in Eerbeek was collected at different time points over the course of three years. After DNA isolation, the PCR-DGGE approach was used to assess the stability of the microbial community in time. DGGE patterns from the different time points were comparable, indicating that the microbial community is not significantly changing in time (Fig. 2). This is reflected by the calculated similarity indices. The bacterial similarity index was 81.9 ± 2.7% and the archaeal similarity index was 75.1 ± 3.1%. These values were in the range of normal temporal community variation, also found in other environments, including the human gastrointestinal tract [Zoetendal *et al.* 2001]. The total microbial community of Eerbeek sludge consists of multiple genera and species that are known or expected to have similar physiological properties, while the observed temporal community stability suggests that a functional equilibrium is reached. This suggests a functional redundancy that can support the elasticity of the mesophilic sludge granule. In interacting consortia, small linear changes in microbial diversity may result in non-linear changes in the conducted processes [Torsvik & Øvreås 2002]. Large number

and diversity of minor populations are likely to contribute significantly to a stable ecosystem function [Fernandez *et al.* 1999].

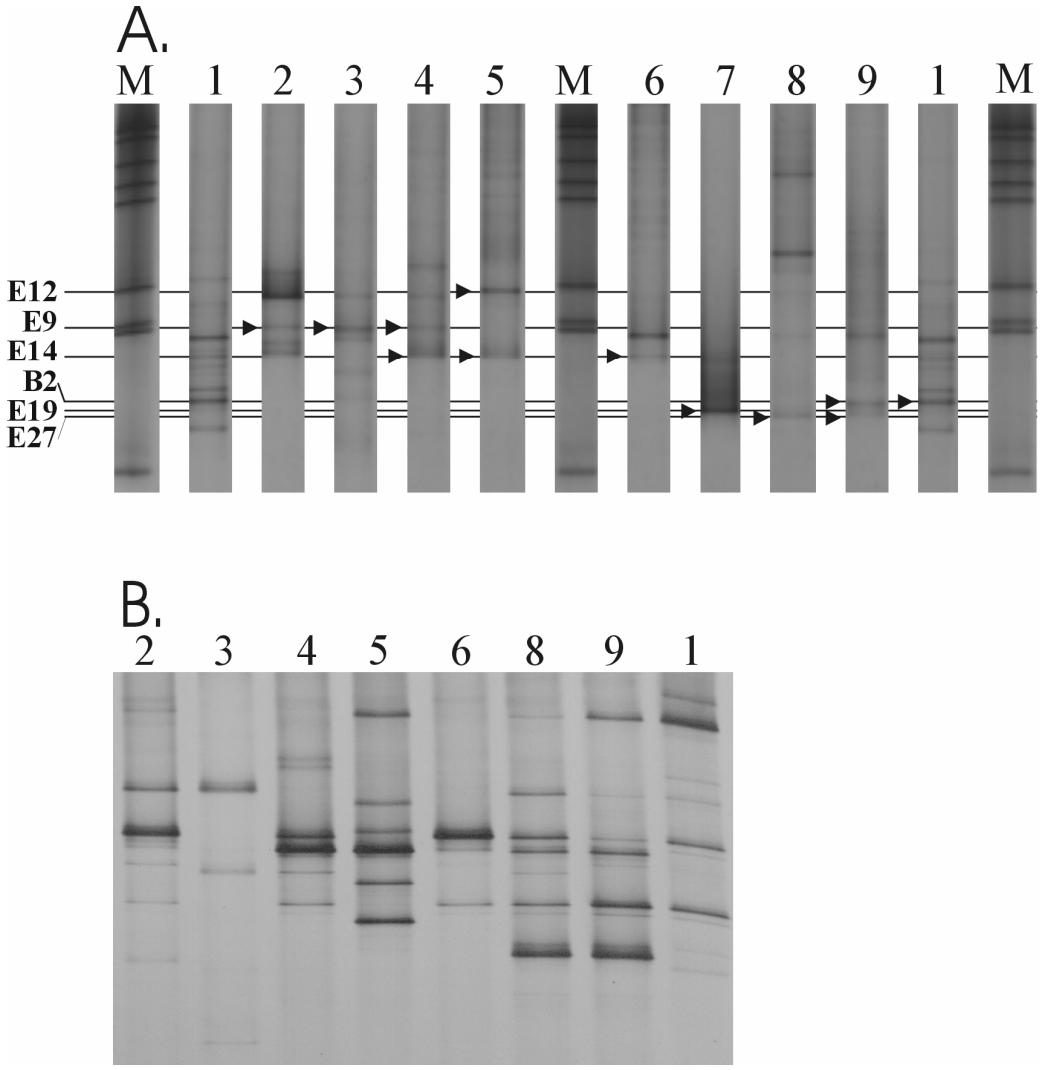


Figure 3: Bacterial (A) and Archaeal (B) DGGE patterns of the enrichments with sludge from the anaerobic wastewater treatment system in Eerbeek, the Netherlands. DGGE patterns from the different enrichments, compared to each other and to the pattern of original Eerbeek sludge. M, marker; 1, total Eerbeek sludge; 2 and 3, butyrate + sulphate enrichment dilution $10^1$ and $10^4$, respectively; 4 and 5, butyrate enrichment dilution $10^1$ and $10^4$, respectively; 6 and 7, propionate + sulphate enrichment dilution $10^1$ and $10^7$, respectively; 8 and 9, propionate enrichment dilution $10^1$ and $10^5$, respectively. Arrows at the left indicate positions of some of the retrieved and sequenced amplicons.

## Analysis of enrichment cultures

Microbial populations were enriched from sludge samples with propionate or butyrate, in the presence or absence of sulphate. This resulted in the following numbers per ml of original sludge: $10^6$ micro-organisms that can utilise propionate, $10^8$ that use propionate and sulphate, and $10^5$ that grow with butyrate with or without sulphate. After several transfers of the enrichment cultures, the lowest and highest dilutions with growth from the four different series were used for DNA isolation and subsequent 16S rRNA gene-targeted PCR-DGGE analysis. Comparison of the resulting DGGE-fingerprints of the original sludge sample and the enrichment cultures indicated that the different substrates resulted in the selective enrichment of distinct microbial populations, but also some common bands were found (Fig. 3). 16S rRNA gene sequences of organisms dominating the enrichment cultures were

determined (Table 2). As indicated in Fig. 3A, clone E9 (92% similarity with uncultured bacterium C1_B004) can be found in the lowest and the highest dilution with growth of the butyrate enrichments with sulphate, as well as in the lowest dilution with butyrate as substrate. Hence, this unknown organism might be involved in the butyrate oxidation. Clone E14 (97% similarity with *Desulfovibrio sulfodismutans*) was retrieved from the butyrate enrichments, but also from the lowest dilution with growth of the propionate and sulphate enrichment. This sulphate-reducing bacterium may have butyrate-oxidising capabilities in syntrophy with methanogens. The propionate enrichments showed a common band (clone E27: uncultured bacterium SJA-172; 94% similarity). The presence of this uncultured bacterium in all propionate dilutions with growth suggests that it might be involved in the oxidation of propionate. Another organism (clone B2: *Propionibacterium lymphophilum*; 93% similarity), present in the highest propionate enrichment dilution with growth seems to be very dominant, since it can also be detected in the total Eerbeek sludge pattern. *Propionibacterium lymphophilum* is normally involved in the production of propionate, but since the sequence similarity is very low (93%), conclusive assignment of this physiological function is hypothetical.

Table 2: Identity of cloned 16S rRNA gene amplicons (approximately 1.45 kb) retrieved from enrichments with sludge from the anaerobic wastewater treatment system in Eerbeek, the Netherlands (% = percentage of similarity between cloned 16S rRNA gene and the closest relative in the NCBI database). Sources of clones E1-E11, butyrate + sulphate enrichment dilution $10^4$; E12-E18, butyrate enrichment dilution $10^4$; E19-E24, propionate + sulphate enrichment dilution $10^7$; E26-E30, propionate enrichment dilution $10^5$, respectively.

| Clone | Closest relative in database | % | **Accession number** |
|---|---|---|---|
| | *Delta-Proteobacteria* | | |
| E2 | Uncultured bacterium Btol | 95 | AY426462 |
| E8 | Uncultured bacterium Btol | 94 | AY426472 |
| E14 | Desulfovibrio sulfodismutans | 97 | AY426458 |
| E19 | *Syntrophobacter fumaroxidans* strain MPOB | 98 | AY426461 |
| E21 | Desulfovibrio alcoholovorans | 99 | AY426464 |
| E23 | *Syntrophobacter fumaroxidans* strain MPOB | 98 | AY426465 |
| E27 | Uncultured bacterium SJA-172 | 94 | AY426467 |
| | *Firmicutes* | | |
| E12 | *Syntrophomonas sp*. MGB-C1 | 96 | AY426456 |
| | *Chloroflexi* | | |
| E6 | Uncultured bacterium tbr1-2 | 91 | AY426471 |
| E9 | Uncultured bacterium C1_B004 | 92 | AY426473 |
| E10 | Uncultured bacterium C1-28 | 97 | AY426455 |
| E15 | Uncultured bacterium SJA-68 | 95 | AY426459 |
| E24 | Uncultured bacterium mle1-42 | 97 | AY426466 |
| | *Spirochaetes* | | |
| E3 | Uncultured bacterium SJA-168 | 99 | AY426468 |
| E5 | Uncultured bacterium SJA-69 | 95 | AY426470 |
| E20 | Uncultured bacterium SJA-182 | 99 | AY426463 |
| E30 | Uncultured bacterium clone C | 95 | AY426469 |
| | *Flavobacteria* | | |
| E1 | Uncultured bacterium clone BA008 | 98 | AY426454 |
| E13 | Uncultured eubacterium WCHB1-69 | 88 | AY426457 |
| E16 | Uncultured bacterium clone BA008 | 99 | AY426460 |

Integrated data from bioreactor- and enrichment culture analysis

In general, 16S rRNA gene sequences obtained from the enrichment cultures showed higher similarity to known sequences, than those retrieved from the original Eerbeek sludge. Sequences retrieved from the original sludge and the derived enrichment cultures were used to construct a phylogenetic tree (Fig. 4). Organisms from several microbial divisions were found, although the dominant sequences from the enrichments clustered mainly in the δ-subdivision of the Proteobacteria. Remarkably, an unidentified cluster, composed of clones from total Eerbeek sludge and butyrate enrichments with and without sulphate, was found deeply branching within the *Chloroflexi*. While there are no cultured close relatives in the NCBI database to these 16S rRNA gene sequences, in most cases closest uncultured relatives have been identified from anaerobic methanogenic ecosystems. Clone B30, for example, has 99% similarity on the 16S rRNA gene level with the uncultured bacterium SJA-61 from a methanogenic trichlorobenzene-degrading bioreactor [Wintzingerode *et al.* 1999]. It is tempting to speculate that the corresponding organisms are directly or indirectly involved in butyrate degradation. Representative clones, like clone E9, were found to be dominant in almost all the butyrate enrichments. Phylogenetic analysis revealed that clone B16, derived from the total Eerbeek sludge, is closely related to clone E27, present in both propionate enrichments and clustering with *Syntrophobacter wolinii* (Fig. 4). This supports the assumption that this organism oxidises propionate.

Molecular fingerprinting, and cloning and sequencing of prevailing 16S rRNA gene types clearly demonstrated that organisms that remained undetectable in the original Eerbeek sludge dominate the enrichment cultures. While DGGE bands corresponding to syntrophic and sulphate reducing bacteria were found in the pattern of the total Eerbeek sludge, they were not detected as the most dominant organisms. This indicates that it is not necessary that organisms involved in syntrophic fatty acid degradation are present in extremely high numbers. Although most of the enriched organisms were not very abundant in the original sludge, they play a crucial role in the degradation pathway of organic compounds and might thus be important for processes during anaerobic wastewater treatment.

In the study presented here, we aimed at the enrichment of propionate- and butyrate-oxidising micro-organisms. Not all prominent organisms in these syntrophic enrichments have been cultivated before. However, the butyrate enrichment was mostly dominated (18 out of 34 clones) by a *Syntrophomonas* species (Fig 3A: clone E12), and the propionate cultures were highly dominated (31 out of 38 clones from propionate + sulphate and 29 out of 34 clones from propionate alone) by the previously isolated *Syntrophobacter fumaroxidans* strain MPOB [Harmsen *et al.* 1998] (Fig 3A: clone E19). Sulphate reducers were present in the cultures with sulphate, but also in the propionate- and butyrate enrichments without sulphate. These organisms can probably combine the properties of syntrophic growth and sulphate reduction.

Concluding remarks

This study showed that the molecular cloning approaches provided insight into the microbial community composition in anaerobic wastewater treatment systems, which is in line with previous data [Godon *et al.* 1997, Sekiguchi *et al.* 1998, Wu *et al.* 2001]. The integrated application of enrichments and molecular techniques, used in this approach, supports existing knowledge of granular sludge structure and function and helps in the understanding of the complicated interactions between the different

trophic groups. Nevertheless, a better understanding of the metabolic processes and ongoing microbial interactions in anaerobic wastewater treatment systems requires a deeper understanding of the physiological properties of key players.

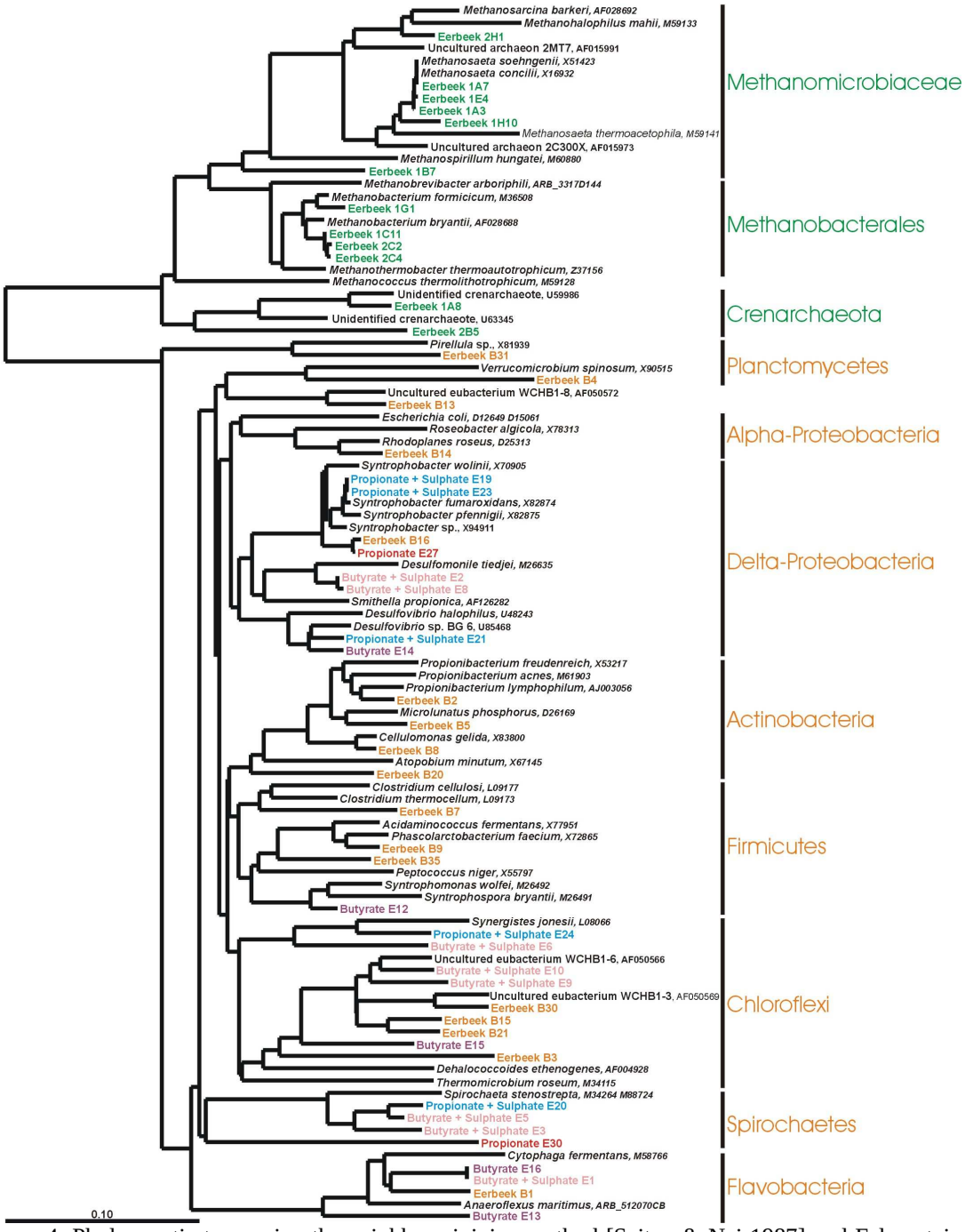


Figure 4: Phylogenetic tree, using the neighbour joining method [Saitou & Nei 1987] and Felsenstein correction (*E. coli* positions 133 to 1086) as implemented in the ARB software package [Ludwig *et al.* 2004], constructed with archaeal and bacterial 16S rRNA gene sequences retrieved from the original sludge (indicated by Eerbeek) as well as enrichment cultures from the anaerobic wastewater treatment system in Eerbeek, the Netherlands.
The following colour codes were used: Green and Orange (Archaea and Bacteria, detected in the original sludge); Pink (butyrate enrichments with sulphate); Purple (butyrate enrichments without sulphate); Blue (propionate enrichments with sulphate); Red (propionate enrichments without sulphate).
Genbank accession numbers are given for reference sequences.

The availability of pure culture isolates will be of crucial importance, as their physiological characterisation will enable the assignment of metabolic function to the sequences in the database. These findings reinforce the notion that combining different molecular techniques and cultivation-based approaches is important for a good understanding of the processes in anaerobic wastewater treatment reactors. Ideally, the link between community structure and function should be determined *in situ*. This is already possible to a limited extend with relatively new techniques such as isotope arrays, microautoradiography and stable-isotope probing [Adamczyk *et al.* 2003, Lee *et al.* 1999, Radajewski *et al.* 2000].
DGGE fingerprinting provides valuable knowledge of dominant phylotypes within complex microbial communities such as those present in anaerobic wastewater treatment systems. The composition of granules seems extremely efficient, but still more information is needed to understand all the processes that take place. Detailed information on the diversity within the different functional groups, however, requires the concerted application with additional approaches, such as selective cultivation methods. With such knowledge, malfunctions may be detected at a very early stage so that process conditions can be changed, and a complete collapse of the anaerobic treatment reactor can be avoided.

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# Chapter 7

## Detection of the microbial diversity in anaerobic wastewater treatment sludge

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## Abstract

A complementary set of molecular and cultivation-based appoaches was used to enhance knowledge about the microbial diversity in an anaerobic paper-mill wastewater treatment system. Most Probable Number series allowed estimating the number of micro-organisms per ml sludge that could use propionate without sulphate ($10^9$), propionate and sulphate ($10^5$), butyrate without sulphate ($10^8$), butyrate with sulphate ($10^5$), glucose ($10^9$) and $H_2/CO_2$ ($10^{10}$). Cloning of 16S rRNA genes, screening of clones with amplified ribosomal RNA gene fragment restriction analysis and partial sequencing revealed that a richness of about 450 species can be expected. From this clone library, with a coverage of about 70%, a selected set of 121 predominant and not closely related 16S rRNA genes was sequenced. Clone sequences were related to a variety of different known species, with expected functions in anaerobic digestion, including fermenters, syntrophic short chain fatty acids oxidisers and sulphate reducing bacteria. However, about 80% of the clones were similar to sequences in the database without close cultured relatives (<97% sequence similarity), but many of these sequences are derived from anaerobic ecosystems as well. It is important to improve the knowledge of such unknown micro-organisms and fast accurate monitoring and identification could be very helpful for that. Therefore, a pilot macro-array was developed and tested. It appeared that combining PCR-made probes from the V1 and V6 variable regions of the 16S rRNA gene could be used for differentiation of relatively closely related organisms. Such tools could prove useful for further research of the microbial diversity and activity in anaerobic ecosystems.

## Introduction

Fast and accurate identification of micro-organisms is required for many purposes, like pathogen detection, taxonomy [Stackebrandt and Goebel 1994] but also for monitoring of biological processes. Conventional detection methods rely on the characterisation of phenotypic traits of pure cultures obtained from specimens after cultivation and isolation of micro-organisms on appropriate laboratory media. These culture-based methods are time-consuming, especially for slow-growing organisms like those that live in syntrophic association with other micro-organisms, and generally restricted to culturable bacteria, which constitute only a small fraction (1-10%) of the global microbial diversity [Amann *et al.* 1995]. At the moment, only about 8200 valid names of cultivated species are described (see for updates http://www.bacterio.cict.fr) [Euzéby 1997]. Up-to-date information regarding the present culturability of micro-organisms from specifically anaerobic sludge is not available, but this culturability might be very well in the mentioned range of 1-10%.

Currently, 16S rRNA gene sequences constitute the largest gene-specific data set, and the number of entries in generally accessible databases is increasing continually (currently about 500,000 sequences of more than 300 bp; www.arb-silva.de), making 16S rRNA gene-based identification of unknown organisms more and more likely [Von Wintzingerode *et al.* 2002]. Therefore, 16S rRNA sequencing is highly suitable for characterisation of the microbial diversity in any given ecosystem of interest. However, despite a new high-throughput pyrosequencing method [Ronaghi *et al.* 1996, Roesch *et al.* 2007], 16S rRNA sequencing is expensive and time-consuming.

Still, it can be regarded a golden standard for the characterisation of microbial communities.

A combination of different approaches, such as selective cultivation methods with molecular detection and identification techniques will give insight into the diversity and characteristics of different functional groups [Roest *et al.* 2005]. Ultimately this combined information will allow the development of an identification DNA micro-array as a rapid molecular biological screening method in the future. DNA micro-arrays are promising for the quantification of microbial genes and therefore highly suited for application in molecular ecology studies [Cho & Tiedje 2002, DeSantis *et al.* 2005, Palmer *et al.*, Wagner *et al.* 2007]. An identification DNA micro-array can function as an early warning system for microbial community changes. Malfunctions of e.g. bioreactors may be detected quickly so that suboptimal process conditions can be changed before the performance deteriorates. It has moreover been shown that direct detection and identification of rRNA from environmental samples is possible without the need of pre-amplification by e.g. PCR, thereby providing an unbiased quantitative insight into the most active populations [Small *et al.* 2001, Koizumi *et al.* 2002, Denef *et al.* 2003, El Fantroussi *et al.* 2003, Peplies *et al.* 2004, Kelly *et al.* 2005].

In the present study, an extended clone library was constructed from anaerobic wastewater treatment sludge. After screening representative clones were completely or partially sequenced. Since it is important to understand more about the functions of micro-organisms and not only detect changes in microbial community composition, a combined application of molecular techniques and cultivation approaches was used to provide additional leads towards the ecophysiology of key populations. Furthermore, the development of a pilot macro-array for the identification of bacteria in wastewater treatment systems is reported.

## Materials and Methods

### Sludge samples

Granular mesophilic sludge samples of a stable full-scale anaerobic paper-mill wastewater treatment system in Eerbeek, The Netherlands, were taken in July 1999, May 2001, March 2002 and June 2002. Details of this treatment plant were given elsewhere [Oude Elferink *et al.* 1998].
In July 2002 also sludge samples from full-scale anaerobic paper-mill wastewater treatment systems in Nieuweschans, Hoogezand and Sappemeer, The Netherlands, were obtained.

### Most probable number (MPN) series

Seven millilitres of the March 2002 Eerbeek sludge were homogenised with a potter tube while flushing with $N_2$-gas. Dilution of the sludge was made in a basal anaerobic bicarbonate-buffered medium without substrates [Stams *et al.* 1993]. From this dilution six MPN (n = 3) series with dilutions from $10^{-5}$ to $10^{-12}$ were started with 20 mM propionate or butyrate with and without 20 mM sulphate; 20 mM glucose; and 1.7 Pa headspace pressure $H_2/CO_2$. The cultures with only propionate and butyrate were pre-grown with *Methanospirillum hungatei* to enhance growth of the syntrophic fatty acid-degrading organisms.

Molecular characterisation

DNA was isolated, based on mechanical disruption by bead beating and phenol/chloroform/iso-amyl-alcohol extraction, from sludge and enrichments according to the method of Oude Elferink [Oude Elferink *et al.* 1997]. Bacterial 16S rRNA gene amplification was done as described previously [Roest *et al.* 2005]. Amplicons were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and cloned in *E. coli* JM109 (Invitrogen, Breda, The Netherlands) by using the TOPO vector for sequencing (Invitrogen) with ampicillin selection and black/white screening, according to the manufacturer's manual. Colonies were picked and grown in LB-medium with ampicillin in deep 96 well plates (Corning, Schiphol-Rijk, The Netherlands). Plates were covered with a gas-permeable adhesive seal (Abgene, Epsom, UK) and incubated at 37°C overnight (o/n). Five micro-litres of o/n culture were diluted in 45 micro-litres of TE buffer, and lysed by incubation at 95°C for 10 minutes. One micro-litre lysate was used directly as template for PCR with primers targeting the T7 and T3 promoters on the TOPO vector. Amplicons were digested simultaneously with restriction enzymes MspI, CfoI, and AluI (Invitrogen). Fragments were separated in 4% low range ultra agarose (BioRad, Hercules, CA) on a SUNRISE Horizontal Electrophoresis System (Gibco BRL), containing 52 wells per row facilitating fingerprint differentiation.

Sequencing and phylogenetic analysis

Selected clones were partially (approx. 500 bp) sequenced (Westburg Genomics, Wageningen, The Netherlands). Similarity searches of partial 16S rRNA sequences derived from clones against sequences deposited in publicly accessible databases were performed using the NCBI Blast search tool at http://www.ncbi.nlm.nih.gov/BLAST/. Alignment and phylogenetic tree construction was performed with the ARB program package [Ludwig *et al.* 2004] as described before [Roest *et al.* 2005]. The obtained neighbour joining tree was used for the selection of 121 representative clones, of which nearly complete 16S rRNA gene sequences were obtained (Westburg Genomics). These 121 nucleotide sequences have been deposited in GenBank (accession no. EF688145 to EF688265). Sequences were aligned and subjected to phylogenetic analysis as described above, using the SILVA 90 database release (March 2007, www.arb-silva.de). Phylogenetic trees were constructed with Felsenstein correction and the bacterial filter in ARB, using parsimony procedures as implemented in ARB (*E. coli* positions 194 to 1172) [Saitou *et al.* 1987]. Rarefaction curves were generated at http://www.aslo.org/lomethods/free/2004/0114a.html [Kemp and Aller 2004b].

Probe generation

Probes were generated by PCR with primers 64F and 104R [Bertilson *et al.* 2002], and 985F and 1046R [Heuer *et al.* 1999], for the 16S rRNA V1 and V6 regions, respectively. Products were purified with a Nucleotide Removal Kit (Qiagen), and the concentration was measured fluorometrically using PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, USA), according to the manufacturers' protocols.

Validation of pilot DNA macro-array with reverse hybridisation

Previous sequencing resulted in 37 clones containing different, but also closely related 16S rRNA gene fragments (accession no. AY426437 to AY426473) [Roest *et al.* 2005]. After an insert PCR with T7 and Sp6 primers, 10 µl (≈ 200 ng) was denatured

by addition of 21 µl formamide, 7 µl formaldehyde and 2 µl 20xSSC, and subsequent 10 minutes incubation at 68°C. DNA was kept single stranded by placing on ice and addition of 40 µl 20xSSC. This DNA was spotted on Natran SuperCharge filters (Schleicher & Schuell, Dassel, Germany), and a group of 10 clones was selected for probe generation by PCR as described. Pooled probes, with a total amount of 25 ng, were denatured by heating at 95°C for 10 minutes. Single-stranded products were labelled radioactively with [$\alpha$-$^{32}$P] dCTP using the Prime-a-Gene kit (Promega, Madison, WI), according to the manufacturer's instructions.
Blots were pre-hybridised for 30 minutes in 100 ml tubes containing 10 ml of QuickHyb™ hybridisation buffer (Stratagene, LaJolla, CA). Overnight hybridisation at 68°C was done in a ThermoHybaid hybridisation oven (Thermo Life Sciences, Basingstoke, United Kingdom). Blots were washed twice for 15 minutes at room temperature in 100 ml 2xSSC; 0.1% SDS, after which the washing buffer was replaced by 100 ml 0.1xSSC; 0.1% SDS for 30 minutes stringent washing at 68°C. Blots were exposed overnight in a Storage Phosphor Screen (Amersham BioSciences, Little Chalfont, Buckinghamshire, United Kingdom), and radiation was visualised using the STORM imager (Amersham BioSciences).

## Results and Discussion

To provide a comprehensive ecophysiological and phylogenetic framework for the design of diversity DNA arrays targeting anaerobic sludge communities, the microbiota of a full-scale anaerobic paper-mill wastewater treatment system was characterised by MPN cultivation and 16S rRNA gene library analysis.

### MPN experiments

MPN series allowed estimating the number of micro-organisms per ml sludge that could use propionate without sulphate ($10^9$), propionate and sulphate ($10^5$), butyrate without sulphate ($10^8$), butyrate with sulphate ($10^5$), glucose ($10^9$) and $H_2/CO_2$ ($10^{10}$). Interestingly, these values differ from previously obtained results [Roest *et al.* 2005], as this time more propionate and butyrate oxidisers were detected. This suggested that the abundance of specific functional groups changed, since in the sample investigated in this study, growth to lower dilutions was found for incubations in the presence of propionate and sulphate compared to a sample taken almost three years earlier [Roest *et al.* 2005]. In line with the data reported here, Oude Elferink and co-authors found in general slightly higher cell numbers per gram of VSS several years before [Oude Elferink *et al.* 1998]. It can however, not be excluded, that the higher numbers found here may be at least partly due to better anaerobic conditions during sludge handling and improved pre-growth of *Methanospirillum hungatei*, but also to better sludge homogenisation in a potter tube.

### Diversity analysis by RFLP

To more comprehensively explore microbial composition and diversity of the sludge, a 16S rRNA gene clone library was constructed and initially analysed by RFLP profiling. This indicated a very diverse community, with 383 different RFLP profiles out of 459 clones analysed. To evaluate to what extend a clone library sufficiently represents the full diversity of a given environmental sample, phylotype-richness estimates can be used [Kemp and Aller 2004b]. Here, two such estimators ($S_{ACE}$ and $S_{Chao1}$) were used to estimate richness of the library, providing an impression of the

total number of phylotypes present in the wastewater treatment system (Fig. 1a, b). Subsequently, the richness estimators were used to evaluate the coverage of the clone library of the 'true' diversity (Fig. 1c, d). The coverage is an estimator of the proportion of phylotypes in a library of infinite size that would be represented in a smaller library [Kemp and Aller 2004a]. Clone library coverage did not yet reach asymptotic values according to RFLP analysis. However, considerable variation can be found within individual genomes due to multiple rRNA gene heterogeneity, as well as sub-species level micro-diversity [Heldtander Köningsson *et al*. 2002], indicating that the asymptotic value will be an overestimation of the total number of species-level Operational Taxonomic Units (OTUs). Practical limitations for RFLP profile discrimination also exist. Even though about 50 profiles were compared next to each other, profiles on different rows of the agarose gel, or on different gels, are difficult to compare, also when appropriate image analysis software is used. Therefore, in the case of doubtful RFLP similarities, all the clones were chosen for partial sequence analysis, probably leading to an overestimation of the actual number of OTUs present in the Eerbeek paper mill wastewater treatment system.

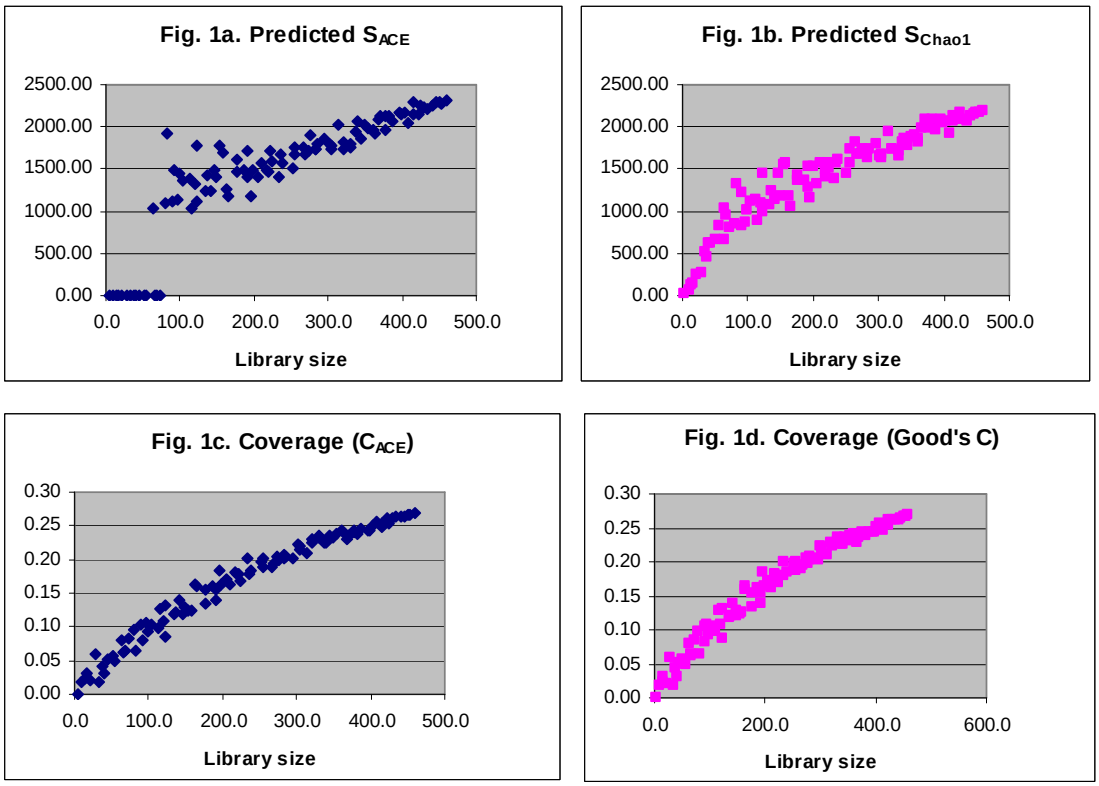



Figure 1: Initial phylotype-richness ($S_{ACE}$ and $S_{Chao1}$) and coverage estimators of bacterial 16S rRNA gene clone library from Eerbeek paper mill wastewater treatment system sludge.

## Diversity analysis by sequencing

Sequence data can give detailed information about the microbial diversity of a sample. Therefore 375 clones were partially sequenced. A wide variety of bacterial phylotypes was identified in the sludge from the Eerbeek paper mill wastewater treatment system. The fact that approximately 80% of the clones were most closely related to sequences of not yet cultured micro-organisms, including some with sequence similarities of as little as about 85%, reinforced the need for increasing efforts towards cultivation and characterisation. Nevertheless, matches with sequences

derived from several anaerobic ecosystems are informative as well and possible functions can be proposed. Some clones represented more pre-dominant micro-organisms, including sequences most closely related to known fermentative micro-organisms belonging to the genera *Cellulomonas*, *Clostridia*, *Oerskovia*, and *Cytophaga*, with the latter mentioned genus known for degradation of biomacromolecules [Cottrell and Kirchman 2000]. Also uncultured *Actinobacteria* were detected. Overall, these genera accounted for approx. half of all ribotypes analysed.

Table 1: Overview of results from clone library-based surveys of sludges from different anaerobic wastewater treatment reactors.

| Reactor | *AlphaProteobacteria* | *BetaProteobacteria* | *GammaProteobacteriaa* | *DeltaProteobacteria* | *Cytophaga-Flexibacter-Bacteroidetes* | *Firmicutes* | *Actinobacteria* | *Planctomyces-Chlamydia* | GNS / *Chloroflexi* | *Spirochaetes* | *Synergistes* | *Thermotogales* | *Thermodesulfovibrio* | Green sulphur bacteria | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fluidised-bed fed by vinasses[a] | 17% | | | | 16% | 46% | 4% | 3% | 5% | 5% | 3% | ND | ND | ND | [Godon *et al.* 1997] |
| Mesophilic sucrose/ propionate/acetate fed[b] | ND | ND | ND | 27% | 8% | 7% | 4% | 10% | 14% | 3% | 1% | ND | 5% | 2% | [Sekiguchi *et al.* 1998] |
| Thermophilic fed with sucrose/ propionate/acetate[b] | ND | ND | ND | ND | ND | 16% | ND | 13% | 18% | ND | 5% | ND | 19% | 7% | [Sekiguchi *et al.* 1998] |
| Mesophilic terephthalate[c] | ND | ND | ND | 78.5% | ND | ND | ND | ND | 7.5% | ND | 0.9% | ND | ND | ND | [Wu *et al.* 2001] |
| Mesophilic citric acid[d] | ND | 7% | ND | 24% | 12% | 57% | | ND | ND | ND | ND | ND | ND | ND | [Collins *et al.* 2003] |
| Mesophilic municipal digester[e] | 4.8% | 5.8% | 1% | 9.2% | 38.8% | 26.2% | 1.5% | ND | 5.4% | ND | 4.4% | 2.4% | ND | ND | [Chouari *et al.* 2005] |
| Mesophilic domestic excess sludge digester[b] | 4% | 7% | 6% | 7% | 21% | 21% | 6% | ND | 14% | 5% | ND | ND | ND | ND | [Ariesyady *et al.* 2007] |
| Mesophilic UASB fed with paper-mill wastewater[d] | ND | ND | 0.5% | 16% | 13% | 16% | 39% | ND | 11% | 2% | ND | ND | ND | ND | This study |

a. Percentages OTU's (> 96% sequence similarity within an OTU)
b. Clone percentages
c. Percentages of clones with a unique electrophoretic position on DGGE
d. Clone percentages screened with ARDRA
e. Percentages OTU's (> 97% sequence similarity within an OTU)
ND. Not detected

As in many anaerobic systems [Sekiguchi *et al.* 1998, Wu *et al.* 2001], the group of green non-sulphur bacteria was abundantly present (approx. 11%). Also, the propionate producing *Propionibacterium* genus, and especially *P. propionicus* was found quite often (7%). Even though the Eerbeek UASB wastewater treatment system is methanogenic, some sulphate is reduced to sulphide [Paasschens *et al.* 1991, Oude Elferink *et al.* 1998]. This is also reflected in the microbial community, because about 12% of the bacteria were affiliated with known sulphate reducers. Approximately 40% of these sequences were most closely related to *Syntrophobacter*, and together with other syntrophic bacteria affiliated with *Desulfotomaculum*, *Syntrophothermus*, and *Syntrophus*, this group of syntrophic key-organisms accounted for about 7% of total clones. As in a previous study [Roest *et al.* 2005], the clone library showed a wide variety of species that were present in the sludge from the UASB reactor under investigation. However, the current clone library is significantly larger and provided as such much better insight with respect to relative abundance of detected phylotypes. This also allowed for a comparison of data obtained in this study on relative abundance of microbial groups with several other molecular inventories of anaerobic wastewater treatment systems, showing relative good overall agreement for most bacterial groups (Table 1). However, it is interesting to note that the phylum of *Actinobacteria* (High-GC Gram-positives), are clearly much more abundant in this UASB bioreactor. The above mentioned genera *Cellulomonas*, and *Oerskovia* cluster all within the *Actinobacteria*. This gives an indication of the importance of macromolecule breakdown in the paper-mill wastewater treatment process.

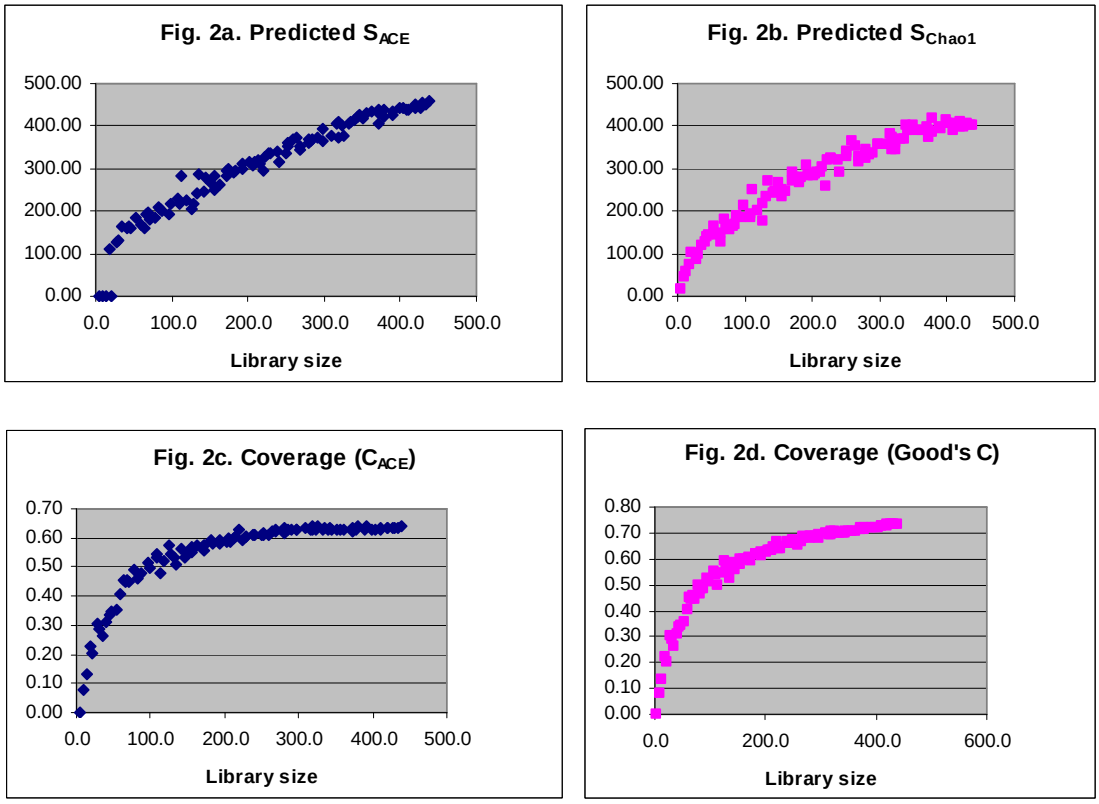
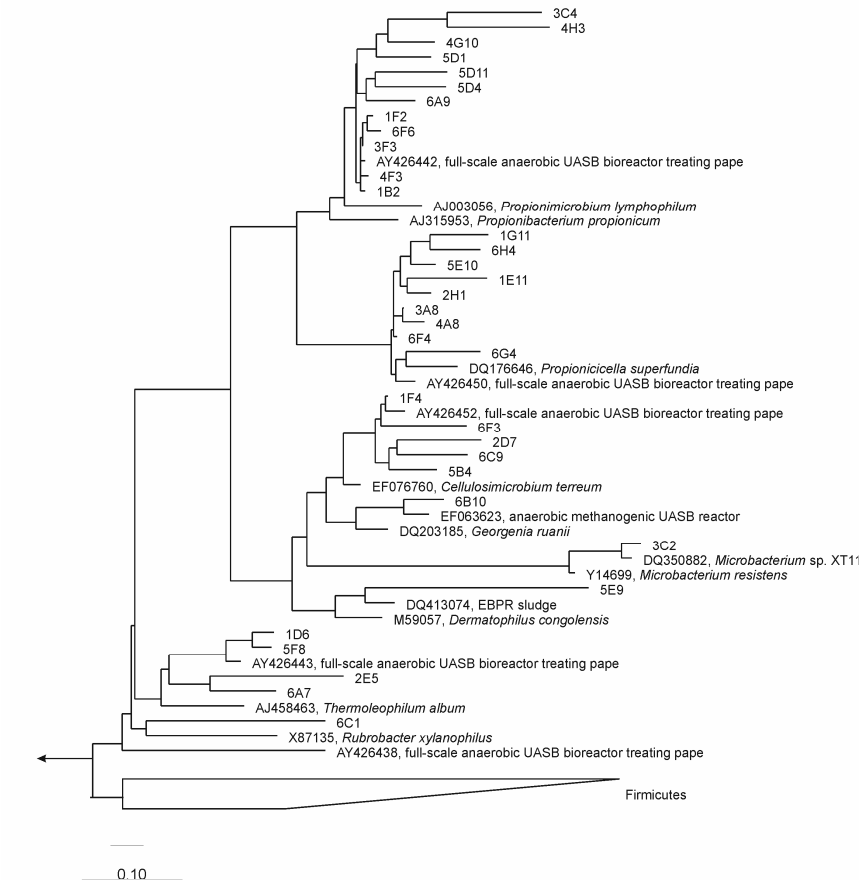
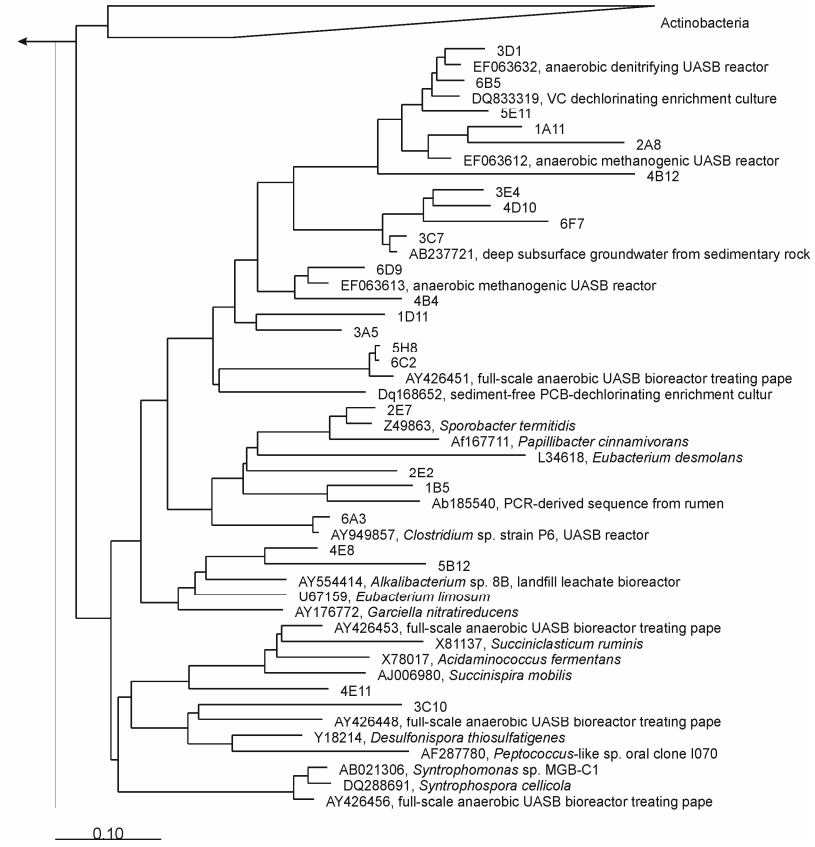



Figure 2: Phylotype-richness ($S_{ACE}$ and $S_{Chao1}$) and coverage estimators of bacterial 16S rRNA gene clone library from Eerbeek paper mill wastewater treatment system sludge after partial sequencing.

A. Phylogenetic tree of nearly full length 16S rRNA actinobacterial gene sequences from Eerbeek.




B. Phylogenetic tree of nearly full length 16S rRNA gene sequences within firmicutes from Eerbeek.



C. Phylogenetic tree of nearly full length 16S rRNA gene sequences of Proteobacteria from Eerbeek.

D. Phylogenetic tree of other nearly full length 16S rRNA bacterial gene sequences from Eerbeek.

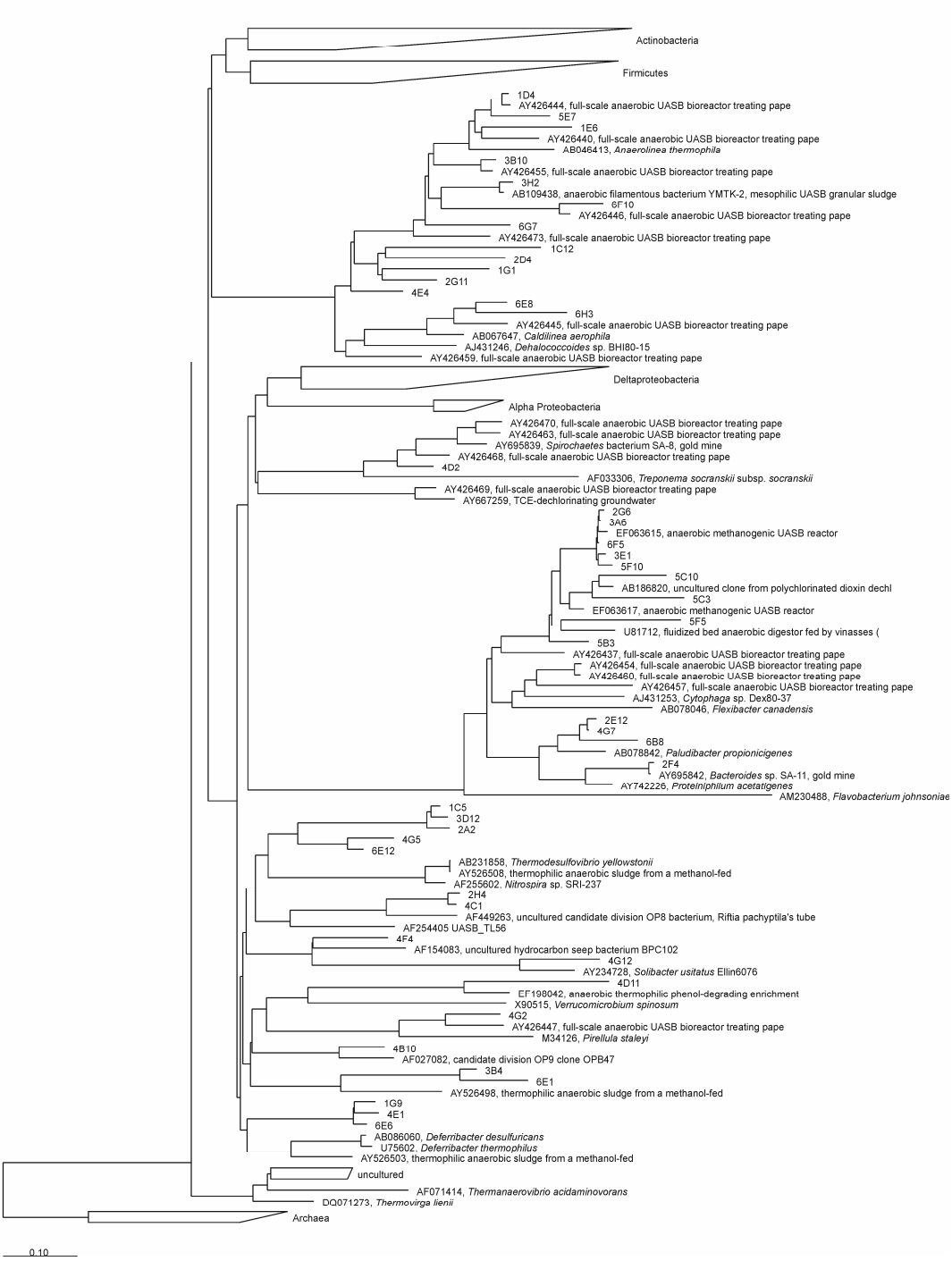



Figure 3: Phylogenetic trees with 121 nearly full bacterial 16S rRNA gene sequences from Eerbeek. Phylogenetic trees were constructed with Felsenstein correction and the bacterial filter in ARB, using the neighbour joining method (*E. coli* positions 409 to 1364) [Saitou *et al.* 1987].

Partial 16S rRNA gene sequences that gave comparable hits in the GenBank database were grouped into OTUs, and rarefaction curves were generated again. This resulted in a significantly lower diversity estimate (Fig. 2). Both the $S_{ACE}$ and $S_{Chao1}$ richness estimators indicated that there were about 450 species in the Eerbeek wastewater treatment sludge present (Fig. 2a, b) and that the clone library had a reasonable coverage of approximately 70% (Fig. 2c, d).

A representative selection of clones from the different phylogenetic groups was nearly completely sequenced and phylogenetic trees were constructed with these 121 sequences (Fig. 3). Since a lot of *Actinabacteria* (Fig. 3a), *Firmicutes* (Fig. 3b) and *Proteobacteria* (Fig. 3c) related sequences were found, separate phylogenetic trees were produced, as well as a phylogenetic tree with other sequences (Fig. 3d). Overall results did not change significantly compared to the grouped partially sequenced clones, but complete and reliable sequence data is pivotal for downstream applications, e.g. for the development of an identification DNA array.

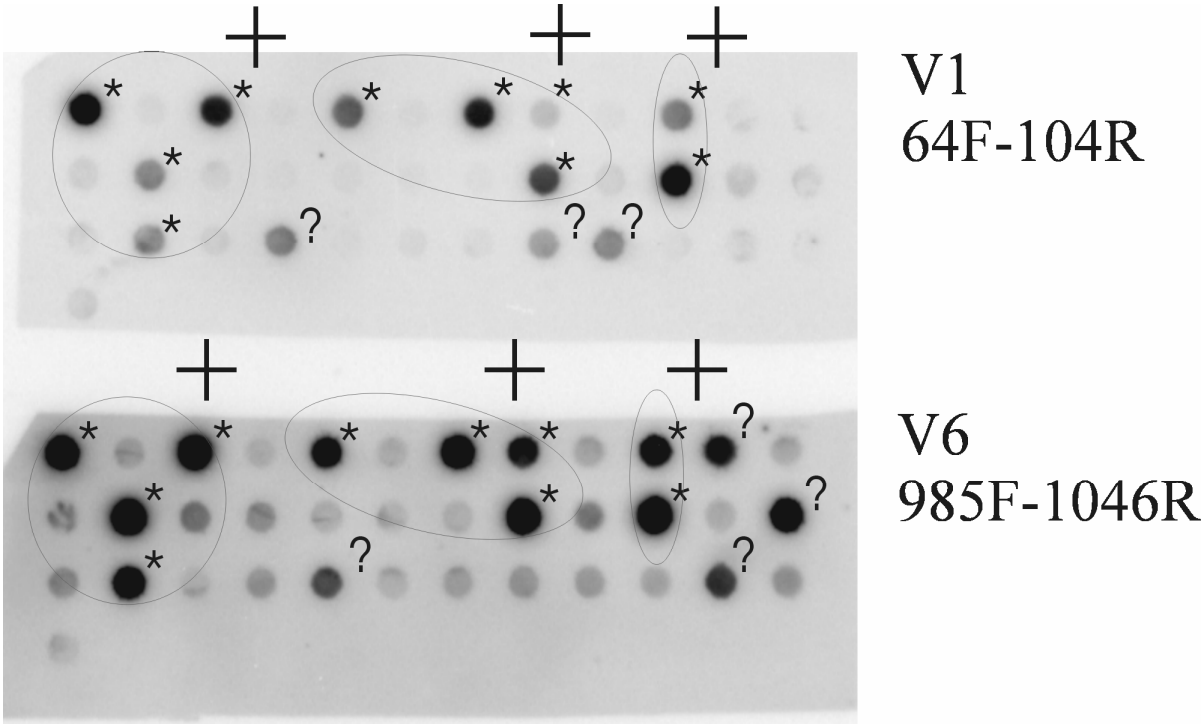



Figure 4: Validation of macro-array with reverse hybridization.

DNA array approach

DNA micro-arrays (also called DNA chips, gene chips or biochips) typically consist of a large number of immobilised DNA fragments (PCR product, oligonucleotides or other DNA fragments) present on a surface, such as a coated glass slide or a membrane [Ye *et al.* 2001]. DNA-arrays can be seen as reverse traditional dot-blots, since the identity of the spotted probes is known and the sample is labelled. Labelled sample nucleic acids will mark the exact positions of those probes on the array where hybridisation occurred. This results in a list of hybridisation events, indicating the presence or the relative abundance of specific DNA or RNA sequences present in the sample. Micro-arrays are already widely used for the detection of transcriptional profiles (expression arrays) or the similarities and differences of genetic contents among different micro-organisms, and they can be used to subtype (fingerprint strains relative to the reference strain) bacterial isolates and for the identification of new diagnostic genetic markers [Call *et al.* 2003]. Furthermore, the search of polymorphisms and mutation detection can be done with micro-arrays. The detection and identification of high numbers of different microbes, especially from complex microbial communities in environmental samples with DNA-arrays, has become increasingly popular during the past 5 years, exemplified by the development and application of generic as well as ecosystem-specific phylogenetic array platforms [Wagner *et al.* 2007]. Here a previously studied clone library [Roest *et al.* 2005] was

used for pilot macro-array experiments. First, the 16S rRNA gene inserts of 37 clones were spotted on a nylon membrane. Subsequently, 10 of these clones, some very closely related to each other (identical V1 or V6 region), were mixed as an artificial sample community. The V1 and V6 regions of the 16S rRNA genes were specifically amplified, labelled and hybridised with the macro-array (Fig. 4). Hybridisation of both variable regions resulted in more than 10 positive events each. However, when these were combined, only the 10 used clones hybridised with both the V1 and V6 regions, yielding confidential and accurate identification of the populations represented in this model community. Fast and accurate differentiation between relatively closely related micro-organisms could prove useful for further research of the microbial diversity and activity in anaerobic ecosystems. Extensive clone libraries, as generated in this study, can facilitate the generation of high-density micro-arrays. Eventually, such high-density identification micro-arrays can be used routinely in anaerobic wastewater treatment operation, monitoring the microbial community dynamics for near online control.

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# Summary and concluding remarks

Anaerobic wastewater treatment has gained popularity and is now one of the key technologies in environmental biotechnology. It is considered the most cost-effective solution for organically polluted industrial waste streams [van Lier *et al.* 2001], and has gained interest due to increasing energy prices and more stringent legislation for the discharge of industrial wastewater, since the 1970's [Lettinga *et al.* 1995]. Nevertheless, while system design has improved and efficient conversions are achieved, fundamental background knowledge on the ruling biological processes is often lacking. **Chapter 1** gives an overview of current knowledge of microbial ecophysiology in anaerobic wastewater treatment systems and focuses mainly on cultivation-independent molecular techniques that can be used to improve understanding of microbial community structure – function relationships that govern wastewater treatment processes. An overview of different applications of anaerobic wastewater treatments was given, followed by a description of tools and techniques for the study of micro-organisms in such ecosystems, including culture-dependent and molecular approaches. Advantages and disadvantages of the various approaches were discussed. A summary of known cultivated key micro-organisms in anaerobic wastewater treatment plants was given, followed by a detailed description of published molecular inventories of the microbial diversity in such systems. With cultivation-independent studies many unknown micro-organisms have been detected. Meta-analysis of these studies revealed that anaerobic wastewater treatment systems are in general dominated by members of the low-GC Gram-positives, the *Bacteroidetes*, and *Delta-Proteobacteria*. *Methanosarcinales* (mainly *Methanosaeta*) dominate usually the archaeal community, but also *Methanobacteriales* and unexpected *Crenarchaeota* have been found regularly in anaerobic wastewater treatment systems. **Chapter 1** concluded with an outlook on emerging integrated approaches, stressing the need for concerted applications of innovative culture-dependent as well as culture-independent techniques. The knowledge on structure and functionality of bioreactor microbiota that can be expected from such novel strategies will be pivotal to a better understanding and predictability of anaerobic wastewater treatment processes and reactor performance.

Population dynamics of anaerobic sludge subjected to batch incubations at different temperatures with different feed mixtures were characterised as described in **chapter 2**. The biochemical results of the incubations were compared with the microbial community shifts using DGGE fingerprinting and subsequent cloning library analysis. This revealed that the bacterial community composition changed from the mesophilic (37°C) to the thermophilic stages (45°C), whereas, the archaeal community showed significant differences between temperature incubations at 45 and 55°C. A total of 324 bacterial clones retrieved from all incubations belonged mainly to two different phyla, *Firmicutes* (68% of the clones) and *Delta-Proteobacteria* (17% of the clones). Monitoring and evaluation of the bacterial population shifts during the incubation at each temperature and feed mixture demonstrated stable community profiles, which might suggest that all detectable members of the community contribute to the degradation of the compounds present in the different incubations, or alternatively that nucleic acids used for the generation of profiles are not sensitive enough to reflect the population changes. The use of RNA instead of DNA might increase this sensitivity, because RNA reflects the activity of microbes better. Instead of feeding single carbon sources individually, thus introducing a bias in favour of the members

of the mixed populations that could utilize that particular carbon source, the mixture of butyrate, propionate and acetate incubated at 37°C demonstrated a comunity shift during the incubation. More specifically, two populations most closely related to a *Syntrophomonas* sp. and *Desulfobulbus propionicus*, respectively, were enriched, suggesting their involvement in the syntrophic degradation of butyrate and propionate in the absence of sulphate, respectively. The presence of *D. propionicus* is actually remarkable, because it is known to degrade propionate only with sulphate. As proposed for *Desulfovibrio* species [Bryant *et al.* 1977], it also might be that *D. propionicus* grows as acetogen in syntrophic association with methanogens. This study indicated that biochemical results compared with the microbial community shifts observed by DGGE profiles could be the way of linking the identity to function of the mixed cultures, especially for the communities predominantly degrading the substrates sequentially.

In **chapter 3** micro-organisms involved in direct and indirect methane formation from methanol in a lab-scale thermophilic (55°C) methanogenic bioreactor were investigated. Reactor sludge was disrupted and serial dilutions were incubated in specific growth media containing methanol and possible intermediates of methanol degradation as substrates. With methanol growth was found till a dilution of $10^8$. However, when *Methanothermobacter thermoautotrophicus* strain Z245 was added for $H_2$ removal, growth was observed up to a $10^{10}$-fold dilution. With $H_2/CO_2$ and acetate, growth was observed till dilutions of $10^9$ and $10^4$, respectively. Dominant micro-organisms in the different dilutions were identified by 16S rRNA-gene diversity and sequence analysis. Furthermore, dilution PCR revealed a similar relative abundance of *Archaea* and *Bacteria* in all investigated samples, except the enrichment with acetate, which contained 100 times less archaeal DNA than bacterial DNA. The most abundant bacteria in the culture with methanol and strain Z245 were most closely related to *Moorella glycerini*. *Moorella mulderi*, a thermophilic homoacetogen, was isolated from a thermophilic bioreactor with methanol as energy source [Balk *et al.* 2003]. *Thermodesulfovibrio* relatives were found with high sequence similarity in the $H_2/CO_2$ enrichment, but also in the original lab-scale bioreactor sludge. SRB have been found more often as hydrogen-utilising *Bacteria* in methanol degradation, although in the presence of sulphate [Balk *et al.* 2007]. It has been proposed that *Desulfovibrio* species grow as acetogens in syntrophic association with methanogens [Bryant *et al.* 1977]. In the $H_2/CO_2$ enrichment *Methanothermobacter thermoautotrophicus* strains were the most abundant hydrogenotrophic archaea. The dominant methanol-utilising methanogen, which was present in the $10^8$-dilution, was most closely related to *Methanomethylovorans hollandica*. Recently a *Methanomethylovorans thermophila* was isolated from the same reactor [Jiang *et al.* 2005]. Compared to direct methanogenesis, results of this study indicate that syntrophic, interspecies hydrogen transfer-dependent methanol conversion is equally important in the thermophilic bioreactor, confirming previous findings with labelled substrates and specific inhibitors [Paulo *et al.* 2003].

In **chapter 4** the microbial diversity of *Bacteria* and *Archaea* was evaluated in two expanded granular sludge bed (EGSB) reactors fed with increasing oleic acid loading rates up to 8 kg of chemical oxygen demand (COD) $m^{-3}$ $day^{-1}$ as the sole carbon source. One of the reactors was inoculated with granular sludge (RI) and the other with suspended sludge (RII) [Pereira *et al.* 2002]. During operation, the sludge in both reactors was segregated in two layers: a bottom settled one and a top floating one. The

composition of the bacterial community, based on 16S rRNA gene sequence diversity, was affected most during the oleate loading process in the two reactors. The archaeal consortium remained rather stable over operation in RI, whereas in RII the relative abundance of *Methanosaeta*-like organisms gradually decreased, starting in the bottom layer. In the range of oleate loads evaluated, 6 kg of COD $m^{-3}$ $day^{-1}$ was found as the maximum value that could be applied to the system. A further increase to 8 kg of oleate-COD $m^{-3}$ $day^{-1}$ induced a maximal shift on the microbial structure of the sludges. At this time point, methanogenic acetoclastic activity was not detected and only very low methanogenic activity on $H_2/CO_2$ was exhibited by the sludges. In a recent study a low methane yield was observed in oleate enrichments and acetate was not mineralized [Sousa *et al.* 2007a]. Another study by the same authors showed an increase of methanogens after prolonged contact of the microbial biomass with long-chain fatty acids (LCFAs) [Sousa *et al.* 2007b]. A previous study by Pereira *et al.* (2003) also showed improved methanogenesis after extended incubation with LCFAs.

The start-up of a full-scale synthesis gas fed gas-lift reactor treating metal and sulphate rich wastewater was investigated and presented in **chapter 5**. 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 at full scale metal precipitation and sulphate reduction occurred in the same reactor. After 7 weeks the full-scale reactor achieved a sulphate conversion design rate of 15 $kg \cdot m^{-3} \cdot day^{-1}$. Zinc sulphide precipitation inside the reactor did not interfere with obtaining a high rate of sulphate reduction. 16S rRNA gene analysis demonstrated that the bacterial communities in both reactors were dominated by the sulphate-reducing genus *Desulfomicrobium*. Archaeal communities of both reactors were dominated by the methanogenic genus *Methanobacterium.* MPN counts confirmed that heterotrophic sulphate-reducing bacteria (SRB) 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 SRB, 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. Not only SRB and methanogens, but even different SRB with similar physiology can co-exist in the same reactor [Dar *et al.* 2007]. This shows that microbial pathways in bioreactors are in parallel rather then in series, contributing to stable ecosystem performance, as described before by Fernandez *et al.* (1999).

To get insight into the microbial community of a full-scale Upflow Anaerobic Sludge Blanket reactor treating paper mill wastewater, conventional microbiological methods were combined with 16S rRNA gene analyses, as described in **chapter 6**. Particular attention was paid to micro-organisms able to degrade propionate or butyrate in the presence or absence of sulphate. Serial enrichment dilutions allowed estimating the number of micro-organisms per ml sludge that could use butyrate with or without sulphate ($10^5$), propionate without sulphate ($10^6$), or propionate and sulphate ($10^8$). Quantitative RNA dot-blot hybridisation indicated that *Archaea* were two-times more abundant in the microbial community of anaerobic sludge than *Bacteria*. The microbial community composition was further characterised by 16S rRNA-gene-targeted DGGE fingerprinting, and via cloning and sequencing of dominant amplicons from the bacterial and archaeal patterns. Most of the nearly full length

(approximately 1.45 kb) bacterial 16S rRNA gene sequences showed less than 97% similarity to sequences present in public databases, in contrast to the archaeal clones (approximately. 1.3 kb) that were highly similar to known sequences. While *Methanosaeta* was found as the most abundant genus, also *Crenarchaeota*-relatives were identified. *Crenarchaeota* have been detected in high numbers and in close proximity of methanogenic *Archaea* in anaerobic bioreactors [Collins *et al.* 2005] and linked to acetate consumption [Collins *et al.* 2006]. The microbial community was relatively stable over a period of 3 years as indicated by the high similarity index calculated from DGGE profiles (81.9 ± 2.7% for Bacteria and 75.1 ± 3.1% for Archaea). 16S rRNA gene sequence analysis indicated the presence of unknown and yet uncultured micro-organisms, but also showed that known sulphate-reducing bacteria and syntrophic fatty acid-oxidising micro-organisms dominated the enrichments.

Molecular techniques and cultivation was used to further enhance knowledge about the microbial diversity in an anaerobic paper-mill wastewater treatment system, which is described in **chapter 7**. Most Probable Number series allowed estimating the number of micro-organisms per ml sludge that could use propionate without sulphate ($10^9$), propionate and sulphate ($10^5$), butyrate without sulphate ($10^8$), butyrate with sulphate ($10^5$), glucose ($10^9$) and $H_2/CO_2$ ($10^{10}$). Cloning of 16S rRNA genes, screening of clones with amplified ribosomal DNA restriction analysis and partial sequencing revealed that a richness of about 450 species can be expected. From this clone library, with a reasonable predicted coverage of about 70%, a set of 121 dominant and not closely related clones was nearly completely sequenced. Clone sequences were related to a variety of different known species, with expected functions in anaerobic digestion like fermenters, syntrophic short chain fatty acids oxidisers and SRB. However, about 80% of the clones was similar to sequences in the database without close cultured relatives, but many of these sequences are derived from anaerobic environments as well. Such high proportions of yet uncultured populations only remotely related to well-characterised isolates are still observed as recently published by for example Sousa *et al.* (2007a). It is important to improve knowledge of such unknown micro-organisms and fast accurate monitoring and identification could be very helpful for that. Therefore, a pilot macro-array was developed and tested. It appeared that combining probes generated by PCR amplification of the V1 and V6 variable regions of the 16S rRNA gene could be used for differentiation of relatively closely related organisms. Such tools could prove useful for further research of the microbial diversity and activity in anaerobic ecosystems.

Anaerobic wastewater treatment systems have been applied for several years and major improvements have been made. Also microbiological techniques and functional application of microbiological methods have led to the isolation of a number of anaerobic micro-organisms from bioreactors. However, the fundamental microbiological background of anaerobic wastewater treatment systems is still not fully understood. The understanding and prediction of microbial community structure – function relationships is still challenging and needs integrated research efforts for resolution. Molecular ecology and physiological data can be obtained together with newly developed technologies, and the combination of classical and modern microbiological methods will definitely improve ecophysiological knowledge of wastewater treatment and other environmental systems.

This thesis presents results from investigations of several full-scale and lab-scale anaerobic wastewater treatments systems. Different systems have been investigated, operated at different temperatures and influents (e.g. LCFA, heavy metal-containing, paper mill wastewaters). When the studies described in this thesis were initiated, knowledge on structure and functionality of microbial communities active in anaerobic wastewater treatments systems was limited. The integrated application of molecular and cultivation dependent analyses of microbiota structure and function of a broad variety of anaerobic wastewater treatment systems described in this thesis has been used to improve insight of the ecophysiology in such reactors. Some general commonalities of anaerobic systems have been found, but also system-specific characteristics. This gives a potential for the identification of general and system-specific indicator populations, allowing improved diagnostics.
Usually the investigated systems were methanogenic. Expected methanogens such as *Methanosarcinales* (mainly *Methanosaeta*) and *Methanobacteriales* were detected, but also *Crenarchaeota* have been found. Further research is needed to further elucidate the exact ecophysiological role of these proposed acetate-using *Crenarchaeota* in anaerobic wastewater treatment [Collins *et al.* 2006]. Besides expected bacterial fermenters, syntrophic short chain fatty acids oxidisers and SRB, a large diversity of bacterial populations has been detected with yet unknown function. Typically, many of these *Bacteria* with unknown function have been found by others in anaerobic bioreactors and other anaerobic environments as well, indicating their functional relevance for such ecosystems. Hence, efforts for their isolation and ecophysiological characterisation should be further increased to improve our understanding of bioreactor functioning. The importance of continued efforts towards isolation and characterisation of such yet uncultured micro-organisms has recently been reinforced by the isolation of novel filamentous anaerobes of the *Anaerolineae* and *Caldilineae*, two novel classes within the phylum of the *Chloroflexi* [Sekiguchi *et al.* 2003, Yamada *et al.* 2006]. This phylum had previously been one of the most pronounced examples of a major phylogenetic lineage without cultured representatives, but presumed importance for the functionality of microbial communities residing in anaerobic bioreactor function [Yamada *et al.* 2005].

Advanced molecular techniques have been developed and used for faster monitoring and identification of the microbes present. Still, further developments of integrated multidisciplinary research are needed to understand and control the useful (and less-wanted) microbes in anaerobic ecosystems, such as engineered wastewater treatment systems. Fundamental and applied questions that remain largely unsolved until now (e.g. sludge granulation or disintegration), can be tackled by such multidisciplinary research teams and the ratio behind successful wastewater treatment systems or unwanted process failures can be resolved further. Continued application of innovative microbiological culture-dependent and culture-independent techniques, and the use of novel strategies will be pivotal to a better understanding and predictability of anaerobic wastewater treatment processes and reactor performance.

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# About the author

Kees Roest (original birth name: Cornelis Roest) was born in Wijk bij Duurstede, the Netherlands, on the 25th of September 1975. After elementary school at 'De Wijngaard' in Wijk bij Duurstede, and secondary education at the Revius Lyceum in Doorn, Kees obtained a BSc degree in Medical Microbiology at the Hogeschool van Utrecht, Department of Laboratory Science in 1997. During his BSc he completed two practical terms. The first of about 7 months at the Netherlands Institute of Ecological Research, Centre for Limnology (NIOO-CL) in Nieuwersluis, The Netherlands, dealt with the diversity of ammonia oxidisers in fresh water sediments. The second practical term was at the Academic Hospital Utrecht (AZU) in the Clinical Microbiological Laboratory for diagnostic microbiological testing of patient materials and had a duration of 4 months.

In 1997, Kees continued his education in Medical Biology at the Vrije Universiteit in Amserdam. This MSc included a practical term of 8 months at the Agricultural Research Department, Institute for Animal Science and Health (ID-DLO) in Lelystad, The Netherlands, now part of Wageningen University and Research centre. During that practical term the genomes of *Campylobacter* and *Salmonella* species were compared. Besides specialisations in oncology and molecular microbiology & biotechnology, a biology teaching orientation course including practicals was done.

While Kees finished his MSc in 1999, he directly started with his PhD research at the Laboratory of Microbiology of Wageningen University. The research was focused on the microbial community analysis in sludge of anaerobic wastewater treatment systems and a combination of culture-dependent and culture-independent approaches was used. Some of the main results of his research are described in this thesis. Also teaching and training in molecular ecology and microbial physiology of MSc students, fellow PhDs, and other visiting scientists was conducted as well as work visits to a range of universities in Turkey, Portugal, USA and Mexico.

After the PhD contract was finished, Kees had quickly a Marie Curie host development fellowship molecular microbiology and environmental technology for the start up of a new molecular microbiology laboratory, including teaching and training, at the Department of Chemical Engineering and Environmental Technology of the Universidad de Valladolid in Valladolid, Spain.

From November 2004 until January 2005 Kees worked with real-time PCR and phylogenetic micro-arrays in the laboratory of Dave Stahl, University of Washington in Seattle, USA.

Kees has set-up another new molecular microbiology laboratory in the Sustainable Environment Research Centre (SERC) of the University of Glamorgan in Pontypridd, Wales, UK, as Marie Curie Transfer of Knowledge research fellow. Microbiological tools and experiments have been used for the monitoring, detection and identification of microbial species in hydrogen and methane producing bioreactors, as well as microbial fuel cells.

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**Roest K.** *et al.* (2007) Microbial community effects of substrate changeover in a two-stage biological hydrogen and methane reactor (*in prep*).

**Roest K.** *et al.* (2007) Molecular monitoring of the microbial community in a stable performing activated sludge bioreactor (*in prep*).

**Roest K.** *et al.* (2007) Design of a macro-array for the detection of bacteria in anaerobic sludge (*in prep*).

# Educational activities

The SENSE Research School declares that Mr. Cornelis Roest has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 45 ECTS*, including the following activities:

SENSE PhD courses:

- Environmental Research in Context
- Research Context Activity: 'Starting and developing a dedicated website and mailinglist for the purpose of an Identification DNA Array Platform'
- Theoretical Ecology
- Euro Summer School: Decentralized Sanitation and Reuse (DESAR)

Other PhD courses:

- Techniques for Writing and Presenting Scientific Papers
- International Fluorescence in situ Hybridization (FISH) course
- English for PhD's
- Bio-Informatics

Activities:

Work visits and seminars at:

- Marmara University, Istanbul Technical University, and Fatih University, Istanbul Turkey
- Universidade do Minho, Braga, Portugal
- Instituto Tecnológico de Sonora, Obregón, Mexico
- University of Arizona, Tucson, Arizona, USA
- Universidad Autónoma Metropolitana-Xochimilco and Instituto Mexicano del Petróleo, Mexico D.F., Mexico
- University of Washington, Seattle, Washington, USA

Organisation of:

- Identification DNA array symposiums and discussion group, Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands
- Identification DNA array platform (http://www.ftns.wau.nl/id-array/index.htm)

Oral Presentations:

- Sulphur meetings, January and September 2000 and November 2001, Galamadammen, The Netherlands
- Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM), 24-27 March 2002, Göttingen, Germany
- Array NL Platform meeting, 27 June 2002, Utrecht, The Netherlands
- Lab on a Chip, 8 – 9 January 2003, Birmingham, United Kingdom
- Nederlandse Vereniging voor Microbiologie (NVvM) meeting, 10 October 2003, Haren, The Netherlands
- PhD/Postdoc meetings, Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands

Deputy director SENSE
Dr. A. van Dommelen

* 1 ECTS (European Credit Transfer System) is roughly equivalent to 28 hours

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