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Detection and quantification of microorganisms in anaerobic bioreactors

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Abstract

The presence of sulfate in anaerobic reactors can trigger competitive and syntrophic interactions between various groups of microorganisms, such as sulfate reducers, methanogens and acetogens. In order to steer the reactor process in the direction of sulfidogenesis or methanogenesis, it is essential to get insight into the population dynamics of these groups of microorganisms upon changes in the reactor operating conditions. Several methods exist to characterize and quantify the microbial sludge composition. Combining classical microbiological and modern molecular-based sludge characterization methods has proven to be a powerful approach to study the microbial composition of the anaerobic sludge.

Introduction

In anaerobic bioreactors organic matter is removed from the wastewater by the concerted action of various groups of microorganisms. Anaerobic treatment of industrial wastewaters in methanogenic bioreactors is used worldwide (Lettinga 1994; Stams 1994). If sulfate is present in the wastewater the final steps in the degradation process can be carried out by acetogens, methanogens, and sulfate reducers. To which extent the reactor process will be methanogenic or sulfidogenic depends on reactor operation conditions (e.g. pH, temperature, loading rate, composition of the wastewater), and the microorganisms present in the reactor sludge. In general sulfate reduction is an undesired process, but sometimes it is used for the removal of sulfur-containing compounds from the wastewater (Oude Elferink et al. 1994). Thus far, it is difficult to steer the reactor process in the direction of complete methanogenesis or sulfidogenesis. In order to get a better grip on the process it is essential to gain more insight into the population dynamics of acetogens, methanogens and sulfate reducers in anaerobic bioreactors. Nowadays, many microbiological techniques are available to study the microbial sludge composition and to monitor population changes. In this paper some of these methods are evaluated for their applicability to study anaerobic bioreactors.

Classical sludge characterization methods

Classical sludge characterization methods are often based on selective growth media. The Most Probable Number (MPN) method is a technique in which serial sludge dilutions are inoculated in selective liquid media or on solidified agar-media. This method can give very useful information on the number of microorganisms that are able to grow on artificial media. However, it should be kept in mind that the MPN method will underestimate the number of microorganisms if the microorganisms are attached to solid substrates or are associated to each other like threaded bacteria such as the acetoclastic *Methanosaeta* sp. (Grotenhuis et al. 1991; Whitman et al. 1992). Another group of microorganisms which will probably be underestimated with the MPN method are bacteria which grow in syntrophic consortia, such as syntrophic propionate or butyrate-degrading acetogens, because their syntrophic partner might not be present in the same high

numbers. Adding a hydrogen-scavenging methanogen to the MPN-dilution medium can help to circumvent this problem (Visser et al. 1993; Wu et al. 1992). Recent studies with granular sludge from a full-scale anaerobic reactor treating papermill wastewater showed that the number of propionate-degrading acetogens was underestimated at least a 1000-fold, if no hydrogen-scavenging methanogens were added to the MPN-dilution medium. Butyrate-degrading acetogens were underestimated at least a 100-fold (Oude Elferink 1998). Direct microscopic analyses are useful as well in the characterization of sludge. A major drawback of most microscope techniques is the fact that the identification of microbes is usually based on cell morphology only, which for most bacteria is not very distinctive. An exception are methanogens, they can be identified with epifluorescence microscopy by detecting the factor F420-dependent autofluorescence (Doddema & Vogels 1978). However, some methanogens, such as *Methanosaeta* do not exhibit autofluorescence (Dolfing et al. 1985). Recently, Surman et al. (1996) have compared the applicability of several light, fluorescence, and electron microscopy techniques such as scanning and transmission electron microscopy (SEM and TEM), atomic force microscopy (AFM), and confocal laser scanning microscopy (CLSM) for the examination of microbial biofilms. All techniques have their special advantages and disadvantages, and the most accurate picture of the true sludge composition can be obtained by combining as many techniques as available. That such a 'combined microscopic approach' can provide a lot of information has been shown by Zellner and coworkers (Zellner et al. 1993), who used SEM, phase contrast microscopy and epifluorescence microscopy of methanogens to study biofilm formation in anaerobic fixed bed reactors.

Molecular-based sludge characterization methods

In the past decades, new methods have been developed which allow the direct identification of microorganisms in the sludge by using for example biomarkers, specific antibodies, or nucleic acid probes.

Membrane lipids and their associated fatty acids have been used to characterize complex microbial consortia (Guckert et al. 1985; Parkes 1987). Bacteria (eubacteria) can be characterized by the patterns of their methylated phospholipid ester-linked fatty acids, known as (PL)FAME-patterns or PLFA profiles. Methanogens are characterized via their phospholipid-derived ether lipids (PLEL). Although FAME-patterns can be useful for the characterization of unknown bacterial isolates (Böttger 1996), they cannot be used for microorganisms that lack specific lipid biomarkers (Ringelberg et al. 1994). In addition, some lipid biomarkers may be less specific than previously thought. Especially in complex microbial ecosystems where physiologically different microorganisms may share the same 'specific' lipid biomarker, this can lead to large characterization mistakes. Recent studies have for example shown that the lipid i17:1ω7, an uncommon compound which was considered as specific marker for *Desulfovibrio* sp. (Taylor & Parkes 1983; Vainshtein et al. 1992) was also present in high amounts in some syntrophic propionate degraders of the genus *Syntrophobacter* (Oude Elferink et al. 1998). Lipid analyses are not very sensitive, and environmental conditions (e.g. growth substrate and temperature) may cause changes in the microbial FAME-pattern (Böttger 1996; Davey and Kell 1996). Futhermore, the molar percentages in which a marker PLFA is found within a genus can vary a lot. The molar percentages of the marker 10Me16:0 can for example range from 2 to 16% within the genus *Desulfobacter*, while the marker i17:1ω7 can range from 5% to 33% within the genus *Desulfovibrio*, and at least from 16% to 26% within the genus *Syntrophobacter* (Kohring et al. 1994; Oude Elferink et al. 1998). Bearing in mind these limitations, lipid analyses can still be useful to obtain a quick general impression of the total microbial sludge composition, and to follow sludge composition changes. Lipid analyses have for example been applied to detect and quantify the presence of methanogens (Nishihara et al. 1995; Ohtsubo et al. 1993), sulfate reducers (Dowling et al. 1988; Oude Elferink et al. 1998; Ringelberg et al. 1994; Edlund et al. 1985), and syntrophic propionate degraders (Oude Elferink et al. 1998) in complex microbial consortia. Combining lipid analyses with tracer experiments using for example ^{13}C-labelled precursor molecules seems very promising for future sludge characterization studies. The labelling studies do not only help with the interpretation of the FAME analyses results, but also make it possible to link specific microbial processes with the organisms involved (Boschker et al. 1998).

Immunodetection techniques are based on the fact that bacterial surface cell wall polymers such as proteins and lipopolysaccharides have strong antigenic properties which can be used to raise antibodies. If

antibodies are labelled with a fluorescent dye or gold particles, in combination with respectively fluorescence or electron microscopy, they can be used for the specific detection of bacteria (Harlow & Lane 1988). Immunodetection is a very powerful tool for the identification of microorganisms in sludge. The method is generally very specific, and suitable for quantitative analyses. Nevertheless it should be taken into account that antibodies can cross-react with other non-related strains (Smith 1982). Futhermore, the method is not very flexible, because a pure bacterial culture is needed to raise the specific antibodies (Harlow & Lane 1988). Many immunodetection studies have focussed on methanogens in anaerobic bioreactors (Grotenhuis et al. 1991; Koornneef et al. 1990; Macario et al. 1991a; 1991b; Robinson & Erdos 1985; Sørensen & Ahring 1997). However, immunodetection techniques can also be applied for the identification of sulfate reducers (Singleton et al. 1985; Smith 1982; Lillebaek 1995). Quantification of microorganisms in multilayered biofilms has long been impossible, because it was difficult to ensure that the antibodies reacted with all antigens in the biofilm. Recently, Bauer-Kreisel et al. (1996) were able to circumvent this difficulty by combining mechanical and chemical treatments with an enzyme-linked immunosorbent assay (ELISA) for the quantification of *Dehalospirillum multivorans* in anaerobic reactor biofilm.

Without doubt ribosomal RNA-based detection and identification methods have become most important in the unravelling of the microbial composition of anaerobic sludge. Sequence analysis of the ribosomal RNA or the rRNA-gene has revealed that 16S and 23S rRNA can be used as evolutionary biomarkers (Böttger 1996, DeLong et al 1989; Woese 1987). The 16S and 23S rRNA contains both highly conserved as well as highly variable regions. At present a large database of 16S rRNA sequences is available, and 16S rRNA analysis has become an important tool for the identification of new isolates. Several rRNA based methods have been developed to identify and quantify microorganisms in complex environments. These methods can even be used without cultivation of the microorganisms. Unfortunately there is not always a link between the phylogeny and the physiology of the microorganisms. This hampers the determination of the role of (unidentified) microorganisms in a complex ecosystem (Pace et al. 1986; Akkermans et al. 1995; 1996; 1998). Some of the RNA-based methods that can be used for the analysis of the microbial sludge composition are depicted in Figure 1.

One of these methods is hybridization with rRNA-based oligonucleotide probes (Amann et al. 1995, Stahl 1995, Raskin et al. 1995a). Oligonucleotide probes are short single stranded oligomers of 15 to 40 nucleotides, which can be synthesized chemically. The oligonucleotide probes are complementary to either variable or conserved parts of the rRNA. For detection and quantification of microorganisms the oligonucleotide probes are either radioactive by ^{32}P-labelling, or are chemically linked to fluorescent dyes. Fluorescently or ^{32}P-labelled rRNA hybridization probes have for example been used successfully in anaerobic bioreactors for the detection and quantification of methanogens (Harmsen et al. 1996a, 1996b; Omil et al. 1997; Raskin et al. 1994a, 1994b, 1995a, 1995b, 1996; Sørensen et al. 1997), sulfate reducers (Harmsen et al. 1996; Kane et al. 1993; Oude Elferink et al. 1997, 1998; Raskin et al. 1996), and syntrophic propionate degraders (Harmsen et al. 1996; Oude Elferink et al. 1998). An advantage of fluorescently labelled rRNA probes is that they can be used for in situ hybridization studies (the so called Fluorescent In situ Hybridisation [FISH]-technique), thus making it possible to study the spacial organisation of the microorganisms in the sludge. The localization of certain microorganisms in the sludge can give very valuable information for reactor operating conditions. Furthermore the combined use of different fluorescent dyes offers very interesting possibilities to study the ecology of certain strains within a community of related organisms. Amann et al. (1996) were able to visualize seven distinct genotypes of closely related bacteria in one sludge sample from an aerobic bioreactor. Figure 2 shows an example of the use of the FISH-technique in anaerobic sludge. Metabolically active cells contain sufficient ribosomes to give visuable hybridization signals. The whole experimental procedure can be finished within 3 hours. The technique is suitable for routine monitoring of large numbers of samples, especially if a suitable image analysis system is available. Like any other method, FISH has also limitations. Dormant or metabolically inactive cells contain insufficient ribosomes to show fluorescent signals after hybridization. Moreover, quantification of the number of microorganisms can be a problem when cells have irregular shapes or when they form chains or compact micro-colonies. Insufficient permeability of the cells can be another limitation. Although Gram negative bacteria usually are readily permeabilized with routine chemicals, Gram positive bacteria need additional enzymatic treatment (Amann et al. 1995).

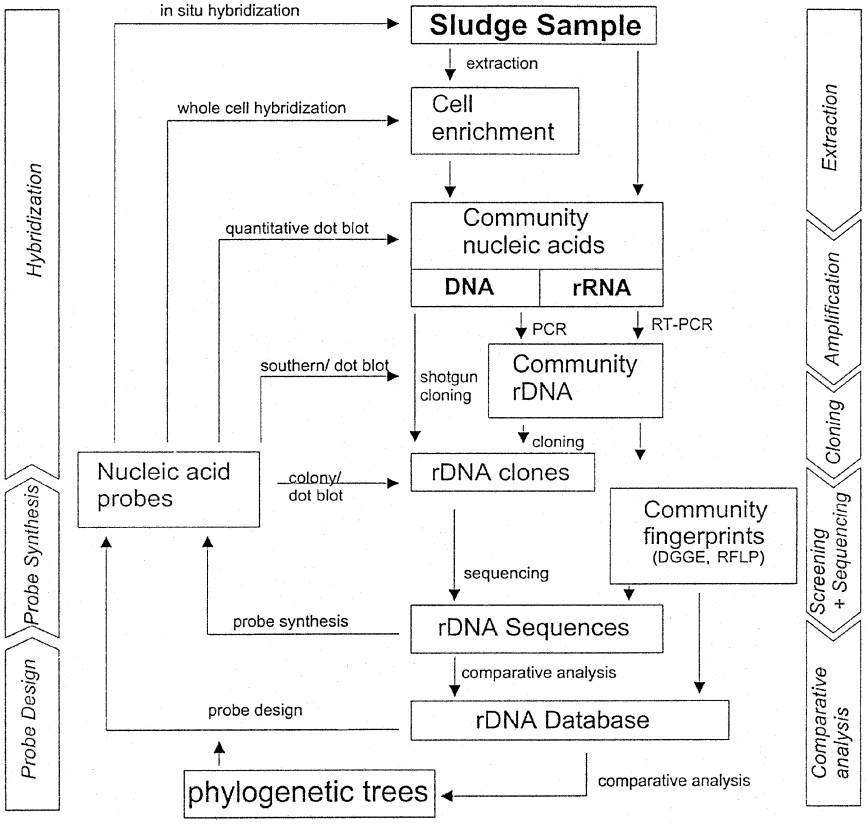

Figure 1. Strategies based on rRNA sequences for the characterization of sludge microbial communities, adapted from Amann et al. (1995), and Hugenholz & Pace (1996). RT, reverse transcriptase; DGGE, denaturing gradient gel electrophoresis; RFLP, restriction fragment length polymorphism.

Finally, the detection limit is not only determined by the use of microscope technique, i.e. at least 10,000 cells/ml are needed to detect one cell, but also by the relative size of the target-population and the amount of autofluorescent particles (false positives).

PCR amplification of rRNA-genes is another rRNA-based detection method. With the polymerase chain reaction (PCR) amplification method a few target rRNA genes can be amplified to make them detectable (Giovannoni 1991). The selection of the PCR primers determines which rRNA gene and which part of the rRNA gene will be amplified, thus determining the specificity of the detection. By combining universal PCR primers with cloning and sequence analysis techniques, it is possible to get information about the microbial sludge composition. For example, by applying the PCR technique in combination with rRNA sequencing a whole range of anaerobic digester microorganisms was identified (Ng et al. 1994; Hiraishi et al. 1995).

To get a general impression of the heterogeneity of the sludge community it is also possible to separate the PCR products with temperature gradient gel electrophoresis (TGGE) or denaturing gradient gel electrophoresis (DGGE). Both methods can be applied to separate PCR products of the same length (up to 650 bp) but with different nucleotide sequences. By amplifying the variable regions of the microorganisms rRNA highly specific fingerprints of a sludge community can be generated. The method has now been widely used to characterize microbial ecosystems (Muyzer et al. 1993; Muyzer & Ramsing 1995). Figure 3 illustrates that TGGE profiles can be used to distinguish differences between the microbial sludge composition of anaerobic sludge from different bioreactors. TGGE profiles of sludge samples from two different sulfidogenic reactors are shown. Both samples give a very different banding pattern, indicating large differences in the microbial populations of both reactors. The TGGE-gel can be used for further characterization of the microbial population. The microor-

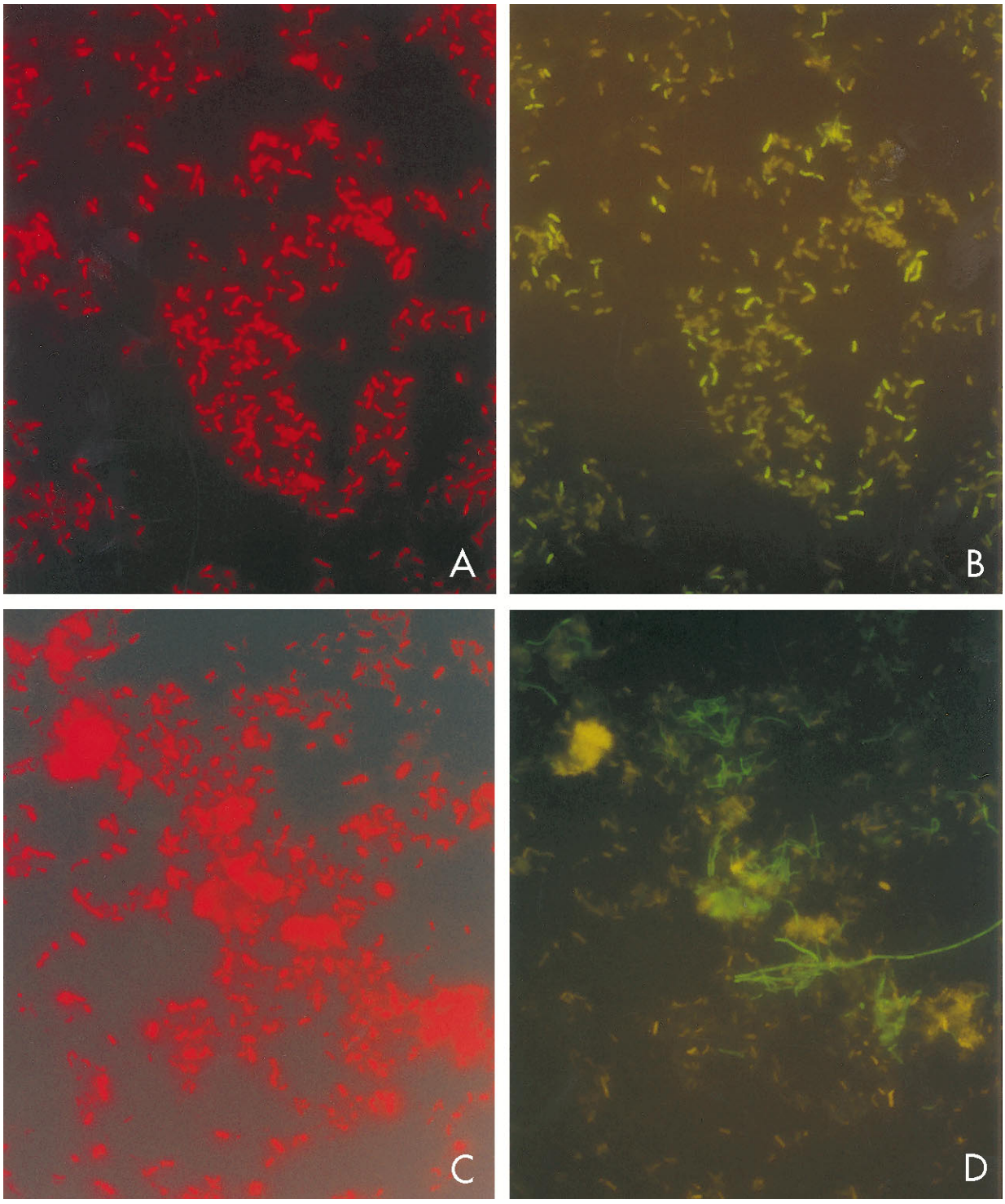

Figure 2. FISH detection of Bacteria, sulfate reducing bacteria, and *Methanobacterium* sp. in a sample of anaerobic sludge. The Bacteria are visualized with bacterial probe EUB338 (A,C), the sulfate-reducing bacteria with probe SRB (B) and the *Methanobacterium* sp. with probe MB (D). The micrographs of the hybridization signals with the EUB338 probe (left) and with the SRB or MB probe (right) are from identical microscopic fields. Probes EUB338 and SRB were described by Amann et al. (1992), and probe MB by E.G. Zoetendal (unpublished).

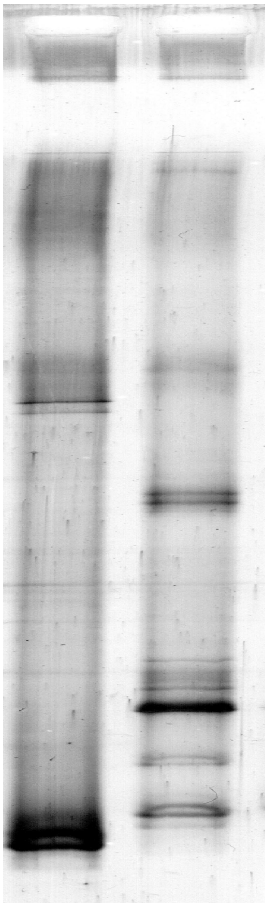

Figure 3. TGGE profile of sludge samples from two different sulfidogenic reactors with different microbial communities. The PCR products were generated by using the eubacterial primers GC968f and 1401r (Nübel et al. 1996). The temperature gradient was 36–45 °C. Gels were stained with silver as described previously (Felske et al. 1997).

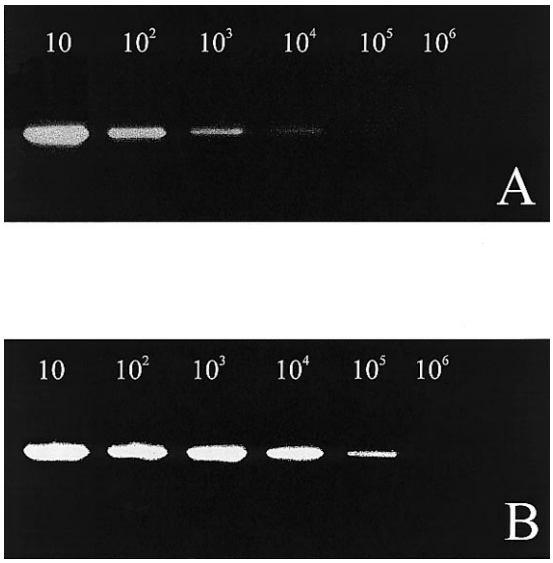

Figure 4. Determination of the ratio between two types of methanogens, *Methanobacterium* sp (A) and *Methanobrevibacter* sp. (B) in anaerobic sludge by MPN-PCR using different DNA dilutions. The primers used for the specific amplification were: *Methanobacterium*, MBf/ARCr (E.G. Zoetendal, unpublished/ Jürgens et al. 1997); *Methanobrevibacter*, MBBf/ARCr (E.G. Zoetendal, unpublished/Jürgens et al. 1997). The specific PCR products both had the expected size of 900 bp (not shown).

ganisms in the sludge can for example be identified by cutting out the bands and sequencing them, or by hybridizing the bands with specific oligonucleotide probes. A combination of DGGE and specific oligonucleotide probes has for example been used to reveal the presence of sulfate reducers in anaerobic sludge (Muyzer et al. 1993). Recently, the TGGE analysis was used in our laboratory for the characterization of a benzoate-degrading methanogenic consortium (G. Zellner, unpubl.).

Several methods have been developed in order to quantify microorganisms with PCR amplification (Ferré et al. 1994). One approach is the use of an internal standard in the amplification of the target DNA (competitive PCR). Usually the internal standard differs only slightly from the target molecule. Both the internal standard and the target molecule are quantitatively amplified. Only an excess of one of the target molecules would inhibit the amplification of the other template. Therefore, the ratio between the signals obtained for both reaction products provides quantitative information on the amount of target DNA originally present in the sample (Ferré et al. 1994). Other methods are based on the principle of labelling one of the PCR primers with a reporter molecule in order to be able to directly or indirectly quantify the PCR products. The Q-PCR System from Perkin Elmer is for example based on such an approach (Martin et al. 1995). One of the most straightforward methods is Most Probable Number PCR (MPN-PCR). The principle is rather simple. Different dilutions of the target rDNA are amplified by PCR and the concentration of the target rDNA that just gives a visuable product on a gel is estimated. From this estimation it is possible to calculate the number of rRNA molecules, and estimate the original number of target organisms in the sample. When different sets of primers are used, e.g. primers for methanogens and eubacteria one first has to determine the amplification efficiency of both primer sets. This can be done by kinetic PCR for bacterial rRNA (Blok et al. 1997). Figure 4 illustrates the quantitative

detection of two types of methanogens in anaerobic sludge by MPN-PCR. Unfortunately PCR-based quantification techniques have some severe limitations. In samples with mixed templates some templates can be selectively amplified. This preferential PCR amplification can introduce a large difference between the actual amount of a group of microorganisms in the sludge, and the measured amount. Although this problem has been well documented (Suzuki & Giovannoni 1996), the extent of the problem remains largely unsolved. Furthermore, PCR-based quantification techniques are influenced by the DNA extraction efficiency, and the PCR efficiency. Recent MPN-PCR studies with anaerobic sludge have shown that the sulfate reducer *Desulforhabdus amnigenus* could still be detected if the ratio between *D. amnigenus* and other sludge bacteria was 1:1,000,000 (Oude Elferink et al. 1997). However, this remains a rough estimation because the rRNA content of microbial cells is not the same for all species and is effected by the growth phase of the cells (i.e. slow-growing or starving cells have a lower rRNA content that fast-growing cells) (Fukui et al. 1996; Gausing 1977; Oude Elferink et al. 1997).

Conclusions and future perspectives

The characterization and quantification of microorganisms in anaerobic reactors is difficult, but essential for optimizing the reactor process. Good results have been obtained by combining classical microbiological and modern molecular-based sludge characterization methods. Especially rRNA and rDNA based techniques have offered new opportunities for the analysis of species and spatial structure of reactor sludge. In general the methanogenic sludge population can now be characterized relatively well. However, for the sulfidogenic and acetogenic sludge population, characterization is still difficult. The study of these latter groups of microorganisms is not only hampered by the presence of many unidentified species in the sludge, but also by the lack of available hybridization probes for those microorganisms which have already been isolated. Therefore, future research should certainly proceed with classical isolation and characterization studies, but the development of more hybridization probes is also essential. For example probes for Gram positive sulfate reducers and syntrophic butyrate degraders would be very useful in this respect.

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