

Corinne Whitby
Torben Lund Skovhus
Editors

Applied Microbiology and Molecular Biology in Oilfield Systems



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Preface

This book provides a combined theoretical and practical approach to molecular microbiology studies in oil reservoirs and related downstream processing plants and is written by some of the world's leading authorities in this area from both academia and the oil and petroleum industry. The book is based on specific case studies and encapsulates the ethos of the 'International Symposium on Applied Microbiology and Molecular Biology in Oil Systems (ISMOS)'. The text explains how each method works and the type of information that can be obtained. This is of direct relevance to reservoir engineers, production chemists, corrosion managers, field technicians and microbiologists, who are working in the oil and petroleum industry. The text begins with [Chapter 2](#), which addresses practical issues relating to sampling from oilfield systems and the types of samples to be collected. Specifically, the focus is on the importance of correct sampling techniques, how to maintain sample integrity, for example, from offshore facilities, until the samples are processed in the laboratory. Details are also given on methodologies relating to DNA/RNA extraction and PCR amplification of nucleic acids from field samples.

[Chapters 3–11](#) introduce each molecular approach in turn and explain the type of information that can be obtained, depending on the specific microbiological or technical questions that are being addressed. For example, if the question is 'which microbial community is present in a sample?' then possible molecular approaches which could be applied include PCR, DNA fingerprinting, fluorescent *in situ* hybridisation (FISH) and cloning and sequencing. If the question is 'how many microorganisms are present in a sample?' then quantitative PCR (qPCR) that targets a specific gene may be applied. In order to address the question of 'which microorganisms are active in a sample?' then approaches such as the use of RNA analyses targeting functional genes may be applied. This categorisation of each technique is rather an oversimplification and indeed information from one method may address more than one of these microbiological questions. For example, while FISH enables the identification and localisation of microbes within their environment, quantitative data on their relative abundance can also be obtained, if specific probes are applied. Details are also given on the recent developments of next-generation sequencing methods and microarrays which enable vast amounts of data to be generated very quickly and will allow us to significantly advance our knowledge of oilfield microbiology in the future. However, the production of such large data sets

requires more sophisticated bioinformatics software and user expertise in order to process it, understand it and apply it, in order to benefit the industry.

Chapters 12–19 give details of the *problems caused by microbes* in oilfield systems such as biocorrosion (MIC) and reservoir souring. The text focuses on how molecular microbiological methods (MMM) offer a novel approach for monitoring the *in situ* microbial communities in order to mitigate potential problems caused by microbes in the future, as well as to determine the efficacy of possible treatment strategies (e.g. nitrate, biocides, scavenging of H₂S, pigging/cleaning and corrosion/scale inhibitors). In addition to the problems caused by microbes, Chapters 20–25 consider the *beneficial implications of specific microbial processes* and how MMM may be used to further exploit these. This includes the use of microorganisms in bioremediation and biorefining and bioprocessing for petroleum oil upgrade, the exploitation of microorganisms in oil recovery, i.e. microbial-enhanced oil recovery (MEOR) and using anaerobic microbial processes for obtaining methane from oil.

Chapters 26–28 describe novel fuels for the future, including the development of biofuels and the removal of naphthenic acids for both heavy oil upgrade and the bioremediation of contaminated tailing pond waters. Finally, in the Appendix, details are provided for the methodologies used by the contributing authors as guidelines for non-specialist users working in the oil and petroleum industry. Currently, there are other methods, such as stable isotopes, proteomics and metabolomics, which may also have the potential to further advance our understanding of microbial ecology and their processes in the sub-surface. Thus, in complex oil systems, where many physicochemical parameters are driving the microbial communities and their activities, it is important to adopt a polyphasic approach which combines molecular microbiological studies, with physiological and biochemical analyses of the microorganisms, alongside a detailed characterisation of their environment. Such a combined approach of analytical technologies will enhance oil production, extend life time of aging equipment/facilities, increase personnel safety and more efficiently protect the environment. Therefore, the energy sector will greatly benefit from the widespread implementation of MMM in the near future.

Acknowledgements

We would like to thank all of the contributors to this book and also to the reviewers for their useful comments and suggestions.

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Part I

Introduction, Sampling and Procedures

Chapter 1

Introduction

Corinne Whitby and Torben Lund Skovhus

Microorganisms are ubiquitous in the environment and are fundamental to the biogeochemical cycling of nutrients in marine, freshwater and terrestrial ecosystems. In the deep biosphere, microbial-mediated transformations are responsible for shaping our natural resources over geological time. However, for many years our knowledge and understanding of microorganisms in sub-surface environments, like oil reservoirs, have been based primarily on culture-based approaches such as plate counts or most probable numbers (MPNs). MPN involves performing serial dilutions of a bacterial culture or an environmental sample into media until the sample is diluted to extinction (Fig. 1.1). Cultures are incubated and assessed by either eye for growth based on turbidity, media colour change or colony/microscopic counting.

There are several major weaknesses associated with MPN including the need for large numbers of replicates at the appropriate dilution to narrow the confidence intervals. Also media biases may select for the fastest growing or most abundant microorganisms which may not represent those species mediating specific catabolic processes in the sub-surface environment. Furthermore, since many microorganisms are difficult to culture in the laboratory, these approaches may lead to a gross underrepresentation of the *in situ* microbial communities present. It is estimated that between 0.1 and 10% of the total communities are culturable (Amann et al., 1995). Also MPN cannot be used to enumerate the microorganisms found in solid samples. Besides the technological constraints with the MPN method it also requires long incubation times (e.g. up to 28 days for some microbes). The MPN approach is laborious for the personnel performing these methods in the field, as several hours may be spent performing the serial dilutions, and if done incorrectly (for instance, by using the same syringe between dilutions) the reported numbers will be inaccurate and of limited value to the operator.

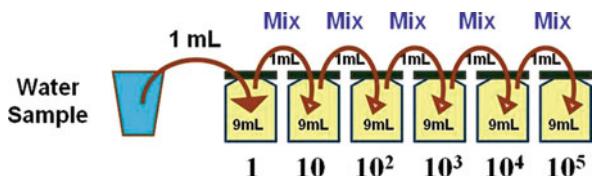
Over the last 25 years the development of molecular microbiological methods (MMM) has completely revolutionised microbial ecology research and the ways microbes are enumerated in technical systems. These methods are based on the

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Fig. 1.1 Most probable number (MPN) technique



direct extraction of nucleic acids from natural samples, e.g. water, soil and sediments (both organic and inorganic materials). These approaches have enabled the previously unculturable microbial groups to be studied and enumerated in a rapid and reproducible manner.

Analysis of nucleic acids is driven by Polymerase Chain Reaction (PCR) approaches which amplify specific gene sequences from target microorganisms. Of particular importance are the macromolecules which form the ribosomes of microbial cells known as ribosomal RNAs (rRNAs). The rRNA has turned out to be an excellent target for determining the evolutionary relationships between microorganisms. Based on comparative rRNA sequence analysis three phylogenetically distinct lineages (or domains) of organisms have been identified which form the tree of life. These domains are the *Bacteria* and the *Archaea* (both consisting of prokaryotes) and the *Eukarya* (eukaryotes) (Fig. 1.2).

Sub-surface microbial community structure is highly diverse containing representatives from each domain. PCR can be applied to target which key functional microbial groups (e.g. sulphate-reducing bacteria) that are present under different environmental conditions and analyse specific metabolic processes that are occurring in the sub-surface environment. In oil reservoirs, microorganisms may readily catabolise the hydrocarbon compounds found in oil and this is exploited during the

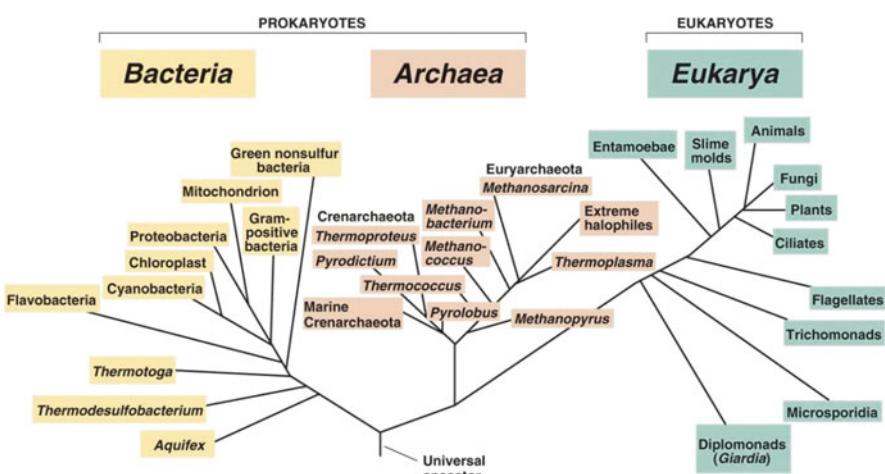


Fig. 1.2 The phylogenetic tree of life (Madigan et al., 2009)

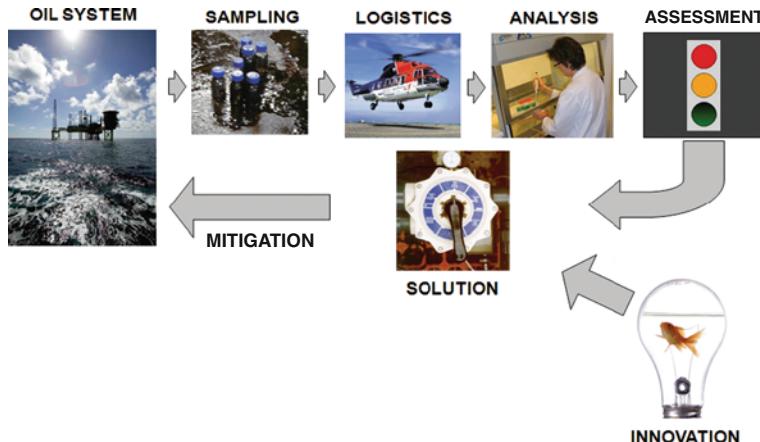


Fig. 1.3 The ISMOS symposium series ethos aims at giving the oil, gas and petroleum industry combined and innovative solutions for problems caused by microorganisms

bioremediation of oil spills. Under certain conditions hydrocarbon biodegradation results in methane production which can be harnessed and this biogas may be used as a fuel source. Microorganisms may also be used to improve and upgrade oil productions, for example, by removing contaminants like heavy metals or sulphur, as well as reducing reservoir porosity and reducing oil viscosity. Some microorganisms can also produce hydrocarbons, e.g. the green microalga *Botryococcus braunii* is able to excrete long-chain (C_{30} – C_{36}) hydrocarbons. Not only are the activities of microorganisms beneficial, but they may also be a hindrance. For example, under some circumstances, such as in fuel storage tanks, microbial growth and hydrocarbon degradation can be problematic. In addition, specific microbial-mediated processes may lead to the production of toxic, corrosive gases like hydrogen sulphide which causes significant economic, safety, environmental and human health costs. Figure 1.3 summarises the processes involved in applying novel and innovative solutions to specific problems in the oil and petroleum industry. Thus, the energy sector will greatly benefit from the widespread implementation of MMM approaches in the future, in daily operations, in public Research and Development projects and new Joint Industry Projects (JIPs).

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Chapter 2

Sampling and Nucleic Extraction Procedures from Oil Reservoir Samples

**Geert M. van der Kraan, Maarten de Ridder, Bart P. Lomans,
and Gerard Muyzer**

Introduction

Today there is a renewed interest towards biological aspects in oil reservoir systems. This interest comes not only from academia but also from the petroleum industry. Fields of common interest are ‘microbial-enhanced oil recovery’ (MEOR), efforts to lower H₂S production and subsequently microbial corrosion (caused by sulphate-reducing microorganisms) and the analysis of microorganisms found in oil wells as additional information source for reservoir conditions.

In the past, the main focus of studies towards oilfield systems has been mostly on the isolation of microorganisms from these special ecosystems (see, for instance, Nazina et al., 2006; Magot et al., 2000). More recently, there has been an increase in the use of molecular microbiological methods (MMM), based on analysis of nucleic acid material (DNA and RNA). To perform such studies, various types of samples can be obtained from oilfield systems. Oilfield production waters (i.e. water produced at the well sites) have mostly been investigated, since produced water is relatively easy and cheap to sample. Still, due to the on average low cell densities in these production water samples (up to 10⁴ cells/ml, in best cases 10⁵ cells/ml) (Mueller and Nielsen, 1996; Nilsen et al., 1996) a filtration step is required to concentrate the cells before molecular analysis can be performed.

Since many microorganisms are attached to surfaces, the production water samples will give only part of the story, since only the suspended free cells are commonly produced along with production water. Also the risk of contamination by the drilling process itself and by the equipment is substantial, see for an example (Juck et al., 2005). Research on both oilfield core material and formation waters gives a more complete view of the indigenous organisms present in a subsurface environment (Vrionis et al., 2005). However, so far only a small number of cores have been analysed, due to the high costs of taking such a sample. In addition, the retrieval of a core sample usually involves the cooperation of an oil company.

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As a result of these difficulties, only a small number of articles can be found in the literature on the study of oil reservoir cores retrieved during the drilling of new exploration or production wells (Azadpour et al., 1996; Belyaev et al., 1983; Spark et al., 2000).

Despite this, it is important to learn more about oilfield ecosystems, since microbial activity not only causes issues like reservoir souring and oil pollution with H₂S, but potentially can also be used to our advantage, e.g. MEOR applications (Grigoryan and Voordouw, 2008; Sen, 2008). Therefore, a correct sampling strategy is a crucial factor for the study of down-hole communities. Here, we will present two case studies in which both sample pretreatment and nucleic extraction are addressed. These case studies demonstrate how to successfully retrieve the genetic material of two sample types: (i) production water and (ii) oilfield cores. The samples were taken from different locations. In addition, we will discuss some points of concern when sampling oilfield systems. Particular focus is given on the filtration of production water and the avoidance of filtration of the oil phase. Also, methods for treatment of cores and taking core fragments after retrieval of a core sample are addressed in detail.

Points of Concern When Sampling Oilfield Brine Waters

Various issues arise when taking brine water samples. Most obvious is usually the time in between the sampling and the actual analysis of the sample, especially with respect to samples taken from offshore locations, like drilling platforms. The arrival time in worst case scenario's can be weeks. Commonly these samples are taken in anaerobic containers, because oxygen intrusion can change the predominance of species in such a community (Moura et al., 2009; van der Kraan et al., 2009). In addition, also the time interval (bringing the sample to the surface) can induce changes since the surface conditions hardly match the down-hole conditions (e.g. in situ pH and temperature). A rapid fixation or storage of the samples at low temperature is essential to avoid a change in the composition of the original community (Rochelle et al., 2006). The second issue is the presence of oil and usually the vast amount of dissolved salts in the water phase, which can go up to salt saturation. High salt concentrations and the presence of oil disturb the DNA extraction (see Table 2.1).

Case Study 1: Brine Water Samples from a Dutch Oilfield and Its Surface Facility Units

In this study, 10 l brine water wellhead samples were taken from various beam pumps and oil–water separator sampling points. Samples were collected in sterile jerry cans. Samples from the oil–water separators were taken from the water phase (close to the oil–water interphase). When sampling the beam pumps, the first water

Table 2.1 DNA extraction from different filtered production water fractions

Sample (name)	DNA amount after extraction (ng/ μ l)	Wavelength 260/280 ^a	PCR result ^b
Filter, released water	nd ^c	nd	–
Cut filter pieces (much)	0.8	0.35	–
Cut filter pieces (less)	0.7	3.91	–
Water from filter	2.1	6.44	–
<i>Centrifuged water:</i>			
Pellet (10 \times)	20.1	1.76	+
Supernatant	1.42	nd	–
<i>Centrifuged backwashed water:</i>			
Pellet (10 \times)	17.3	1.8	+
Supernatant	nd	nd	–

^aThe quality of the genetic material is determined by the absorption ratio at 260 and 280 nm. For DNA/RNA the value should be between 1.8 and 2. Other values indicate that pollution is disturbing the measurement.

^bPCR is an amplification reaction, described in more detail later in this book. If the PCR reaction is positive, a final indication is given that the DNA extraction worked well and that it is pure enough to allow amplification.

^cnd value is not realistic.

fraction is discarded. This to exclude sampling of water that has been contained in the upper part of the piping system, which might hold another community due to this containment (Basso et al., 2005). The sampling location was an oilfield located in the western part of the Netherlands. The jerry cans were filled to the top and sealed with screw caps to avoid oxygen intrusion. Subsequently, the samples were taken to the laboratory and filtered directly to minimise the chance of community changes. For the collection and concentration of the cells, two times 4 l of brine water (in duplicate) was filtered using hollow fibre filters (Fig. 2.1).

The lamellas of these filters contain pores with the size of 0.22 μ m, which allow the liquid to pass through and retain the bacterial cells, which usually have a size between 1 and 2 μ m. Hollow filters offer an advantage over traditional filters since they allow a larger liquid volume to pass through before the filter gets clogged. This is due to their large filter surface area, which allows a more representative filtration (Somerville et al., 1989). During filtration procedures, filtration of the oil phase must be avoided, because oil blocks the filters and disrupts the extraction of DNA from the filtered cells. Avoidance of oil filtration can be achieved by allowing a natural separation between the oil and the water phase by natural flotation combined with a slow filtration by pumping the water phase from the bottom of the jerry can. Optionally in some studies a better release between the oil phase and cells that are attached to the oil phase is enforced by the addition of a surfactant (e.g. Tween).



Fig. 2.1 Hollow fibre filter (e.g. from Spectrumlabs, Mediakap-5). The filter lamella is clearly visible in the cartridge. This filter is suitable to filter larger volumes of water than traditional filtering approaches and can be stored without touching the filter, thus reducing the chance of contamination with allochthonous microorganisms

Pretreatment of Filters After Filtration with Brine Water Samples

After filtration a few choices can be made regarding the DNA extraction. The main purpose is to release the cells from the filter, which can be done in various ways. First, the filter cartridge, in particular the filter lamella, retains some liquid after filtration, which might have a high cell density. Second, the filter can be back-washed with a sterile buffer solution to remove retained cells from the filter. Third, the filter with cells attached can also be analysed.

If the production water contains a lot of salt (e.g. >50 g/l) the released cell suspension can be centrifuged to remove most of the salt and traces of oil, which might disturb the DNA extraction. One filter of each of the earlier described sampled environments was taken, while the second filter was kept frozen as a backup. The filters used in this study released about 3 ml of suspension after opening. Opening of the hollow fibre filter cartridges was performed in a fume hood under a flame to avoid contamination from the air. The 3 ml of liquid was divided over three sterile Eppendorf tubes. Also the filter lamellas were back-washed with sterile buffer releasing even more cells. Finally, the filter lamellas were cut into small pieces using sterile scissors. Liquid samples were centrifuged for 1 min at 13,200 rpm. Subsequently, 90% of the supernatant was removed, thereby achieving a 10 times concentration of the biomass and a removal of potential PCR inhibitors. The cell pellet was resuspended into the remainder of the supernatant. Four types of material now served as a basis for DNA extraction: (1) released suspension, (2) back-washed suspension, (3) centrifuged cell/particle pellets and (4) small cut filter pieces.

Alternative Sampling Using Specific Commercial Concentrator Kits

If the sample location is too far from the laboratory, then an on-location filtration can be applied. This can be performed with specific commercial kits. One type of kit has proven itself to be specifically convenient for sampling brine waters. The kit contains filters that hold chemicals to lyse cells and to denature proteins. The water is filtered and the cells that the water contains fall apart when they come in contact with the filter due to the present compounds. The DNA is preserved in the filter and proteins are denatured. Subsequently, the filter can be frozen and taken back to the laboratory for further analysis. An example of such a kit is the FTA Concentrator Kit produced by WhatmannTM.

Furthermore, DNA preservation is prolonged extensively if kept below 0°C. Pieces of these filters can be used directly for microbial community analysis using molecular techniques. Since such a procedure was not necessary here, this sampling route has not been applied. The major concern with this approach is that lysis of cells is not a constant factor. The cells of various species vary in their resistance towards cell lysis and cells of some species might not lyse and therefore will not be detected in the molecular analysis. This is due to differences in their cell wall composition (Gram negative or Gram positive). Extraction methods may therefore give different results (Hong et al., 2009). Another alternative that can be considered is bringing the filter set-up (pump, hoses, filters, etc.) to the sample location. This might involve also a power generator to supply the electrical pump with energy.

Taking Oilfield Core Fragments Without Contamination

Often researchers do not always have influence on the retrieval of the core sample which is commonly performed by the oil company. It is important that contamination during retrieval of the core remains limited and with respect to the preservation of the genetic content should be frozen as quickly as possible. Various methods exist to estimate core sample contamination. To discuss these in detail would be beyond the scope of this chapter. Mostly they involve chemical tracers indicating drilling mud intrusion. Core samples are after retrieval checked for the intrusion of drilling mud in the core itself using a microscope. Also microbial tracers can be used exposing a core to ‘positive control’ microorganisms (for more information on contamination checks, see Colwell et al., 1992; Zhang et al., 2005).

During the transport of a core sample, it is advised to keep the temperature below 0°C. This can be achieved by storing the core in dry ice (solid CO₂). To be sure that the sample that will be analysed has not been subjected to any kind of laboratory contamination, the core itself can be cut in a fume hood or a sterile cabinet using a sterilised chisel. It is also possible to cut the core while keeping an active gas burner next to it; this provides an air updraft, preventing contamination from the air. Pieces of the inner part of the core can now be obtained that have not been

in contact with the outside atmosphere or drilling mud. It is important to keep the core at a temperature below 0°C. This can be performed by leaving the core itself contained in solid CO₂. Also some laboratories are equipped with a cold-room. The obtained core samples now have to be crushed; this is done with a sterile pestle and mortar. The subsamples are kept below 0°C in liquid nitrogen (N₂) during the grinding (Zhang et al., 2005); the obtained sand-like material is now ready for DNA extraction using commercial extraction kits.

Case Study 2: Sampling and Treatment of Different Core Samples

Core 1: Unconsolidated Sandstone from an Oilfield in Gabon (Africa)

Our laboratory received a frozen core sample from an oilfield in Africa. The core consisted of unconsolidated sandstone and was obtained from a depth of 1,154 m. The in situ temperature and pressure of the oilfield were 43°C and 120 bar, respectively. The porosity of the core was 24–32% with a permeability of 1.0–3.5 Darcy. The core was salt saturated. Small pieces of core were taken from four different spatial positions. Also a piece of the outer core was taken as a control, this to check if the outer area of the core had a different species composition indicating contamination. Core pieces were crushed under liquid nitrogen using a sterile pestle and mortar (Fig. 2.2). The pestle and mortar were sterilised before usage by autoclaving

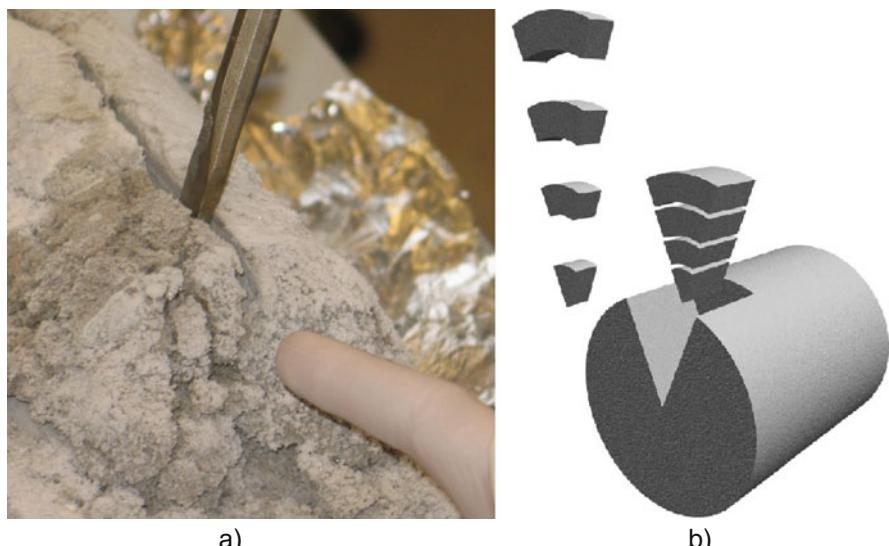


Fig. 2.2 (a) Image of a core sample, (b) Spatial sampling of the core (schematic)

at 160°C for 4 h. DNA extraction on the crushed material was performed using a commercial DNA extraction kit (MoBio soil DNA extraction kit).

Core 2: Sandstone from an Oilfield in Rotterdam (The Netherlands)

Our laboratory received two core samples from an oilfield located in the western part of the Netherlands. Both cores were retrieved from separate sample locations coming from a depth of around 2,000 m. The core material resembled coal ashes; it held a very loose structure. In this case grinding was not needed. Subsamples were taken out of three different areas in the cores using a sterile spatula. The upper layer of the material was scraped off before taking the 1 g samples, to rule out contamination from the air. During these procedures, the core was kept below 0°C.

The first attempts to retrieve DNA from these core samples using the standard protocol provided by the manufacturer of the DNA extract kits were unsuccessful. We assumed that the released DNA could adsorb strongly to the core material which resembled a kind of active carbon, which is known as an effective sorbent. We therefore modified the standard protocol and in addition tried different DNA extraction kits (see Appendix).

Standard DNA Extraction Method (Brine Water Samples and Core 1)

Nowadays commercial DNA extraction kits are widely available. Usually they are based on a series of extractions and removal of substances that can inhibit future DNA amplification reactions. In most cases there is also a protein removal step included. To lyse the cells bead beating is used. The starting material containing the cells is placed in a tube containing beads and is subsequently subjected to heavy shear stress thereby breaking the cells (beating). The commercial kits commonly involve the following steps:

- A cell lysis step in which the cells are disrupted releasing the genetic content into the solution
- A removal step in which PCR inhibitors are removed from the solution
- A protein degradation step
- Several cleaning steps
- A step in which the genetic material (DNA, RNA) is concentrated

All the above-described samples in the brine water study and the first core were prepared and subjected to DNA extraction. In these two particular studies DNA was extracted using the soil DNA extraction kit (Mo Bio Laboratories Inc, Carlsbad)

according to the manufacturer's protocol. This kit was chosen since the crushed core sample resembles sandy soil and the brine water samples contained particles.

Subsequently the amount of DNA from all extractions was quantified using a Nanodrop 1,000 Spectrophotometer (see Table 2.1 for an example of a performed DNA extraction). The obtained DNA was then used in further studies to analyse the microbial community.

Alternative and Improved DNA Extraction Methods

Usually the soil DNA extraction kit (MoBio) is sufficient to obtain a good DNA extractions from various samples (see Table 2.1). However, for some samples, such as the core samples from the Rotterdam oilfield, a pretreatment, or the use of a different extraction kit is necessary. In this case the Powersoil DNA extraction kit (Mobio)TM was tried. However, this kit does not result in the desirable extraction efficiencies. To improve the DNA extraction procedure using the Powersoil extraction kit, various pretreatment steps were tried. Addition of "so-called" chaperone compounds to the soil sample prevents the genomic DNA from absorbing to the core material by shielding charges. Two effective substances are skimmed milk (a concentrated mixture of proteins) and poly-deoxyinosinic-deoxycytidylc acid (poly-dIdC) which competes with the DNA for adsorption. In our case study, two of the three pretreatments proved successful, i.e. (i) use of different washing steps and (ii) the addition of skimmed milk. To cover all different DNA extraction methods would be beyond the scope of this chapter, but various other DNA extraction methods are available (see for an example Pel et al., 2009), in which separation is performed based on the nonlinear response of long, charged polymers like DNA. Another example is van Doorn et al., (2009) in which internal amplification controls are used.

Conclusions

Overall conclusions that can be drawn are (1) Samples need to be preserved either by fixation or by freezing as soon as possible to prevent community changes; (2) production water can best be filtered over hollow fibre filters to avoid rapid clogging of the filter and contamination with allochthonous microorganisms. The overall conclusion with respect to the two case studies presented is that most effort should be put in to cleaning the samples thoroughly to get rid of various extraction and PCR inhibitors, before a DNA extraction is started. If samples from different environments have to be compared, a consistent DNA extraction protocol is required to be able to draw sound conclusions from the comparison.

The applied pretreatments can be different in any case when samples from oil-fields are taken. In the case of the brine water samples, the centrifuged cell pellets gave the best result with respect to the DNA yield and the amplification step. Dependent on the type of core material, a choice should be made, as to which additional pretreatment is selected if a direct extraction is not successful. In the case

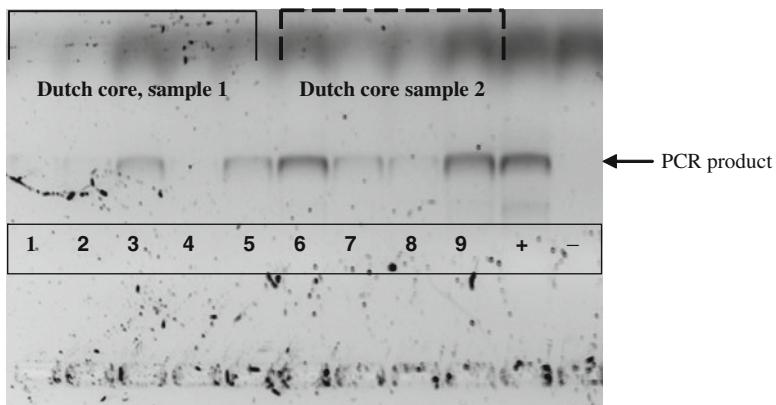


Fig. 2.3 Agarose gel electrophoresis of PCR products obtained with DNA extracted from two core samples using different pretreatments. Lanes 1, 5 and 6: washed samples; lanes 2 and 7: addition of poly-dIdC; lanes 3 and 9: addition of skimmed milk; lanes 4 and 8: no pretreatment; + is the positive control reaction; – is the negative control reaction

of the Dutch core samples, sample washing and addition of skimmed milk proved to be the most effective for DNA extraction (Fig. 2.3). Eventually in both case studies the DNA was successfully extracted and studied.

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Part II

**Application of Molecular Microbiological
Methods to the Oil Industry**

Chapter 3

Application of Molecular Microbiological Methods to the Oil Industry to Analyse DNA, RNA and Proteins

Sean M. Caffrey

Overview of Molecular Microbiological Methods (MMM)

Microorganisms have a tremendous impact on the deep biosphere. They are fundamental components of the global carbon, sulphur, nitrogen and energy cycles (Trudinger and Swaine, 1979). Microorganisms are also responsible for shaping our natural resources, for example, the conversion of light to heavy petroleum reserves is a consequence of biodegradation (Head et al., 2003). While microorganisms can negatively affect oil reserves by increasing oil density, sulphur content, acidity, viscosity and metal content of the oil (Aitken et al., 2004), microorganisms have also been used to improve oil production. Bacterial biomass production has been used to reduce reservoir porosity, biosurfactants can reduce oil viscosity and biogenic methane production has been proposed as a method to release energy from unrecoverable hydrocarbon resources (Gray et al., 2009; Ollivier and Magot, 2005). Regardless of whether the effect is positive or negative, it is essential to ascertain the members, numbers and activities of the microorganisms present in any resources we hope to effectively manage.

Biodiversity in most environments is dominated by uncultured microorganisms (Amann et al., 1995). Many microbial species are difficult to culture and study in isolation because standard laboratory culturing conditions fail to facilitate growth, these microorganisms are dependent on other organisms for critical processes or some microorganisms can readily enter nonculturable states. In some environments, such as soil, it has been estimated that as many as 99% of the endogenous species are uncultivable with existing methodologies (Amann et al., 1995). This means that any survey of microbial communities relying primarily on culture-based techniques, such as plate counts or most probable numbers (MPNs), will severely underestimate the existent microbial diversity. The severe limitation of culture-based environmental microbiology has made the development of molecular methods targeting RNA, DNA and proteins a necessity.

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Metagenomic techniques bypass culturing steps by sequencing the DNA isolated directly from environmental samples; this allows metagenomics to provide a nearly comprehensive representation of an environment's microbial biodiversity, see Table 3.1 for an overview of metagenomic methods.

For microbial community analysis, either the total environmental DNA or an enriched fraction of the DNA extracted from the environment can be utilised. PCR amplification is the technique most commonly used to selectively enrich a particular or set of genes present in the environment. The use of PCR amplification to enrich the target genes eliminates a major problem of metagenomic (environmental) sequencing, i.e. limiting amounts of difficult to process samples. Even if it is possible to obtain large volumes of sample material, samples containing oil, fine clays or other contaminating material can make DNA extraction problematic. As long as the sequences are known, PCR primers can be developed that will target genes of individual species, groups of related species or even all microorganisms. The targeted genes could be phylogenetic markers or functional genes. Targeting functional genes will provide information not only about the presence of species in the environment but also of the activities that occur there. The dissimilatory sulphite-reducing genes (*dsrA*) are often used as targets to assess the presence of sulphate-reducing bacteria and/or sulphate reduction in an environment (Karr et al., 2005). The appreciation that the 16S rRNA gene can act as an evolutionary chronometer (Woese, 1987) and can therefore provide a method of species identification (Lane et al., 1985) has made the 16S rRNA gene of primary interest in determining the phylogenetic structure of an environment, and therefore sequenced-based metagenomic studies interested in phylogenetic analysis often target this gene.

Techniques employing PCR primers for the 16S rRNA gene target the conserved regions and amplify either the entire gene or a portion of the gene containing one of the nine variable regions (Giovannoni et al., 1990; Dams et al., 1988). Furthermore, primers targeting the 16S rRNA gene can be tailored to amplify the gene from a small selection of related microorganisms or can be universal and amplify nearly all eubacterial and archaeal species (Baker et al., 2003; Morales and Holben, 2009). Although universal PCR primers are intended to amplify sequences from all microorganisms equally, this is typically not the case and therefore the amplification process will introduce some bias in the community analysis (Baker et al., 2003; Lueders and Friedrich, 2003). Additionally, not all microorganisms contain the same number of rRNA gene copies in their genome and since rRNA gene copy has been correlated with growth rate in some soil bacteria, slow-growing, difficult to culture bacteria maybe underrepresented when the 16S rRNA genes are used for community analysis (Klappenbach et al., 2000).

Once the PCR-amplified sequences (amplicons) are prepared, an estimate of the microbial diversity can be obtained from post-PCR fingerprinting techniques, such as DGGE (Denaturing Gradient Gel Electrophoresis) or T-RFLP (Terminal-Restriction Fragment Length Polymorphism). DGGE gels are capable of separating PCR products with very similar length, but different GC content. The number of bands on the gel provides an indication of species diversity. How the banding pattern changes over time or between samples suggests how the community

Table 3.1 Overview of metagenomic techniques

Technique	Description	Species present	Species number	Function
DGGE	Fingerprinting technique, PCR – amplified genes are separated on a denaturing gel; bands can be excised and sequenced	<i>Pro:</i> allows rapid visualisation of microbial diversity; illustrates community dynamics; identifies all organisms in sample including unknown microorganisms by sequencing extracted bands <i>Con:</i> poor results when diversity is high; bands must be excised individually and sequenced for community members to be identified; non-quantitative; limited by efficiency and specificity of universal primers	Not quantitative	Can be used to analyse diversity of functional genes (i.e. <i>dsyAB</i>)
Amplicon sequencing	Pyrosequencing of 16S rRNA amplicons (pyrotags) or functional genes	<i>Pro:</i> identifies all community members; high throughput can identify nearly all present species <i>Con:</i> limited by efficiency and specificity of universal primers	Not quantitative	Although not routinely performed, amplicons of functional genes can be analysed
Random shotgun sequencing	Pyrosequencing of total DNA	<i>Pro:</i> all or any phylogenetic markers can be analysed minimising bias from any one method <i>Con:</i> expensive and requires more starting material than amplicon sequencing; data storage and bioinformatics analysis are complicated	Can be semi-quantitative if whole genome amplification is not required	<i>Pro:</i> functional genes may be analysed; providing a comprehensive profile of metabolic processes in the environment <i>Con:</i> large amount of DNA required; short reads make linking functional genes to specific organisms difficult; need for sequencing depth and bioinformatics support can be expensive

Table 3.1 (continued)

Technique	Description	Species present	Species number	Function
qPCR	Fluorescent dyes and a calibration curve or end point analysis used with PCR primers along with a calibration curve to determine the starting gene copy number in a sample	<i>Pro:</i> highly sensitive <i>Con:</i> amount of data available produced depends on post-PCR analysis technique used	<i>Pro:</i> high sensitivity and produce quantitative or semi-quantitative results <i>Pro:</i> high throughput	<i>Pro:</i> quantitative <i>Con:</i> target must be identified ahead of time and sequence must be known; a limited number of simultaneous targets
FISH	A fluorescent oligonucleotide probe hybridises directly to RNA inside cells	<i>Pro:</i> allows species identification and the observation of spatial interactions between microorganisms <i>Con:</i> target must be identified ahead of time; a limited number of simultaneous targets can be analysed	<i>Pro:</i> localisation in addition to quantification <i>Con:</i> limited throughput <i>Con:</i> labour intensive	<i>Pro:</i> allows function identification and the localisation of functions occurring in sample <i>Con:</i> target must be identified ahead of time; a limited number of simultaneous targets
Microarray	A glass slide coated with fragments of specific genes; labelled ribosomal RNA, mRNA, cDNA, DNA are hybridised to the microarray, signal identifies when the target nucleic acid is present	<i>Pro:</i> relatively high throughput and semi-quantitative <i>Con:</i> can only determine species presence if probe for organism presents on array	<i>Pro:</i> semi-quantitative <i>Con:</i> only determines species presence if probe for organism presents on array; cross-hybridisation can limit quantisation of low intensity signals	<i>Pro:</i> relatively high throughput and semi-quantitative <i>Con:</i> can only determine gene presence if probe for gene presents on array; function inferred from sequence homologies of expressed genes
SIP	Radioactively labelled substrates are incorporated into active microbial community members	<i>Pro:</i> only identifies active species; not high throughput <i>Con:</i> not high throughput	<i>Pro:</i> can quantitatively identify active species <i>Con:</i> not high throughput	<i>Pro:</i> can quantitatively determine metabolite utilisation <i>Con:</i> not high throughput

structure is changing (Muyzer and Smalla, 1998). For species identification, individual bands can be excised and sequenced. Although a popular technique, DGGE suffers from several technical limitations such as the inability of resolving samples with high levels of diversity. The limited amount of sequence information provided is a more fundamental limitation, since the sequence (and phylogenetic information) is only available for the bands that are excised and sequenced. T-RFLP analysis uses primers containing different fluorescent labels to amplify the 16S rRNA gene from the community DNA (Luna et al., 2009). The amplicons are then digested with restriction enzymes to release one of the labelled ends. These fragments can be separated on a gel for visualisation and purified for sequencing. Directly sequencing all the PCR amplicons eliminates this problem, but the high cost of generating clone libraries for capillary sequencing typically limits the size of the clone libraries to less than 500 clones and consequently only allows the highly abundant community members to be identified (Dunbar et al., 2002). The development of second-generation sequencing technology has dramatically increased the throughput and decreased the cost of DNA sequencing, allowing hundreds of thousands of sequences to be generated (Huber et al., 2007). The main limitation of the second-generation sequencing technologies has been the short read lengths. While the capillary sequencing of clone libraries can generate sequences of the entire rRNA gene, second-generation sequencing can only target one or several of the rRNA's variable regions. Clone libraries containing fragments of the extracted environmental DNA also have the advantage that they can be used as a platform for functional analysis (functional metagenomics), using techniques like heterologous gene expression (Handelsman et al., 2002).

Unless the 16S rRNA gene sequence can identify the organisms as being closely related to a well-studied organism, the 16S rRNA gene sequence will not provide information on the role this organism plays in the microbial community. Sequencing all the DNA (total DNA) extracted from an environment can circumvent this problem. Random shotgun sequencing of total environmental DNA will not only retrieve the sequences of the 16S, rRNA gene and other phylogenetic markers, such as *recA*, but also determine the sequences of all the genes, providing an indication of the genetic potential present in an environment (Tringe and Rubin, 2005). Total DNA sequencing can determine if important functional genes, such as dissimilatory sulphite reductase genes, are present in the environment. The type and diversity of the functional genes present can provide an indication of the biological processes shaping the environment being studied (Kunin et al., 2008). As with 16S rRNA gene amplicon sequencing, the cost of generating clone libraries of the total DNA to facilitate capillary sequencing can be cost prohibitive. Even utilising second-generation sequencing techniques can be costly, as random shotgun sequencing will require a larger number of sequencing runs, greater volumes of starting material and will have a much greater cost associated with data storage and analysis than is required for amplicon sequencing.

Although the aforementioned techniques are capable of determining detailed microbial community structures, they are generally not fully quantitative and therefore do not provide information on the numbers of the community members

(Smith and Osborn, 2009). Quantitative PCR (qPCR), FISH (Fluorescence in situ Hybridisation) and microarrays are tools that can quantify the number of particular microorganisms in the environment. Like traditional PCR, qPCR can target either the 16S rRNA gene, other phylogenetic markers or functional genes. However, unlike PCR, qPCR uses end point analysis to determine the relative quantities of the genes present in or between environments. The FISH technique utilises fluorescently labelled probes that target genes in the environment, as long as their sequences are known. Typically the 16S rRNA genes or an important functional gene is targeted in FISH analysis. By measuring the amount of fluorescence incorporated by the probe binding to its target, FISH allows for the identification and enumeration of the microorganisms in an environment. With the use of fluorescence microscopy, FISH can also illustrate the spatial localisation of these microorganisms (Bayani and Squire, 2004). During the manufacture of microarrays, probes targeting thousands of genes are fixed to glass slides. For the analysis of environmental samples, microarrays can contain either probes targeting 16S rRNA genes (Phylochips) or probes targeting functional genes (functional gene arrays/geochips) (He et al., 2007; Schonmann et al., 2009; Zhou, 2003). Microarrays have the advantage of providing semi-quantitative information and unlike the aforementioned methodologies, microarrays are a higher throughput technology and can target a large number of genes in a single experiment. Microarrays do suffer from the same limitation as qPCR and FISH; the sequences of the target gene must be known before probes can be designed and only organisms and genes for which probes are developed will be identified. Consequently, unknown and untargeted organisms/genes will be missed; this is not a problem inherent in metagenomic sequencing technologies.

Simply determining which microorganisms are present in an environment does not provide what might be the most relevant information – *which microorganisms are active?* Although proteins are the active molecular entities, the high-throughput study of protein expression in environmental samples (metaproteomics) is still an emerging field (Maron et al., 2007). Consequently, RNA expression (transcriptomics) is typically used to assess gene expression. Once RNA is isolated from environmental samples and converted to cDNA, microarrays, PCR and metagenomic sequencing techniques can be utilised (Gilbert et al., 2008; Parro et al., 2007). Microarray and PCR methodologies suffer from the same limitations when used for transcriptomics as when they are used for community analysis. Arrays made for functional or phylogenetic markers will only provide information about genes for which probes have been generated. SIP (stable isotope probing) is an approach for linking microorganisms to specific functions within an environment (Radajewski et al., 2003). SIP involves introducing a stable isotope-labelled substrates into a microbial community and following the flow of the labelled substrates by isolating diagnostic biomarkers such as fatty acids and nucleic acids which have incorporated the label. The molecules in which the isotope label appears provide identifying information about the organism that used the substrate. For example, by analysing the incorporation of labelled nucleotides into the 16S rRNA of bacteria, the actively growing members of the community can be determined.

The emergence of metagenomics has facilitated for the first time a truly comprehensive examination of microbial community structures and functions. The molecular tools now exist to examine an entire community's structure and functions using both low-and high-throughput techniques. The detailed information provided by these methodologies will enable informed decisions regarding resource management to be made. The following chapters give a more detailed description of each individual molecular microbiological methods (MMM) and how the technique can be applied to oil systems in relation to the specific biological questions being addressed.

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Chapter 4

Which Microbial Communities Are Present?

Importance of Selecting Appropriate Primers and Probes for Use in Molecular Microbiological Methods (MMM) in Oilfields

Ketil Bernt Sørensen

Introduction

Molecular microbiology techniques play an increasing role in the oil industry. Most of the current applications are based on either Fluorescence in situ Hybridisation (FISH) or polymerase chain reaction (PCR) or some variation thereof. These types of approaches require the use of oligonucleotide primers and probes (i.e. short fragments of DNA that are complementary to the target DNA/RNA of the microorganism of interest). In the case of FISH, the probes are fluorescently labelled in order to identify the target cells. Before undertaking either FISH or PCR approaches, it is important to select the most appropriate primers or probes for targeting the microorganisms of interest in a given environment. If appropriate primers/probes are unavailable in the literature, then new primers/probes should be developed based on the gene sequences currently available in the sequence databases. Generally, primers/probes may target either the small subunit (SSU) rRNA genes or the specific functional genes. This chapter provides a brief introduction to both of these aspects. Further detailed information is provided in subsequent chapters.

Primers and Probes that Target the 16S rRNA Gene

All prokaryotes contain ribosomes that are encoded by the 5S, 16S, and 23S rRNA genes. In 1987, Carl Woese described the 16S rRNA gene as ‘an evolutionary clock’ and since then it has become the primary gene of interest in determining the phylogenetic diversity of prokaryotes. This is due to the fact that the 16S rRNA gene has both highly conserved, variable, and hypervariable regions. PCR primers which target the conserved regions will amplify the 16S rRNA genes from nearly all bacterial or archaeal species and are sometimes termed ‘universal bacterial’ or

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Table 4.1 Examples of general 16S rRNA-targeted FISH probes and PCR primers used for studies of prokaryotic communities

Name	Application	Target	Nucleotide sequence (5'-3')	References
UNIV1392	FISH	Most <i>Prokaryotes</i>	ACGGGCGGTGTGTRC	Olsen et al. (1986)
ARCH915	FISH	Most <i>Archaea</i>	GTGCTCCCCGCCAATTCCCT	Stahl and Amann (1991)
EUB338	FISH	Most <i>Bacteria</i>	GCTGCCTCCCGTAGGAGT	Amann et al. (1990)
BAC8F	PCR	Most <i>Bacteria</i>	AGAGTTTGATCMTGGCTC	Godon et al. (1997)
BAC1492R	PCR	Most <i>Bacteria</i>	GNTACCTTGTACGACTT	Godon et al. (1997)
ARC344F	PCR	Most <i>Archaea</i>	ACGGGGCGCAGCAGGCGCGA	Raskin et al. (1994)
ARC958R	PCR	Most <i>Archaea</i>	YCCGGCGTTGAMTCCAATT	Delong (1992)

‘universal archaeal’ primers (Baker et al., 2003; Morales and Holben, 2009). Several such universal primers and probes are available in the literature (examples are given in Table 4.1) and can be employed if one intends to characterise or enumerate the general community of microorganisms in a sample using the 16S rRNA gene as a target. Universal 16S rRNA primers/probes have been applied with great success in virtually any environment within the Earth’s biosphere, including locations as diverse as wastewater treatment plants, acid mine drainage, deep-sea subsurface sediments, and oil reservoirs (de los Reyes et al., 2002; Sørensen and Teske, 2006; Tan et al., 2009; Zhu et al., 2003).

It is also possible to design primers/probes that target the variable and hyper-variable regions of the 16S rRNA gene. This facilitates the development of group-specific, genus-specific, and species-specific primers/probes. Such specific primers allow the amplification of the 16S rRNA gene from a small selection of closely related microorganisms. In the case of group-, genus-, and species-specific primers and probes, it is important to note that although the organisms in a phylogenetic group contain relatively similar 16S rRNA molecules, they may be functionally highly diverse. For example, the phylogenetic group *Deltaproteobacteria* contain sulphate- and sulphur-reducing, nitrate-utilising, aerobic, and iron-reducing microorganisms. Examples of specific phylogenetic FISH probes are given in Table 4.2.

Primers that Target Specific Functional Genes

Although analysing the phylogenetic diversity in a given environment is of great interest in molecular microbial ecology studies, for the oil industry it is often more important to target a specific catabolic process and the key functional groups of

Table 4.2 Examples of 16S rRNA-targeted FISH probes used for studies of specific phylogenetic groups of microorganisms

Name	Application	Target	Sequence (5'-3')	References
ALF1b	FISH	Most <i>Alphaproteobacteria</i> , some <i>Deltaproteobacteria</i> , <i>Spirochetes</i>	CGTTCGYTCTGAGCCAG	Manz et al. (1992)
BET42a	FISH	<i>Betaproteobacteria</i>	GCCTTCCCACATCGTTT	Manz et al. (1992)
GAM42a	FISH	<i>Gammaproteobacteria</i>	GCCTTCCCACATCGTTT	Manz et al. (1992)
SRB385	FISH	Most <i>Desulfovibrionales</i> and some other <i>Bacteria</i>	CGGCGTCGCTGCGTCAGG	Amann et al. (1990)
SRB385Db	FISH	<i>Desulfobacterales</i> , <i>Desulfuromonales</i> , <i>Syntrophobacterales</i> , <i>Myxococcales</i> , and other <i>Bacteria</i>	CGG CGTTGCTGCGTCAGG	Rabus et al. (1996)

microorganisms involved. A functional group is defined as a group of microorganisms that share an important metabolic function in the specific system, e.g. the ability to convert sulphate to hydrogen sulphide or to generate methane. In many cases these functional groups do not form a coherent phylogenetic group. For example, sulphate reducers encompass several phylogenetic groups including, among others, the *Deltaproteobacteria*, gram-positive *Clostridia*, and archaeal thermophiles. At the same time, each of these phylogenetic groups contains numerous non-sulphate reducers. As a consequence it is not possible to target the entire group of sulphate reducers by applying 16S rRNA-based primers or probes. Instead, one has to turn to functional genes, i.e. genes that encode some enzyme with an important function in the organism.

Functional groups such as sulphate-reducing prokaryotes (SRP), nitrate-utilising bacteria (NUB), and methane-producing archaea (methanogens) all contain one or more key genes that are necessary to generate the enzymes involved in their metabolism. Thus, all sulphate reducers contain genes for the enzymes dissimilatory sulphite reductase (DSR) and adenosine 5'-phosphosulphate reductase (APS). These enzymes catalyse key steps in the conversion of sulphate to sulphide. Methanogens contain genes for methyl coenzyme M reductase (MCR), which is necessary for the production of methane. When properly enumerated, these genes represent a convenient measure for the number of sulphate reducers and methanogens, respectively, in a sample.

Studying and enumerating functional groups of microorganisms are done by applying PCR primers that are specific for the signature genes of the particular

group. Compared to genes for 16S rRNA, the functional genes are much more variable in their sequence, and it is often not possible to design a primer set that can simultaneously target all known members of a functional group. Depending on the nature of the system under investigation, previously published primers may or may not detect all the functional genes and thus microorganisms that are present. In this respect, oil systems represent an extreme environment with high temperature and high pressure, and the microorganisms that are present are often very different from those in natural environments. Therefore, primers for functional enzymes that work well, for example, in mesophilic, marine sediments are often not suitable for oil systems. An example of this is given elsewhere (see [Chapter 10](#)).

Conclusions

As is the case for any other analytical technology, the molecular approach also has its own pitfalls and limitations. Thus, the use of suitable primers is a prerequisite for successful application of PCR to detect or enumerate specific genes in a sample, and suitable probes must be used for FISH in order to visualise and count the microorganisms correctly. A necessary first step in setting up a microbiological surveillance programme of specific troublesome groups such as SRP or methanogens in an oil system is to characterise the microorganisms that are present. This initial system review will determine which types of microorganisms are abundant and thereby which primers or probes are suitable for the system. When performed by trained personnel, the system review and subsequent evaluation and validation of primers and probes should not pose any major challenge.

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Chapter 5

Which Microbial Communities are Present?

Application of PCR-DGGE: Case Study on an Oilfield Core Sample

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Introduction

In this case study a PCR-DGGE analysis has been performed on an oilfield core sample. In many scientific articles PCR-DGGE analysis is often referred to as one analysis technique, but in fact it is a combination of the PCR and DGGE techniques performed in succession (Muyzer et al., 1993). When PCR and DGGE methods are combined, this analysis can provide a quick fingerprinting tool in which sampled environments can be compared. An additional advantage of DGGE is that it also provides information on the genetic content of a sample in the form of relatively small pieces of DNA or RNA sequence data. This can be either data on the microbial diversity in the form of 16S rRNA gene fragments or information on metabolic activity in the form of sequence data of partial functional gene fragments. The 16S rRNA gene codes for a compound that is part of the cellular machinery, which translates DNA into protein. The sequence is a conserved region, which makes comparison of gene sequences possible. A PCR-DGGE analysis results in an image in which each different environment displays a specific banding pattern.

PCR-DGGE Fingerprinting

When performing a PCR-DGGE analysis, a particular gene or gene part is first selectively multiplied using PCR. Primers (small single-stranded DNA molecules holding commonly 20 bp, also known as oligonucleotides) are added to a pool of environmental DNA (extracted from an environment of interest, see [Chapter 2](#)). These primers are homologous to chosen regions of the DNA (chosen such that this region is in range of the targeted gene). During denaturation of the DNA, the primers hybridise to this chosen homologous position and serve as a starting point for an elongation reaction performed, by an enzyme capable of replicating DNA

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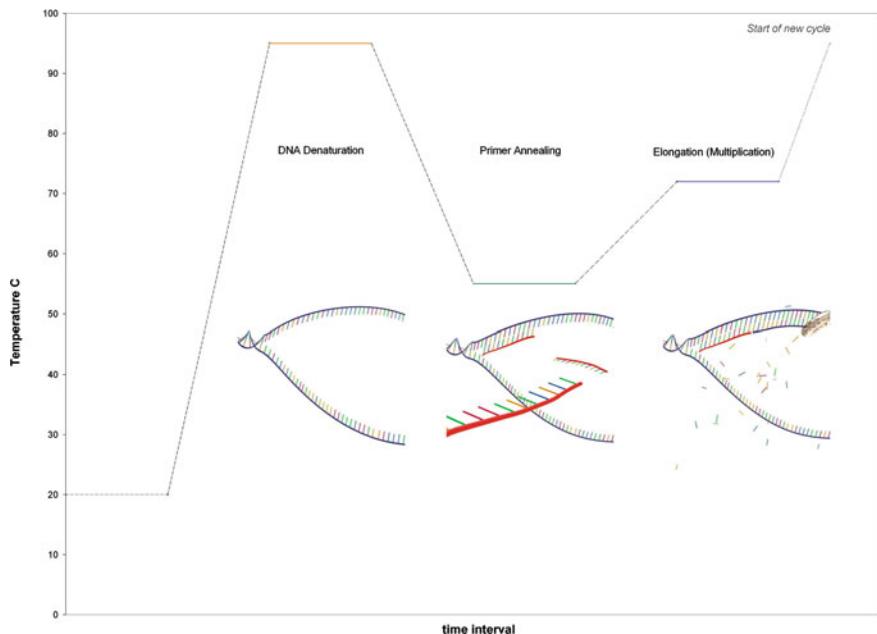


Fig. 5.1 Different steps of a PCR cycle (denaturation, primer annealing and elongation)

(DNA polymerase). The gene (fragment) is now copied. Going through a series of such multiplications (amplifications) the gene of interest is copied exponentially. Following the PCR reaction up to 10^{11} – 10^{12} gene copies, in general, of the targeted gene are created (this of course depends on the amount of original genes added to the PCR mix at the start). This provides an idea on the sensitivity of the PCR technique. But since PCR is so sensitive, contamination can be an issue, i.e. primers binding preferentially to DNA from a contamination source rather than from the target DNA. Contamination is especially important when the target DNA is present in low concentrations.

The PCR Cycle

A PCR amplification procedure consists of a constant repetition of changes in temperature, allowing different steps/reactions to occur. A typical PCR cycle is as follows (Fig. 5.1):

- *Melting (denaturation) of the DNA:* the temperature is increased to 95°C, at this temperature the DNA loses its helical structure and becomes single stranded.
- *Primer annealing:* the temperature is lowered to (commonly to 55°C) allowing primer attachment (The temperature of annealing depends on the properties of

the oligonucleotide and is therefore variable; the temperature of 55°C given here is a basic assumption.)

- *Elongation:* the temperature is then raised again to 72°C, allowing the DNA polymerase to copy the DNA from the annealed primers as a starting point.

After elongation, a next cycle commences with the temperature increased again to 95°C. But now the newly copied DNA also serves as a template, therefore PCR is exponential and can boost low DNA quantities up to several orders of magnitude. A common PCR amplification reaction is between 30 and 35 (of the above-described) cycle repetitions.

The DGGE Analysis

Following the PCR reaction, all the amplicons (PCR products) of interest will be of the same length. In a DGGE analysis, gene fragment separation is not based on gene size, but on the DNA sequence of the amplified fragment or more precisely on the ‘GC richness’ of a gene fragment. Commonly DNA and RNA consist of four bases, Adenine (A), Thymine (T), (Uracil (U) only in RNA), Cytosine (C) and Guanine (G). RNA is different than DNA in structure and function. RNA is single stranded and it is the intermediate step in the translation from DNA to proteins. A gene located on the DNA is first transcribed to an RNA molecule. This RNA molecule is subsequently translated into protein by cellular components called ribosomes. In DNA, the specific base pairs A–T(U) and G–C are formed. The G–C base pair in a double-stranded DNA molecule holds three hydrogen bridges and the A–T(U) base pair, however, holds only two hydrogen bridges (Fig. 5.2). This difference in hydrogen bridging causes changes in the so-called melting (denaturing) behaviour of the double-stranded DNA molecules. More G–C base pairs consequently results in a firmer bonding between the two complementary strands.

These differences in hydrogen bridging are exploited in the DGGE technique in which separation of sequences is based on this G–C base pair richness and the melting behaviour of the different multiplied gene fragments during the PCR. DGGE is an electrophoresis technique. The electrophoresis gel commonly used in DGGE analysis consists of polyacrylamide, which holds a matrix-like structure. This matrix holds chemicals (urea and formamide) that denature DNA. In the gel, a gradient from weak to strong is placed in the gel using these denaturants. A DGGE gel is cast between two glass plates. The glass plates are put in a holder leaving a small space in between. First, acrylamide solutions with denaturants are prepared. Acrylamide polymerase initiators are added and a gradient is cast between the glass plates. A denaturant-free fraction is cast on top of the gradient, the stacking gel. In this stacking gel, spaces are blocked for the acrylamide creating slots. The acrylamide now polymerises between the glass plates (this takes approximately several hours to complete).

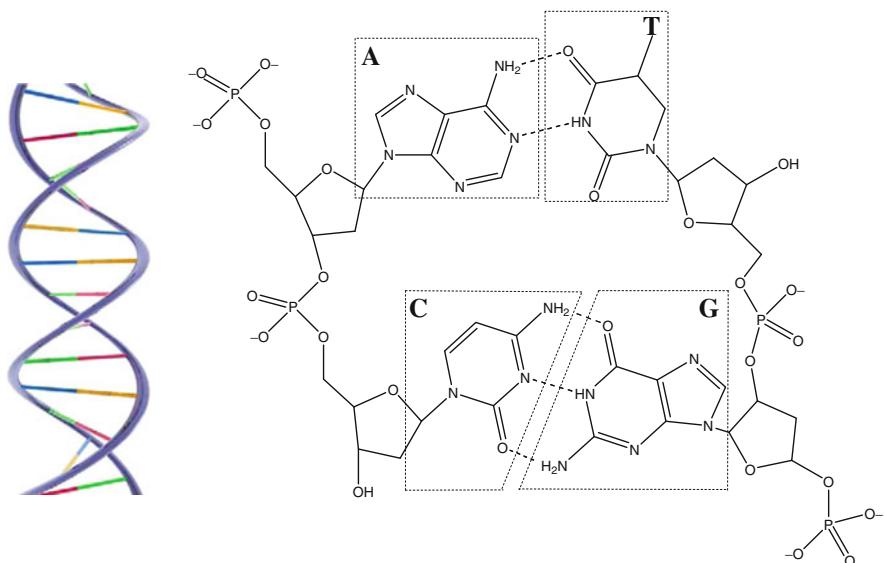


Fig. 5.2 DNA structure. (a) Artist impression of the double helix structure of DNA (simplified). (b) Schematic representation of the DNA base pairing and hydrogen bridges, with Adenine, Thymine, Guanine, Cytosine. Clearly shown is that the AT base pair holds only two H-bridges, while the GC base pair holds three. The connecting molecule bonds and structures are commonly known as the DNA backbone and consist of ribose and phosphate groups. The extra hydrogen bridge in the GC base pair is the key to the separation of PCR products in the DGGE technique. More GC base pairs in the gene fragment comply with more resistance to the denaturants

Products (gene fragments) from a PCR reaction are placed on a gel and are pulled through the gel using an electrical potential difference. As the (charged) gene fragments migrate downwards in the gel, they come across an increasing concentration of urea and formamide (DNA has a negative charge). These denaturants compete with the double-stranded DNA fragments for hydrogen bridges, since they contain unprotonated NH₂ groups holding an excess of electrons. If this competition becomes too strong, the DNA denatures (melts) and becomes a single-stranded molecule. To prevent a complete separation between the two strands, one of the primers holds a GC clamp. This is a 40-bp long sequence comprising only G–C base pairs. The clamp is added to all the multiplied gene fragments. This GC clamp does not dissociate thereby holding the two strands together. The high GC richness of the GC clamp makes it resistant to the denaturants and keeps the two complementary strands together after denaturation. A DNA molecule with two single strands held together by the GC clamp, migrates more slowly through the gel than a double-stranded DNA molecule, since it is more hindered by the matrix of the gel. As with the GC clamp, the more G–C base pairs a gene fragment has, the more resistant it is towards denaturation. Consequently it will migrate further down in the gel (Fig. 5.3). Not only the GC richness is determined by the melting behaviour of a gene fragment but also the distribution of the GC pairs through the gene

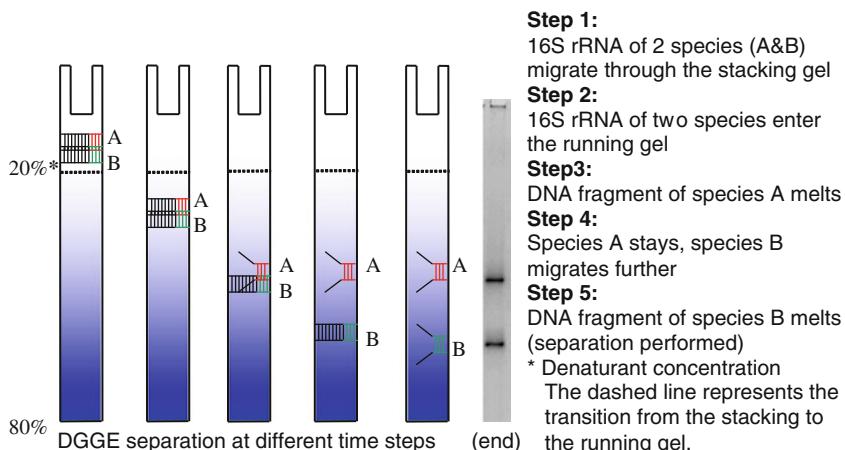


Fig. 5.3 Schematic overview of a DGGE lane followed through time including the end result. The grey tone indicates the concentration of denaturant at that location in the gel

fragment is of importance. If, for example, a gene consists of a ‘GC-rich’ domain and an ‘AT-rich’ domain, the ‘AT-rich’ domain will melt earlier than the GC-rich domain, creating a melted fragment, which also retards the migration through the gel, compared to a fragment that holds the same amount, but evenly distributed amount of GC base pairs.

The DGGE technique pulls the different gene fragments apart from each other based on the GC richness of a molecule, giving a characteristic banding pattern (fingerprint) when the gel is stained with a fluorescent dye and photographed. Examples of these dyes are ethidium bromide (photographed using UV light), SYBR Safe and SYBR Green (Invitrogen) (photographed using a blue light imager). Before a good separation is obtained, the separation protocol has to be optimised. In order to retrieve the actual sequence information contained in the band, it needs to be physically excised from the gel. The DNA now preserved in the band can be identified by using it as a template for reamplification by PCR and performing sequence analysis. When 16S rRNA gene sequence fragments have been amplified usually one band represents a single species although a species might show up as two bands. The separation resolution of DGGE, however, is limited since a better separation is performed on short gene fragments. The resolution of DGGE technique is lowered by usage of longer gene fragments, because of complementary melting domains.

Possible Improvements on the PCR Programme

If genomic DNA is retrieved from an environmental sample, it can hold in principle a diverse community. The sample can display one or multiple types of 16S

rRNA genes. If a PCR-DGGE analysis is performed on 16S rRNA gene fragments, the primers are designed to target the full bacterial or archaeal kingdom. This is done by locating annealing sites using databases containing many sequences, e.g. Probecheck. Annealing sites are sequences that are identical to all the targeted species. Still it is possible that the primers will anneal slightly better on certain 16S rRNA genes compared to others. This will cause some species to be overrepresented in the final PCR product pool. To circumvent this ‘preferential amplification’, a ‘Touchdown PCR programme’ can be implemented in the PCR programme (Don et al., 1991). During a ‘touchdown PCR’ analysis the annealing temperature, which usually is 55°C, is lowered during the first 20 PCR cycles with 0.5°C/cycle starting at a temperature above the annealing temperature. This is to improve the specificity of annealing (at first species that match 100% with the primers will be targeted). Since the primers anneal better with different 16S rRNA genes at different temperatures, this allows a more equal amplification of species in the first 20 cycles of the PCR. This touchdown will, however, not solve the problem completely. PCR can therefore still give a biased image of a community and even leave out species that are not amplified.

RNA Analysis Using PCR-DGGE

In contrast to PCR-DGGE performed on DNA, which is indicative for the presence of microbes in an environmental sample, a PCR-DGGE performed on RNA (e.g. *ribosomal*-RNA and *messenger*-RNA) provides insight in the active part of the total microbial population present. RNA is single stranded and subsequently more unstable than DNA. The RNA is therefore first ‘copied’ to DNA, creating cDNA (a DNA version of the RNA molecule, containing the code of the original RNA molecule) using an enzyme called reverse transcriptase. These cDNA gene fragments then can be analysed with DGGE, similar to a common DNA analysis, as described here. When looking at RNA, information can be obtained on expression levels of genes when conditions of an ecosystem are changed (Dar et al., 2007) (e.g. pollution with heavy metals, oil). RNA expression is much more sensitive to this kind of change than DNA; subsequently information that can be obtained from RNA can be more detailed than information from DNA. With RNA analysis a smaller subset of the population can be targeted. But since RNA is an unstable molecule, it is hard to keep the RNA intact. Therefore, RNA analysis is more difficult than DNA analysis.

Benefits of the PCR-DGGE Technique

The major advantage of PCR-DGGE is that the fingerprints (banding patterns) correspond to different environments, allow a direct and quick comparison of these

environments on one gel, by approximation, i.e. the 16S rRNA gene fragments of similar species migrate the same distance in a gel before denaturation. This allows a high-throughput screening of many samples, since bands can also be excised and sequenced. A better choice can be made, if an environment is further studied using, for example, clone library construction.

Limitations of the PCR-DGGE Technique

Although the PCR-DGGE analysis technique is rather straightforward and quick, the technique has limitations: The DNA gene fragments are not inert rods, they interact with each other in the gel. This makes comparison of different DGGE gels difficult. Often a cluster analysis is required to compare the fingerprints. This is especially the case when the results are on different DGGE gels. Only dominant species will hold sufficient amounts of original 16S rRNA gene copies to eventually show up as bands on a gel. So often, only sequence information on the dominant bands can be retrieved. This dominance, however, can be an artefact of the PCR itself as explained by the preferential amplification. That is PCR-DGGE cannot reliably used as a quantitative method. It can also occur that bands co-migrate to the same positions in the gel; this retards the possibility of retrieving a clean sequence from the band and questions the ‘one band one species’ concept. Also it can occur that a single species produces two or more bands on a gel. These ‘shadow’ bands can give a false-positive idea about the diversity of an ecosystem (Janse et al., 2004). Shadow bands (doublets) can also be an effect of degenerate primer pairs. Degenerate primers imply that the used primers are a mixture of highly similar sequences, except one or two base pairs which are different (Kowalchuk et al., 1997). This is usually done to broaden the range of species that can be detected using the primer pair. The disadvantage is that the species on a DGGE gel can show up as double bands. The double band artefact can also be caused by the fact that there is more than one RNA operon for a specific gene in the genome of a species (Nubel et al., 1996). Finally, DGGE is limited by the short sequence length of the gene fragments that can be recovered from the DGGE bands. This never allows a full comparison with full 16S rRNA genes as they are stored in databases (e.g. the GenBank database).

Case Study 1: PCR-DGGE on an Oilfield Core Sample

In this case study a PCR-DGGE analysis on 16S rRNA gene fragments has been performed on four different segments taken from an African oilfield core sample. Overall core properties are retrieved from a depth of 1154 m. The ambient temperature and pressure were 43°C and 120 bar, respectively. The core was salt saturated. An attempt has been done to get a quick insight in the predominant *Bacteria* and

Archaea that are found in the core and to see if the different locations (outer shell to centre) in the core display differences in their microbial community. From the taken core segments genomic DNA was isolated (see [Chapter 2](#)). In this case, 16S rRNA gene fragments were amplified in a PCR reaction using primer pairs for Bacteria and Archaea, amplifying all the 16S rRNA genes of the different species present in the samples. Bacteria and Archaea have to be analysed separately since they represent two very different branches of life. No primer pair exists to target both groups. These PCR products were then placed on two DGGE gels. After the DGGE analysis the gels were stained using a dye that binds to genomic material and photographed revealing fingerprints (banding patterns) corresponding to the sampled environment.

In order to retrieve sequence data contained in these bands, which now constitute the partial 16S rRNA gene fragments of one single species, the bands were cut from the gel and placed in separate sterile vials holding 15 µl of highly purified sterile water. The DNA molecules diffuse out of the band in the water phase overnight (at 4°C). One microlitre of this water now holding gene fragments of presumably a single species was used as a template for a PCR (the success of DGGE band reamplifications is 80%). The specific gene belonging to one species is now the only sequence that was amplified and the PCR product only contain copies of that sequence. After this reamplification, the gene fragment was purified, getting rid of the primers and free nucleotides. Reamplification is required to retrieve enough material for sequencing. This reamplification can be performed with primers that do not have a GC clamp. After reamplification and purification, sequences can be sent to a commercial company for sequencing.

Results

After the PCR-DGGE analysis, bands were excised and a total of 45 sequences were obtained. Sequences of these bands were obtained and compared with known sequences using the GenBank database using the BLAST algorithm (Altschul et al., [1990](#)). All of the sequences showed similarity in their 16S rRNA gene sequences to microorganisms previously isolated or detected from petroleum reservoirs and hypersaline environments. The microorganisms could be grouped into different genera: *Halanaerobium*, *Halomonas*, *Chromohalobacter* and *Orenia* were the bacterial genera. *Haloferox* was the only archaeal genus. These genera are part of the four bacterial phyla (Proteobacteria, Firmicutes and Halobacteriales) and the only archaeal phyla (Euryarchaeota), which are known to accommodate halophilic organisms. Sequences of known and described species (organisms which hold a 16S rRNA gene sequence similarity between 97 and 100% when compared to the 16S rRNA gene sequences that have been previously described) were selected and their characteristics (temperature, salinity) were deduced from the literature and subsequently compared with the core environment. These criteria can give some

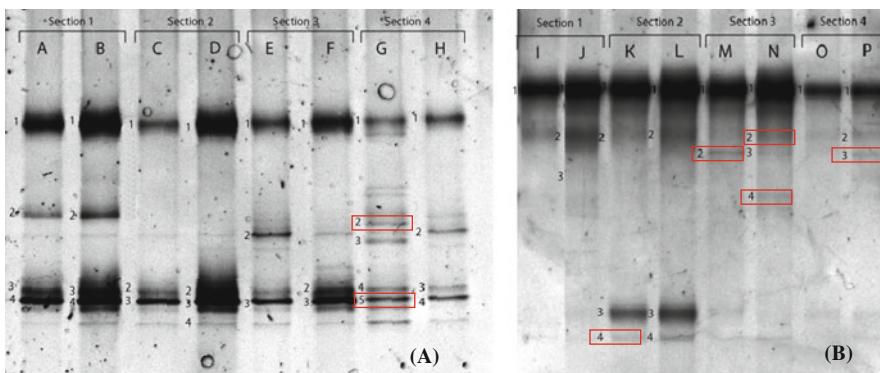


Fig. 5.4 DGGE patterns of core samples DNA. (a) Bacterial DGGE using the bacterial primer pair BAC341F+GC-BAC907Rm with a urea-formamide gradient of 20–80%. The numbers indicate the bands that were cut out and subsequently sequenced. (b) Archaeal DGGE using the archaeal primer pair Parch-519 fm and ARC-915R+GC with an urea-formamide gradient of 30–70%. The numbers indicate the bands that were cut out and subsequently sequenced. Bands that were not successfully sequences are shown by the boxes. For both DGGEs, the sequence results are explained in the text. (Images were made by staining the gels using a Sybrsafe fluorescent dye (Invitrogen) and they were illuminated using a C-box blue light imager and photographed)

indication towards the indigenous nature of the microorganisms present in the core. It should be noted, however, that 16S rRNA gene sequence similarity does not directly correspond to a metabolism (Fig. 5.4).

All the sequences belonging to the described closest relatives are able to sustain growth at the in situ temperature of the reservoir. This implies that our sequences belong to species that have a similar physiology. Looking at salt tolerance, the four closest relatives were able to thrive at salt saturated levels. Five microorganisms could be classed as being halophilic (i.e. requiring NaCl for growth). Only one microorganism was found that could be classed as being halotolerant (i.e. not requiring NaCl for growth). Furthermore, nine organisms could either anaerobically ferment sugars or be classified as being facultative anaerobic (e.g. being able to grow anaerobically by denitrification). Based on the results, eight organisms could be classified as being potentially indigenous to the reservoir. These are the bands corresponding to Halanaerobiales species based on the extreme salt tolerance, the obligately anaerobic nature and the acknowledged presence of these microorganisms in high-saline oilfields. The bands based on the halomonodaceae can also possibly be classified as being indigenous to the reservoir. Interestingly, there was a general lack of methanogens, SRB (sulfate-reducing bacteria) and IRB (iron-reducing bacteria), being common inhabitants of oil reservoirs. This lack of SRB supports the hypothesis that SRB are introduced by human activity although also other explanation for their absence can be given, such as the probability that they are not yet known (Table 5.1).

Table 5.1 Overview of found closest relatives and their accession numbers

Bands	Closest relative in GenBank	Sequence identity ^a	Accession	Environment
(A) Bacterial DGGE using primer pair BAC-341F+GC and BAC-907Rm				
A1,B1,C1,D1,E1, F1,G1,H1	<i>Halanaerobium praevalens</i>	100%	AB022035	Great Salt Lake, Utah
A3,A4,B3,B4,C2,C3,D2 D3,D4,E3,F2,F3,H3,H4	<i>Chromohalobacter israelensis</i>	100%	AM945672	Tunisian solar saltern
E2,H2	Uncultured bacterium clone E5M13F	99%	EU796033	WWTP
G3	<i>Paracoccus</i> sp. 10-1-100	100%	EU376960.1	Desert of Xingjiang
G4	<i>Halomonas elongata</i>	95%	AJ295147.1	Solar Saltern
(B) Archaeal DGGE using primer pair Parch-519 fm and ARC-915R+GC				
I1,J1,K1,L1,M1,N1, O1,P1	<i>Orenia salinaria</i>	96%	Y18485	Mediterranean anaerobic saltern
J3,L4	<i>Orenia salinaria</i>	92%	Y18485	Mediterranean anaerobic saltern
J2,K2,L2	<i>Halanaerobium kushneri</i>	98%	HKU86446	Oil brine
J2,K2,L2	<i>Halanaerobium acetoethylicum</i>	98%	HAU86448	Oil rig filter
N3,P2	<i>Halanaerobium</i> sp. AN-BI5B	94%	AM157647	Deep-sea halocline
L3	<i>Haloarchaeon MSNC</i> 16(6)	99%	FJ868735	Hypersaline ponds, Camargue
K3	<i>Haloferax</i> sp. FC28_21	92%	EU308262	Greek solar saltern

^aSequence similarities of known nearest neighbours should be preferably above 97%. However, in some cases this is not possible, see specific example in this table.

Conclusions

The microbial diversity of this hypersaline petroleum reservoir (Gabon, Africa) was analysed using the method of PCR-DGGE. A total of 45 sequences was obtained using PCR-DGGE targeting bacterial and archaeal 16S rRNA. The sequences were examined using a combination of sequence analysis (GenBank database) and phylogenetic analysis (ARB software). Concerning this study, the following conclusions could be made:

- All found microorganisms should be (based on 16S rRNA gene sequence similarity), able to sustain growth at the in situ temperatures of the reservoir.
- The found *Halanaerobiales* species are probably indigenous to the reservoir, based on the description of the described closest relatives regarding high salt tolerance, the capability of anaerobic fermentation and the indicated presence at high-saline oilfields. It should be mentioned, however, that 16S rRNA gene sequences do not provide direct information on the metabolic properties of an organism.
- The found species that were related to the known species *C. israelensis*, *H. elongata* and *H. halodenitrificans* can also possibly be classified as being indigenous to the reservoir, based on the high salt tolerance.
- The fact that communities from the different segments taken, yields little differences, indicates that microbial communities are more or less stable over the depth range.
- With the archaeal primers, bacterial genera were also detected, this indicates that these primers are not archaeal specific. This artefact has been found in other studies (Vissers et al., 2009).

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Chapter 6

Which Microbial Communities Are Present?

Application of Clone Libraries: Syntrophic Acetate Degradation to Methane in a High-Temperature Petroleum Reservoir – Culture-Based and 16S rRNA Genes Characterisation

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Introduction

The presence of microorganisms in petroleum reservoirs has been established about 100 years ago. Microbiological, radioisotope, molecular biological, and biogeochemical techniques have been used to investigate microbial diversity and activity in the oilfields. These techniques were applied separately, and the composition of the microbial community and its geochemical activity remained poorly understood. By culture-based methods anaerobic microorganisms reducing sulphate, thiosulphate, $\text{Fe}^{(3+)}$, or elemental sulphur, as well as fermentative bacteria, acetogens, and methanogens have been isolated from petroleum reservoirs (Magot et al., 2000; Orphan et al., 2000; Bonch-Osmolovskaya et al., 2003).

By 16S rRNA gene techniques, microbial communities of high-temperature oilfields in California (Orphan et al., 2000), Western Siberia (Bonch-Osmolovskaya et al., 2003), and China (Nazina et al., 2007; Shestakova et al., 2006; Li et al., 2006) have been investigated. Formation waters were found to contain 16S rRNA genes of the known thermophilic (*Thermococcus*, *Thermotoga*, *Petrotoga*, *Thermoanaerobacter*; *Methanothermobacter*, *Methanococcus*, and *Methanoculleus*) and mesophilic microorganisms, as well as of uncultured archaea and bacteria.

The distribution and activity of microorganisms in a high-temperature Dagang oilfield (China) were characterised earlier (Nazina et al., 2006, 2007, 2008). Analysis of the 16S rRNA genes from methanogenic enrichments growing in the medium with acetate revealed the predominance of phylotypes of H_2 -utilising methanogens in the clone library; no phylotypes of acetoclastic methanogens were retrieved (Shestakova et al., 2006). These data did not agree with the results of radioisotope analysis which confirmed the formation of $^{14}\text{CH}_4$ from

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$^{14}\text{CH}_3\text{-COONa}$. It was suggested that syntrophic associations were responsible for acetate degradation to methane in the oilfield. The aim of this work was to characterise the microbial community of the high-temperature horizons of the Dagang oilfield (P.R. China) by 16S rRNA gene clone library analysis, culture-based and radioisotope methods and to study microbial interactions during acetate degradation to methane.

Case Study: Physicochemical and Microbiological Characteristics of the Dagang Oilfield

The Dagang oilfield is situated in the Hebei province (P.R. China). The studied sandstone oil-bearing horizons were located at the depth of 1,206–1,435 m below sea level; the temperature was 59°C. The natural formation water had low salinity (5,612 mg/l) and pH of 7.1–7.6; it contained acetate (<5 mg/l) and hydrocarbonate (433–670 mg/l). Accompanying gas contained methane (95–98%), its higher homologues (0.8–1.8%), nitrogen (0.5–3.3%), and carbon dioxide (0.06–0.77%).

North block of the Dagang oilfield was exploited with the MEOR technology (microbial enhancement of oil recovery) based on the stimulation of the stratal microflora activity by the pumping of a water–air mixture supplemented with nitrogen and phosphorus mineral salts into the oil stratum through injection wells. During the biotechnological treatment, bicarbonate content in formation water increased from 0.4–0.6 to 0.7–1.8 g/l, sulphate from 0 to 12–72 mg/l, acetate from 5 to 160.7 mg/l, formate from 0 to 67.4 mg/l, and *iso*-butyrate from 0 to 98.2 mg/l (Nazina et al., 2007, 2008).

The number of cultivable fermentative bacteria varied from 10^2 to 10^7 cells/ml, of sulphate reducers from 0 to 10^2 cells/ml, and of methanogens from 0 to 10^4 cells/ml. Methanogens were revealed both in media with H_2+CO_2 and acetate as carbon and energy sources. Microscopic analysis revealed the presence of rod-shaped cells in both media for methanogens. The rate of sulphate reduction was in the range of 0.06–205.8 $\mu\text{g SO}_4^{2-}/\text{l/day}$. The rates of methanogenesis from labelled bicarbonate and acetate varied from 0.037 to 6.776 and from 0.002 to 3.16 $\mu\text{g CH}_4/\text{l/day}$, respectively (Nazina et al., 2007, 2008).

16S rRNA Gene Clone Libraries

Archaeal and bacterial 16S rDNA clone libraries were generated from total DNA collected from formation water outside (A1066-1 – 403 archaeal clones) and inside the zone of the biotechnological treatment (North Block) (ANB – 181 archaeal clones, BNB – 453 bacterial clones) and from acetate-degrading methanogenic association (AAc – 95 archaeal clones, BAc – 46 bacterial clones) (Tables 6.1 and 6.2).

Phylogenetic analysis of archaeal clones by direct sequencing (59 + 17 + 41 clones) and PCR with the *Methanothermobacter*-specific primer (343 + 143 + 54 clones) revealed the predominance of H_2 -utilising methanogens of the order

Table 6.1 Closest relatives of archaeal phylotypes in the clone library from formation waters and methanogenic enrichments of the Dagang oilfield

Phylum	Closest identified relative/accession number	Type sequence	Numbers of clones in library		
			1*	2*	3*
<i>Methanobacteria</i>	<i>Methanothermobacter thermautotrophicus</i>	ANB-1	59 + 343 ^a	17 + 143 ^a	41 + 54 ^a
	Delta H (AE000666)	ANB-21			1
	<i>Methanobacterium formicicum</i>				
	DSM 1312 (M36508)	ANB-217 ANB-2	1		
	Uncultured archaeon clones LCA (AB084240) and HDBW-WA07 (AB237740)			3	
	<i>Methanosaeta thermophila</i> PT, DSM 6194 (AB071701)	ANB-7			3
	<i>Methanomethylolovorans thermophila</i> LZFAW (AY672821)	ANB-170			2
	Methanogenic archaeon NOBI-1 (AB162774)	ANB-154		2	
	Uncultured archaeon clones Ou2O-50 (AJ556495) and C1_R046 (AF419643)	ANB-14 ANB-67		10	
	Total		403	181	95

1.* Natural formation water; 2.* formation water from the zone of the biotechnological treatment; 3.* methanogenic association on acetate.

^aClones were analysed by the PCR with the *Methanothermobacter*-specific primer. Clones without any sign were analysed by sequencing DNA.

Table 6.2 The most representative bacterial phylotypes in the clone library from formation water of the Dagang oilfield

Phylum	Closest identified relative (accession number)	Type sequence	Numbers of clones	Similarity (%)
Alphaproteobacteria	<i>Sphingomonas aerolata</i> NW12 (AJ429240)	BNB-1	33 + 333 ^a	98–99
	<i>Afipia</i> genosp. 14 (U87785)	BNB-23	2	99
	<i>Labrys methylaminophilus</i> JLW10 ^T (AY766152)	BNB-75	2	100
Betaproteobacteria	<i>Variovorax</i> sp. TUT1027 (AB098595)	BNB-13	2	99
	<i>Curyibacter gracilis</i> IAM 15033 ^T (AB109889)	BNB-545	5	98–99
	<i>Polaromonas aquatica</i> CCUG 39797 (AM039831)	BNB-451	3	99
	<i>Thanera aromatica</i> S100 (AF438167)	BNB-478	1	99
	<i>Pseudomonas nitroreducens</i> 0802 (AF494091)	BNB-27	28	97–99
Gammaproteobacteria	<i>Thermanaerobacter keratinophilus</i> 2KX1 (AY278483)	BNB-173	1	99
	<i>Carboxydothermus ferrireducens</i> 5d265 (U76364)	BNB-276	1	96
	<i>Thermacetogenium phaeum</i> PB (AB020336)	BNB-181	1	95
	Uncultured <i>Desulfotomaculum</i> clone (EF077227)	BNB-38	8	87
	<i>Fervidobacterium</i> sp. (AY882754, EF565822, M59177)	BNB-4	10	92–98
Clostridia	<i>Thermotoga subterranea</i> SL ₁ ^T (U22664)	BNB-514	1	99
	<i>Dicyoglomus thermophilum</i> DSM 3960 ^T (X69194)	BNB-48	5	99
	<i>Sphingobacteriaceae</i> bacterium Tibet-IIK55 (DQ177471)	BNB-223	2	97
	<i>Rhodococcus erythropolis</i> DFA9 (AB180237)	BNB-52	1	99
	<i>Thermodesulfobacteriovorans</i> sp. TSL-P1 (AB021304)	BNB-172	1	93

^aClones were analysed by the PCR with the *Sphingomonas*-specific primer.

Methanobacteriales in these communities (Table 6.1). Phylotypes related to organotrophic methanogens *Methanosaeta thermophila* (three clones), *Methanomethylovorans thermophila* (two clones), and uncultured members of the order *Methanomicrobiales* (two clones) were detected only in the zone of the biotechnological treatment. Bacterial phylotypes in the clone library retrieved from the zone of the biotechnological treatment (BNB) were represented by 30 phylogenetic groups belonging to *Proteobacteria*, *Clostridia*, *Thermotogae*, *Dictyoglomi*, *Bacteroidetes*, *Actinobacteria*, and *Nitrospirae*. Phylotypes of Proteobacteria of the genera *Sphingomonas*, *Afipia*, *Labrys*, *Phyllobacterium*, and *Bradyrhizobium* (Alphaproteobacteria); *Variovorax*, *Curvibacter*, *Polaromonas*, *Thauera*, *Hydrogenophilus*, *Delftia*, *Leptothrix*, and *Achromobacter* (Betaproteobacteria); and *Pseudomonas* (Gammaproteobacteria) predominated in the clone library.

Members of these genera are mesophilic aerobic organotrophs; they probably arrived into the reservoir with cooled injection water. The group of clostridia was the second numerous one; it comprised thermophilic bacteria with fermentative metabolism (*Thermoanaerobacter*, *Carboxydotothermus*), sulphate reducers, and syntrophic bacteria (*Thermacetogenium* and *Desulfotomaculum*). Thermophilic bacteria with fermentative metabolism belonging to other phylogenetic subdivisions (*Fervidobacterium*, *Thermotoga*, *Dictyoglomus*) were also revealed. Bacterial phylotypes (46 clones) from the acetate-degrading methanogenic association belonged to *Caldanaerobacter subterraneus* (43 clones), *Thermoanaerobacter keratinophilus* 2KXI (2 clones), and *Thermotoga subterranea* SL1 (1 clone).

Isolation of Pure Cultures and Reconstruction of Syntrophic Acetate Degradation to Methane

Two pure cultures of H₂-utilising methanogens were isolated from thermophilic methanogenic enrichments growing on media with acetate and H₂+CO₂ (strains KZ3 and KZ24, respectively). Strain KZ3 was related to the species *Methanothermobacter thermautrophicus* (Delta H, AE000666); another strain, KZ24a, to *Methanothermobacter wolfeii* (DSM 2970, AB104858) (99% similarity of 16S rRNA genes). Attempts to isolate acetate-utilising methanogens were unsuccessful. During 7 years of investigation of the Dagang oilfield, no *Methanosarcina*- or *Methanosaeta*-like cells have been observed in enrichment cultures.

On media with pyruvate, 10 strains of anaerobic rod-shaped bacteria were isolated from the methanogenic association. All strains were phylogenetically related to *Thermoanaerobacter ethanolicus* (99.6% similarity of 16S rRNA genes). The strains used thiosulphate as an electron acceptor in the presence of glucose; this feature was not previously known for this species. For the reconstruction of syntrophic growth on acetate pure cultures of the methanogen *Methanothermobacter* (strains KZ3 and KZ24a) and *T. ethanolicus* (strains 1017-7b and 1017-7d) were combined. Methane was produced from ¹⁴CH₃-COONa only by binary cultures of *Methanothermobacter*-*Thermoanaerobacter*, while no individual culture was capable of methanogenesis under these conditions (Fig. 6.1).

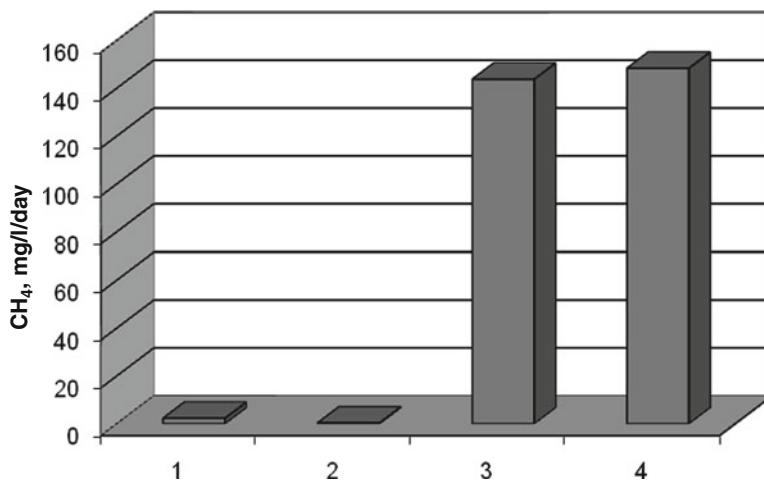


Fig. 6.1 Methane formation from acetate ($100 \text{ mg/l} + 60 \mu\text{g} \text{ }^{14}\text{CH}_3\text{-COONa/l}$) at 60°C within 24 days by pure culture of *M. thermautotrophicus* (1), *T. ethanolicus* (2), co-cultures of *M. thermautotrophicus* – *T. ethanolicus* (3), and a methanogenic association (4)

Discussion

Microbial community from a high-temperature Dagang oilfield was investigated by 16S rRNA gene analysis, microbiological, and radioisotope methods. This community included aerobic organotrophs, as well as anaerobic fermentative bacteria and archaea, sulphate-reducing and syntrophic bacteria, and methanogenic archaea. Methanogenesis was the main terminal process in formation water of the Dagang oilfield. By radioisotope methods, methane production from both $^{14}\text{CH}_3\text{-COONa}$ and $\text{NaH}^{14}\text{CO}_3$ was revealed. The rates of methanogenesis were comparable to the values reported for other high-temperature oilfields exploited with water flooding (Nazina et al., 1995; Bonch-Osmolovskaya et al., 2003). Previous 16S rRNA gene analysis of methanogenic enrichments has shown the predominance of H₂-utilising methanogens *Methanothermobacter* sp. The genes of acetate-utilising archaea were not revealed.

Two mechanisms of acetate degradation to methane are known. At high acetate concentration, thermophilic methanogens *Methanosarcina thermophila* and *Methanosaeta thermophila* carry out direct fermentation of acetate to methane. Methanogenesis from acetate in these organisms is catalysed by an acetoclastic reaction in which the methyl group of acetate is reduced to methane: $\text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CH}_4 + \text{CO}_2$ ($\Delta G^\circ = -36 \text{ kJ/mol}$). However, such acetoclastic methanogens have not yet been isolated from high-temperature oilfields. The second mechanism originally proposed by Barker in 1936 (from Schink, 1997) consists of two reactions. In the first reaction, acetate is oxidised to form hydrogen and CO₂, by syntrophic acetate-oxidising bacteria: $\text{CH}_3\text{COO}^- + 4 \text{ H}_2\text{O} \rightleftharpoons 2 \text{ HCO}_3^- + 4 \text{ H}_2 + \text{H}^+$ ($\Delta G^\circ = +104.6 \text{ kJ/mol}$). In the second reaction, CO₂ is converted to

methane by lithotrophic methanogens: $4 \text{ H}_2 + \text{HCO}_3^- + \text{H}^+ \rightleftharpoons \text{CH}_4 + 3 \text{ H}_2\text{O}$ ($\Delta G^\circ = +135.6 \text{ kJ/mol}$). Thus, syntrophic acetate degradation is possible only when syntrophic and hydrogen-consuming microorganisms cooperate.

The process of syntrophic thermophilic acetate degradation resulting in methane formation was first observed in the course of operating the laboratory reactor for decomposition of lignocellulose-containing solid waste (Zinder and Koch, 1984; Lee and Zinder, 1988). To date, two syntrophic associations of acetate oxidisers with hydrogenotrophic methanogens are known (strain AOR, *Thermacetogenium phaeum*) (Lee and Zinder, 1988; Hattori et al., 2000). Syntrophic acetate degradation with methane formation in high-temperature oilfield was previously suggested by Davydova-Charakhch'yan and co-workers (1992), who isolated a H_2 -utilising methanogen from a thermophilic methanogenic association grown on acetate, but the acetate-oxidising component of the association was not studied. Moreover, all the thermophilic methanogens isolated from oil reservoirs were hydrogenotrophs (Magot et al., 2000; Nilsen and Torsvik, 1996; Orphan et al., 2000; Bonch-Osmolovskaya et al., 2003). Although methane formation in enrichment cultures with acetate was observed at the temperatures of 70, 80, and 92°C, attempts to isolate acetate-utilising methanogens were unsuccessful (Nilsen and Torsvik, 1996).

Our analysis of archaeal 16S rRNA genes (679 clones) demonstrated that H_2 -utilising methanogens of the order *Methanobacteriales* (*M. thermautotrophicus*, 658 clones) predominated in the clone libraries obtained from formation water and acetate-degrading methanogenic associations of the Dagang oilfield. The phylotypes of acetate-utilising methanogens were revealed neither in the primary methanogenic enrichments nor in the original formation waters. Five methanogenic phylotypes of the order *Methanosarcinales* were found only in the ANB clone library from the formation water where high acetate concentration resulted from MEOR technology.

Over 30 phylogenetic groups of thermophilic and mesophilic bacteria were revealed in the formation water. The representative phylotypes belonged to the classes of *alpha*-, *beta*-, *gamma*-, and *deltaproteobacteria*, *Clostridia*, *Thermotogae*, *Dictyoglomi*, *Bacteroidetes*, *Actinobacteria*, and *Nitrospirae*. Bacteria of the genera *Fervidobacterium* and *Thermotoga* are common inhabitants of high-temperature oilfields of France, United States, Africa, Japan, and Western Siberia (Fardeau et al., 1997; Orphan et al., 2000; Bonch-Osmolovskaya et al., 2003). The phylotype of a sulphate-reducing bacterium *Thermodesulfobacter* (*Nitrospirae*) found in the clone library was also retrieved in the clone libraries from other oilfield waters (Li et al., 2006) and in methanogenic enrichments (Nazina et al., 2006).

The phylotypes of syntrophic bacteria closely related to *Thermovirga lienii* (fam. *Syntrophomonadaceae*) and *T. phaeum* were detected; the latter was previously revealed in methanogenic enrichments (Nazina et al., 2006). Pure cultures of H_2 -utilising methanogens (*M. thermautotrophicus*) and anaerobic organotrophic bacteria (*T. ethanolicus*) were isolated from methanogenic enrichments grown on the medium with acetate. The *Methanothermobacter–Thermoanaerobacter* co-culture was found to degrade acetate to methane. Although the *Thermoanaerobacter–Caldanaerobacter* group of bacteria is common in underground oil horizons, their ecological function has been obscure.

In the present work, it was demonstrated for the first time that in the presence of methanogenic archaea bacteria of the genera *Thermoanaerobacter*–*Caldanaerobacter* may be involved in syntrophic acetate oxidation to H₂ and CO₂; the latter is then reduced to methane.

Involvement of bacteria of the *Thermoanaerobacter*–*Caldanaerobacter* group in the terminal processes of decomposition of oil organic matter together with methanogens improves our understanding of their function in the community. Acetoclastic methanogenesis is probably not pronounced in high-temperature oilfields, so that direct acetate decomposition to methane contributes insignificantly to the overall methanogenesis. Thus, culture-based and molecular studies enabled us to specify the composition of the microbial trophic chain, to estimate the geochemical activity of microorganisms, and to obtain qualitatively new information concerning the terminal stages of oil biodegradation in high-temperature oilfields.

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Chapter 7

Which Microbial Communities Are Present?

Using Fluorescence In Situ Hybridisation (FISH): Microscopic Techniques for Enumeration of Troublesome Microorganisms in Oil and Fuel Samples

Lars Holmkvist, Jette Johanne Østergaard, and Torben Lund Skovhus

Introduction

Enumeration of microbes involved in souring of oilfields and microbiologically influenced corrosion (MIC) with culture-based methods, usually yield inadequate and contradictory results. Any cultivation step will almost certainly alter the population structure of the sample and thus the results of cultivation analysis are not a good basis for mitigation decisions. The need for methods that are cultivation independent has over the past 10 years facilitated the development of several analytical methods for determination of bacterial identity, quantity and, to some extent, function, applied directly to samples of the native population. In this chapter, we demonstrate the features and benefits of applying microscopic techniques to a situation often encountered in the oil and petroleum industry: control of microbial growth in fuel storage tanks. The methods described in this chapter will focus on direct counts of specific groups of microorganisms with microscopy and these are based on the detection of genetic material and not on culturing.

Drawbacks with Culturing Bacteria

While great advances have been made in media and cultivation/enumeration techniques, it is now generally accepted that only 1–10% of all bacteria are cultivable by these classical microbiology methods (Amann et al., 1995). Industries that have previously used cultivation-based methods for microbiological surveillance are therefore looking for methods that include a larger fraction or, better yet, the entire population of troublesome microorganisms.

The solution is within reach due to advances in molecular microbiology research and microbial ecology made during the last decade (Amann et al., 1995 and Skovhus

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et al., 2009). Several methods are presently available that can detect entire populations of microorganisms without the limitations of cultivation. In addition, these cultivation-independent techniques can be applied much faster (within a few hours to a few days) compared to traditional cultivation-based techniques (with up to 30 days of cultivation), resulting in a potentially faster response time to take action of mitigating problems in the industry (Kjellerup et al., 2006).

Counting All Prokaryotes in Oilfield or Fuel Samples with DAPI

The 4',6-diamidino-2-phenylindole (DAPI) method quantifies all intact microorganisms containing DNA (both living and inactive cells) in almost any type of liquid sample such as injection water, produced water and fuel samples (Larsen et al., 2005 and 2006). The information on total cell number per millilitre of sample is valuable in cases where growth of microorganisms in general has to be inspected and monitored over time. Further, based on previous data of total cell numbers from many different systems, it is possible to evaluate whether a total cell number of microorganisms is within a low, medium or high range. The DAPI procedure can be completed in less than half a day. This means that numbers often can be reported the same day as the sample arrives in the laboratory. The liquid sample is filtered whereby microorganisms (bacterial, archaeal and fungal cells) are collected

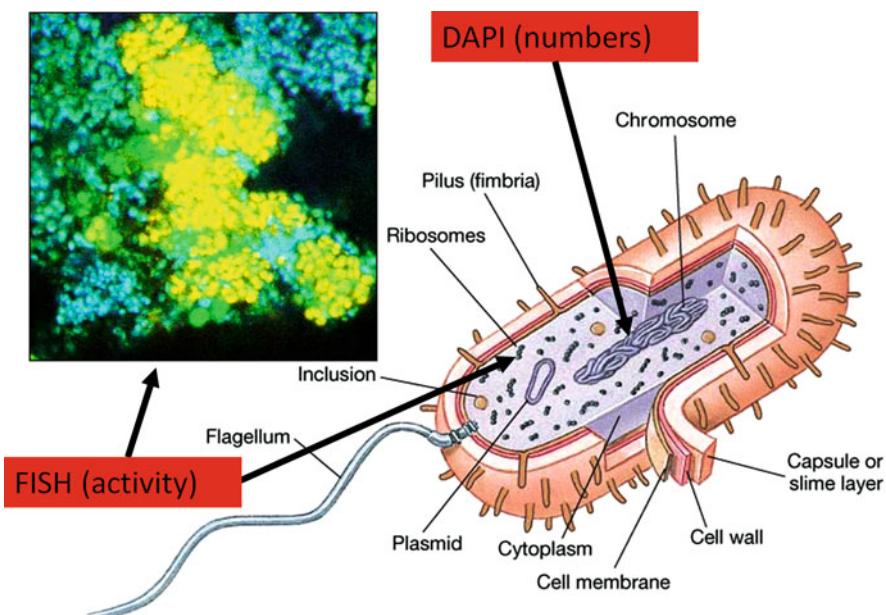


Fig. 7.1 A schematic drawing of a bacteria cell and a microscopic picture of active cells (*bright cells*) stained with the FISH technique. RNA in the ribosomes and the DNA in the bacteria are target sites for the fluorescent dyes used in the FISH/DAPI method

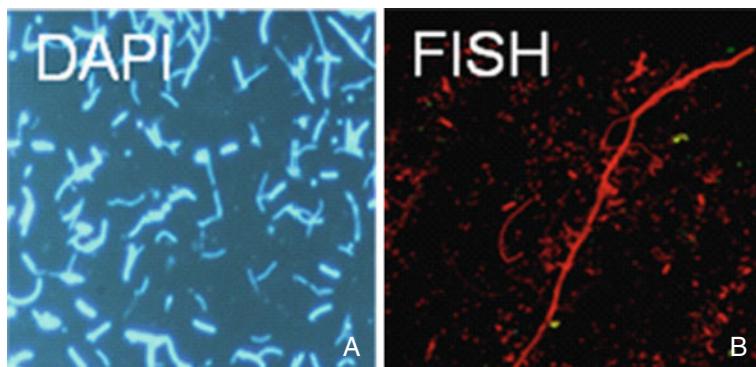


Fig. 7.2 Microscopic pictures of cells stained with fluorescent dye from (a) the DAPI method and (b) the FISH technique

on a filter. These are subsequently stained with a fluorescent dye (*4',6-diamidino-2-phenylindole*) that binds to the DNA in the cells (Fig. 7.1) and then washed to remove excess dye.

The cells are finally counted directly in an epifluorescence microscope where they are clearly viewed (Fig. 7.2).

Counting All Active Prokaryotes with Quantitative FISH

In some cases, it is desirable to know the level of active/alive microorganisms in an oil or fuel sample, because this information gives a more accurate measure of the survival or activity of potentially troublesome microorganisms in a system. For this purpose the FISH technique should be applied. The FISH technique (Fluorescence *in situ* Hybridisation) is a robust way of quantifying the fraction of active microorganisms in all kinds of process water, such as injection water, cooling water and produced water, and linking them to the activity of known bacterial groups such as *Bacteria*, *Archaea* and sulphate-reducing bacteria (SRB).

The genetic material inside microorganisms is unique for each strain. Using the sequence of the 16S rRNA in the small subunit (SSU) of the ribosome in prokaryotes, gene probes can be designed to target different phylogenetic groups of microorganisms or individual species (see Chapter 4 by Sørensen, this volume). Adding a fluorescent dye to the probe enables detection of cells that have been targeted by the specific gene probe. The preparation steps for FISH are different to the DAPI protocol. However, cells stained for FISH are counted in the same manner as in direct bacterial counts (DAPI). Using different fluorophores, it is possible to stain more than one group at a time, and if a general bacterial stain is included such as DAPI, it is possible to obtain quantitative information from FISH (% specific group of the total bacterial population). This approach is called qFISH (quantitative FISH).

Case Study: Application of the FISH/DAPI Method to Control Microbial Growth in Diesel Tanks Using Biocides

MIC of oilfield facilities occurs to varying degrees within topside structures and downhole facilities. Studies show that many of the microorganisms isolated and further characterised from oily waters may thrive under extreme and broad environmental conditions. For example, some exist under high temperatures (Beeder et al., 1994) and some are able to degrade very complex hydrocarbon chains of crude oil or fuels (Anderson and Lovley, 2000). This is most probably because many of the microorganisms found within oilfield facilities originate from oil reservoirs, where they are adapted to the extreme physical and chemical conditions of these habitats. Growth of microorganisms inside equipment containing oily waters such as pipelines, diesel tanks or other fuel storage and transport systems is often discovered by high numbers of planktonic cells from the water phase. However, these levels of planktonic cells originate from biofilms on the inner surfaces of the production system. Over time, the biofilms ultimately grows in thickness and complexity that most often result in plugging of filters, degradation of fuel and eventually MIC. For these reasons, it is important to detect, monitor and control at least the levels of planktonic microorganisms in fuel systems, on a regular basis, in order to follow the development in numbers of troublesome microorganisms and take action if numbers reach high and critical levels.

In the following case story, FISH/DAPI was successfully applied on samples taken in different diesel tanks on the Gorm platform in the Danish sector of the North Sea. Water from the diesel tanks was collected by Maersk Oil each month from May to September 2009 with the purpose of monitoring microbial growth and, in the long run, to be able to prevent and control microbial growth to avoid filter plugging. This study illustrates how the FISH/DAPI data typically can be applied in an effective monitoring programme offshore.

The steps from sampling over analysis and mitigation are illustrated in Fig. 7.3. The samples for FISH/DAPI were first collected in sterile IQ Kit[©] flasks and then delivered from the Gorm platform to the Danish Technological Institute (DTI) where they were analysed within a few days. Upon sample arrival in the laboratory, the samples were immediately fixed and hybridised, where after cells could

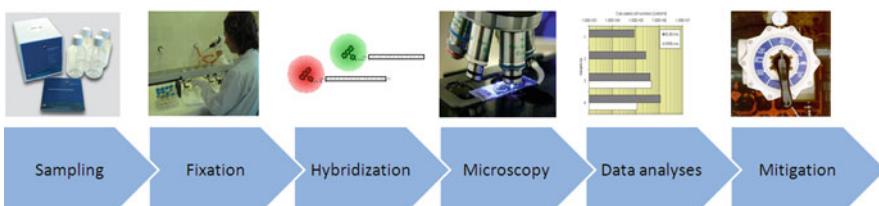


Fig. 7.3 A diagram showing the different steps in obtaining FISH/DAPI data from sampling with IQ Kit[©] to data acquisition. The new knowledge from the FISH/DAPI results can be used to plan mitigation strategies with, for example, biocides

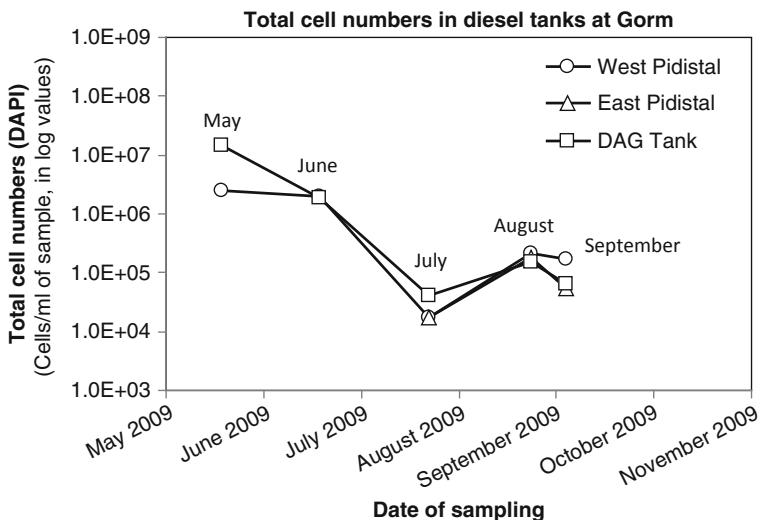


Fig. 7.4 Graph showing total cell numbers obtained with the DAPI method in three diesel tanks at Gorm from May to September 2009. Numbers are in logarithmic values

be visualised and counted in a microscope. After data acquisition, it was decided whether the tanks should be treated with a biocide or not.

The study showed that total cell numbers (DAPI), as well as the numbers of active *Bacteria* (FISH) in May, were rather high ranging from 10^6 to 10^7 cells/ml (Figs. 7.4 and 7.5). Thus, it was necessary to initiate a biocide programme to immediately reduce the numbers and the activity of troublesome microorganisms in the tanks. Consequently, Maersk Oil treated the tanks with biocide (the active products in the biocide were magnesium nitrate and isothiazoline) in June 2009 (Figs. 7.4 and 7.5). After the biocide treatment, the FISH/DAPI result showed that the treatment had an immediate effect on total cell numbers (DAPI) and numbers of active *Bacteria*, that were reduced in number by about 2–3 orders of magnitude. More biocide was dosed in both July and August to keep the number of microorganisms low. Samples for FISH/DAPI were taken right after each treatment in order to follow the level of microorganisms. During the summer time from July to August, the total number of cells as well as the number of active *Bacteria* increased in the three diesel tanks. This was most likely due to a rise in the temperature of the tanks. A final treatment with biocide was performed in September 2009 that resulted in a further reduction in the total cell numbers in all tanks, but only the numbers of active *Bacteria* were reduced in the East Pidistal (Fig. 7.5).

The case study clearly demonstrates that the FISH/DAPI method is well suited to circumstances where microbial growth in liquid samples (e.g. the storage tanks) has to be monitored on a regular basis. This is mainly because the FISH/DAPI method is a rapid analytical method. It only takes a few days for the procedure to be completed and data produced and delivered to the end user. In contrast, it typically lasts about

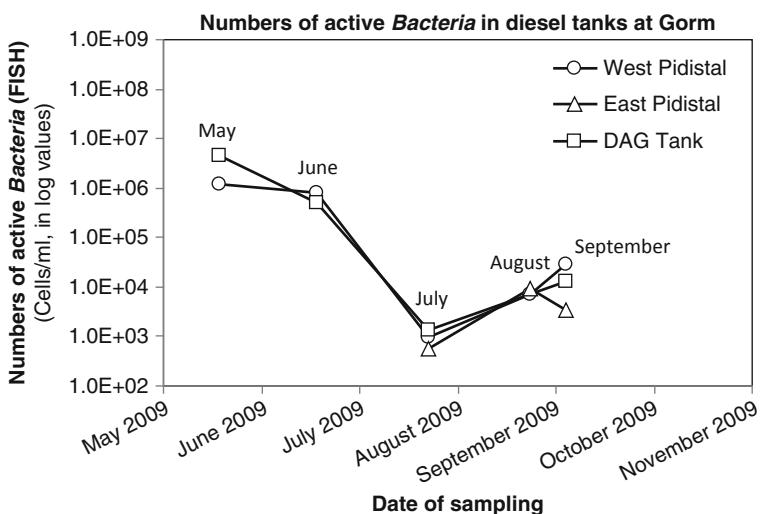


Fig. 7.5 Graph showing number of active *Bacteria* obtained with the FISH technique in three diesel tanks at Gorm from May to September 2009. Numbers are in logarithmic values

1 month with the traditional cultivation-based methods to obtain the results. Hence, in the case described above it would not have been possible to do a fast monitoring of the high numbers of microorganisms based on cultivation. In many cases, preventing plugging and MIC in oil and petroleum systems is a question of making the right safety precautions. This study shows that the risk of system failure could be reduced when the FISH/DAPI method was applied for monitoring purposes.

The FISH/DAPI method is overall a unique tool that gives valuable and robust information on all types of water–oil-based industrial systems, where it can visually detect the various microorganisms (e.g. *Bacteria*, *Archaea* and fungi), help in the evaluation of filter plugging and finally be a tool for risk assessment of MIC. However, testing with the FISH/DAPI method on a large number of different samples from the oil industry has shown that this method is not optimal when solid samples are to be analysed such as scales, wax and corrosion products. Also the method will not be optimal on samples containing oil droplets and microorganisms with autofluorescence. In these cases, the qPCR method is the preferred method.

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Chapter 8

Which Microbial Communities Are Present?

Sequence-Based Metagenomics

Sean M. Caffrey

Introduction

Analysis of the 16S rRNA gene sequences isolated from environmental samples has demonstrated the extraordinary microbial diversity missed by traditional culture-based methodologies (Tringe and Rubin, 2005). Figure 8.1 summarises several metagenomic sequencing approaches.

Although there are many culture-independent methodologies capable of assessing microbial diversity discussed in this volume, none of these tools can identify all the microorganisms or functions in a high-throughput way. PCR, FISH and microarrays can only identify organisms or genes for which probes have been prepared and always miss unknown genes. When using universal primers for the 16S rRNA gene, DGGE and other post-PCR techniques use DNA amplified from nearly all the microorganisms in the sample, but this technique is limited by band resolution and species identity is only determined for bands that have been individually excised and sequenced. Advances in sequencing technology over the past decade have increased sequencing throughput over 50-fold when compared to Sanger-based sequencing, making it possible to produce gigabases of sequencing information in just days (MacLean et al., 2009). The corresponding decreases in sequencing costs have made it feasible to employ enough sequencing runs to sequence the total DNA extracted from environmental samples, circumventing many of the limitations of other techniques used in community analysis. Using a single 454 pyrosequencing run, Sogin et al. (2006) sequenced the 16S rRNA gene amplicons generated from eight environments and produced 118,000 sequence tags, more than any Sanger-based sequencing study to that date (Sogin et al., 2006; Tringe and Hugenholtz, 2008). Subsequent studies have produced greater than 900,000 16S rRNA gene sequence tags. The development of next-generation sequencing platforms has led to the production of three main competing systems: 454 sequencing (Roche Applied

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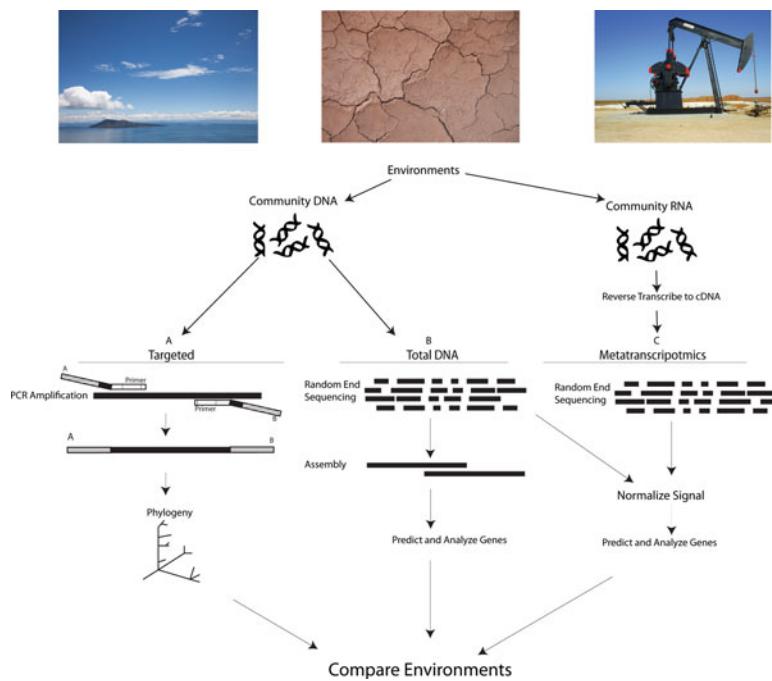


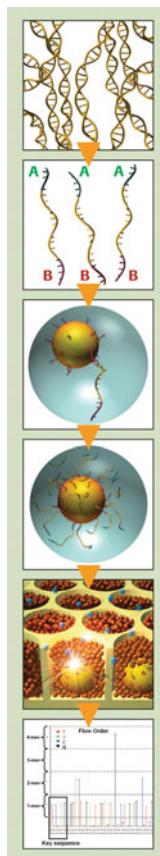
Fig. 8.1 Metagenomic sequencing. (a) Targeted amplicon sequencing. Gene of interest is targeted by PCR amplification prior to sequencing. (b) Total DNA sequencing. All the genes in the sample are sequenced. (c) Metatranscriptomics. Extracted RNA is converted to cDNA and then sequenced

Science; Basel), Solexa technology (used in the Illumina Genome Analyzer, San Diego) and the SOLiD platform (Applied Biosystems, Foster City, CA, USA).

At over 400 bps, the read length of the 454 titanium sequencing chemistry is much longer than 36 bp reads produced by either Solexa or Illumina (Mardis, 2008). Accordingly, 454 sequencing is the most appropriate platform for metagenomics studies because the longer read lengths make the bioinformatics of metagenomics analysis simpler. Longer read lengths improve BLAST homology searches and assembly of long centigs. The longer reads also make phylogenetic linkage of genes with their host organisms easier and improve species identification from 16S rRNA sequence tags (Wommack et al., 2008).

The 454 pyrosequencing work flow begins with library creation (Shendure and Ji, 2008) (Fig. 8.2). DNA is either sheared to fragments of less than 800 bp segments and then ligated to short adapters (A and B adapters – specific for both the 3' and 5' ends), or PCR amplicons are generated that include the two flanking adapter sequences. The adapters are used for the purification, amplification and sequencing steps. The resulting library is then bound to DNA capture beads with each bead containing only an individual single-stranded DNA library fragment. The beads are then subjected to Emulsion PCR amplification, where each bead is amplified in isolation but in parallel with the rest of the library. The amplification results in each bead containing several million copies of its captured sequence. After

Fig. 8.2 454 pyrosequencing workflow (454 Sequencing
© 2009 Roche Diagnostics)



A and B adapters are ligated onto single strand fragments of isolated DNA for total DNA sequencing or the A and B adapters are added during PCR amplification for Targeted sequencing. Fragments with A and B adapters are isolated.

DNA fragments are bound to beads, one fragment per bead.

Colonial amplification of DNA fragments occurs via emulsion PCR inside wells (microreactors).

The amplified fragments are loaded onto a PicoTiterPlate for sequencing. One bead per well. Individual nucleotides are flowed over the wells in a fixed order. Addition of complementary nucleotides to the template strand results in a chemiluminescent signal recorded by the CCD camera.

Signal intensity and positional information allows the software to determine the sequence.

amplification the beads are loaded into PicoTiterPlate sized to only allow one bead per well. Sequencing occurs via sequencing-by-synthesis. Nucleotides are sequentially flowed across the PicoTiterPlate and if the nucleotide is complementary to the template strand in the library it is incorporated by a polymerase. The pyrophosphate generated during the sequencing reaction reacts with APS and ATP in the presence of the sulphurylase and luciferase enzymes to produce a flash of light. This light flash is recorded by a CCD camera and the intensity of the light flash and its position on the plate provide the information required to generate the sequences.

For the sequencing of an environmental sample (metagenomics), either the total extracted DNA or an enriched portion of the total extracted DNA can be used. Sequencing of the total DNA (random shotgun sequencing) provides sequence information not only of the microbial community members but also on all the functional genes present in the environment. Unfortunately, because the sample contains so much information, a significant amount of sequencing must be employed to analyse the sample. This not only is expensive in terms of sequencing runs but will also require a great deal of computing and bioinformatics resources. Even when

a large number of sequencing runs are used, the rare community members in the environment may be missed. An alternative to total DNA sequencing is a portion of the DNA can be enriched with PCR amplification. Typically, primers are generated that target phylogenetic markers like the 16S rRNA gene or conserved genes like *recA*, but functional genes like the dissimilatory sulphite reductase genes could be targeted. Amplifying a small portion of the DNA means less sample is needed, fewer sequencing runs are required to identify rare species and bioinformatic analysis is much simpler. Also by adding unique bar codes to the PCR primers many samples can be sequenced simultaneously. Of course by sequencing only a targeted portion of the DNA less information about the microbial community present in the environmental sample is obtained.

Amplicon (PyroTag) Sequencing

The combination of conserved and variable regions in the 16S rRNA gene makes it the ideal target for surveying an environment's microbial diversity (Woese, 1987). PCR primers targeting the conserved regions can be specific for a group of microorganisms (such as sulphate-reducing bacteria) or universal and attempt to amplify all bacterial and archaeal species in the environment. Although no universal primer set will amplify every single microbe or amplify each organism equally (without bias) (Leuders and Friedrich, 2003), PCR amplification minimises many of the problems inherent in metagenomic sequencing. Since only nanograms of starting material is required (Miller et al., 2009; Sogin et al., 2006) PCR amplification allows the examination of environments from which it is difficult to obtain samples or extract DNA. Although 454 sequencing produces the longest reads of any second-generation sequencing technology (Shendure and Ji, 2008), 400 bp will only cover a portion of the 1,542 bp long 16S rRNA gene. Fortunately, universal primers amplifying the 16S rRNA gene containing the V6+V7 regions have been shown to produce species richness estimates comparable to those generated from full length sequences (Youssef et al., 2009). The use of pyrosequencing to sequence a portion of the 16S rRNA gene is known as 16S tag pyrosequencing and generates pyrotags. Pyrotag sequencing has been widely used and its effectiveness has been demonstrated in several studies (Callaway et al., 2009; Dowd et al., 2008; Huber et al., 2007; Huse et al., 2007; Sogin et al., 2006).

Methodologies

PCR amplification for pyrotag sequencing requires some special considerations. The PCR primers must be modified to contain the flanking A and B adapters required for the 454 sequencing process (Fig. 8.3) and bar codes can be added to facilitate pooling multiple samples into a single sequencing run (multiplexing). Samples can be multiplexed using unique 5 bp (or longer) bar codes that follow the 5' adapter and precede the primer sequence. Sequences belonging to each sample can then be identified from the bar code present in the resulting sequence.



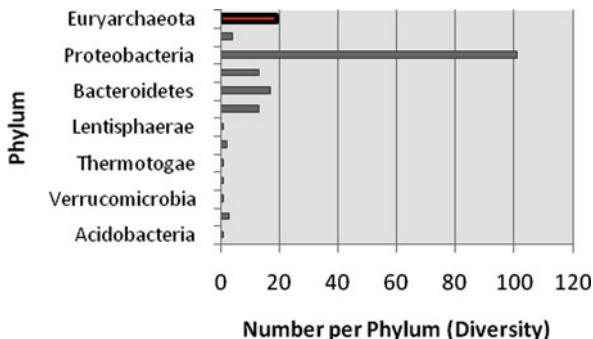
Fig. 8.3 Primers for amplicon sequencing. The primers consist of either A or B adapters which are used in the sequencing process, a MID sequence that acts as a bar code and allows multiplexing of samples during sequencing, and the primer region which is specific for the gene of interest

A typical pyrotag analysis procedure is generally organised in the following manner (Andersson et al., 2008; Dowd et al., 2008; Miller et al., 2009): (1) A quality control stage removes any short or noisy reads (i.e. reads with imperfect primer sequences). Chimeras, sequences erroneously derived from more than one template, are also eliminated. (2) Primer and adapter sequences are trimmed from the amplicon sequence. (3) OTUs (operational taxonomic units), also called phytotypes, are then created. OTUs are defined by DNA sequence similarity. Sequences with greater than 97% similarity are typically assigned to an OTU. This OTU is thought to represent a single species, sequences with greater than 85% similarity are grouped into the same genus and those with greater than 80% similarity into the same phylum (Schloss and Handelsman, 2005). (4) Sequences are classified by blasting the OTUs against 16S rRNA databases to identify the OTU's phylum, genus or even species. (5) The community analysis that follows the quality control stage can include estimation of species richness (Chao1 estimator, ACE), population evenness and diversity across communities (Shannon Index) (Bohannan and Hughes, 2003). Although many tools exist to facilitate the analysis of pyrotags, the Ribosomal Database Project has developed a data pipeline (the Pyrosequencing Pipeline) that will take raw pyrotags and will classify OTUs and generate biodiversity reports (Cole et al., 2009). Obtaining detailed metadata is as important as collecting good samples. The metadata provides information about the sample's environmental context and processing. Important metadata information about the sample's environment can include the location, temperature, pH, water chemistry, depth and pressure. It is also important to record processing and handling information such as DNA extraction method and storage conditions. Using ordination techniques, like principle component analysis (PCA), the effects of changes in metadata variables, such as temperature and depth, or species richness and diversity can be determined (Lozupone and Knight, 2005).

Applications

Although, to date, no comprehensive pyrotag survey of a petroleum environment has been published, a large Genome Canada-funded project 'Metagenomics for Greener Production and Extraction of Hydrocarbons' at the University of Calgary will be sampling many hydrocarbon resource environments over the next 4 years. As a pilot

Fig. 8.4 Estimate of species diversity from produced water sample extracted from a low temperature oilfield



for this project, the Pyrosequencing Pipeline was used to process pyrotags representing the V4 region of the 16S rRNA gene generated from DNA extracted from produced waters of a low temperature oilfield (unpublished). The pyrosequencing generated 22,693 reads averaging 238 bp of sequence. The analysis of this data identified 285 unique genera in 13 phyla (Fig. 8.4).

The sample was dominated by Proteobacteria, particularly alpha- and delta-proteobacteria. This single pilot study produced more sequence data than 2 years worth of DGGE analysis. As this project progresses, many more hydrocarbon sites will be sampled and pyrotag sequences of the V6–V8 hypervariable regions will be sequenced using titanium pyrosequencing technology.

There have been numerous comprehensive pyrotag studies conducted in other environments. Miller et al. (2009) used bar coded pyrotag sequencing to examine two alkaline hot springs (Miller et al., 2009). This analysis produced 34,000 sequences from 39 samples and identified 391 OTUs. The steep temperature gradients in alkaline hot springs are of interest to many microbiologists because they allow an assessment of the role environmental variation plays in structuring diversity. Cyanobacteria and Chloroflexi accounted for nearly 70% of the sequences obtained from the two sites. Despite differing in water chemistry and rate of temperature change over distance, the sites demonstrated similar decreases in richness and diversity with increasing environmental temperature. Temperature was a stronger predictor of community structure than geographic location or water chemistry. Surprisingly, results from this study indicated that the observed lineages of cyanobacteria and *Chloroflexi* were not co-adapted along the thermal gradient as expected, but instead demonstrated a negative correlation in abundance of the organisms along the thermal gradient.

Conclusions

The incredible sequencing depth delivered by pyrotag sequencing is providing unprecedented access to even the rarest microbial community members. Since this technique requires relatively little starting material it is particularly relevant for

hydrocarbon environments where samples may be far underground and difficult to obtain or oil saturated and difficult to process. This methodology not only provides the identity of the microbial community members in the sample and a sense of their relative abundance; by using ordination techniques (PCA) environment parameters can be correlated with the microbial community structure. Therefore, the effects that alterations to an environment's physical and chemical parameters will have on the indigenous microorganisms can be modelled. Consequently, a knowledge of the microbial community present in an environment can facilitate informed management decisions by modelling the interactions between the resources physical and chemical parameters and the indigenous community. For example, knowledge of the community structure and environmental parameters can be used to predict the likelihood of sulphide production in an oilfield and how sulphide production might change with the introduction of sulphate-rich flood water.

Total DNA Random Shotgun Sequencing

Although sequencing the total environmental DNA, can be useful for circumventing several of the problems inherent in pyrotag sequencing, such as PCR amplification bias, its utility for purely phylogenetic analysis is compromised by its requirement for greater amounts of starting material (DNA) and the increased amount of sequencing necessary to recover the rare species. This increases costs substantially. Also by adding bar codes to the PCR primers, over 100 PCR amplified samples can be run on a single 454 titanium run (Hamady et al., 2008), whereas, depending on sample complexity, only a handful of total DNA samples can be analysed per titanium run. Considering the sizable investment in time and resources that total DNA metagenomics sequencing entails it may be advisable to prescreen the environments with 16S pyrotag sequencing. This will allow for the sample's scientific interest and estimate the sample's complexity knowing an environment's microbial diversity prior to total DNA sequencing will help to determine the amount of sequencing required to adequately analyse that sample's metagenome.

However, describing the phylogenetic diversity of microbial communities is only the first step. A greater challenge is to assign ecological roles to the community members. The biological process assumed by the uncultured microbiota is an untapped resource for biotechnology applications. When the total DNA is sequenced, community analysis can be conducted by finding the 16S rRNA gene sequences in the sample (or other phylogenetic markers). However, the 16S rRNA gene sequences are only a small fraction of the information available. Sequencing the total DNA can provide the sequences of all the genes in the environment and therefore it may be more useful to analyse the activities ongoing in the environment rather than the members of the microbial community. In this case functional genes, such as those for sulphate reduction, are identified for and an environments a metabolic profile can be developed. Ideally if the amount of sequencing is sufficient, the activities ongoing in the environment can be linked back to the community members with phylogenetic anchors (Kunin

et al., 2008). Unless the environment contains very low species diversity or has a prominent dominant species, completely assembling or ‘closing’ genomes will be difficult. Therefore, linking genes to organisms will require binning and in the absence of large contigs a gene-centric analysis is the most effective approach to data analysis.

Methodologies

Due to the considerable amount of DNA required for total DNA sequencing (~5 µg), it is often necessary to employ whole-genome amplification (WGA) to generate sufficient amounts of starting material. Although all amplification techniques will introduce some bias, a recent study comparing several WGA methods found that multiple strand displacement introduced the least bias, while producing the highest yields of DNA (Abulencia et al., 2006; Pinard et al., 2006). Once sequence information is available a typical data processing procedure for total DNA metagenomics sequencing could include (1) quality control: elimination of short and noisy reads, (2) assembly of sequence reads into larger contigs, (3) gene calls and functional annotation on reads and contigs, (4) sorting the contigs into bins (binning) representing related phylogeny and (5) community composition profiling and functional analysis.

Thus far, no assembly programme has been specifically designed to deal with the problems inherent to metagenomics assembly, such as small read size and phylogenetic complexity (Raes et al., 2007). There are two strategies that can enhance metagenomic assembly, pre-binning and the use of reference genomes or sequences. Pre-binning attempts to collect sequences of phylogenetically similar origin together so that the complexity of the sequences to be assembled is reduced. Reference sequences are previously sequenced large segments of DNA from organisms believed to be in the sample of interest. These are used as a backbone on which to assemble the metagenomic reads. Gene calls can be made by both evidence-based approaches that use homology (BLAST searches) to identify genes and ‘ab initio’ techniques that separate genes from non-coding regions using the intrinsic features of coding DNA. ‘ab initio’ techniques have the ability to identify novel genes without known homologs. BLAST comparison tools such as CRITICA (Badger and Olsen, 1999) and Orpheus (Frishman et al., 1998) are useful for the homology approach of gene finding, while ‘ab initio’ tools include GLIMMER (Delcher et al., 1999) and Genemark (Besemer and Borodovsky, 1999). MetaGene is a gene finding tool that has been specifically designed to work the shorter, fragmented nature of metagenomic data (Noguchi et al., 2006). Binning is the process of associating the reads, contigs and genes generated from sequencing with the organisms from which the sequences were derived. Although assembling reads into large contigs containing multiple phylogenetic anchors is the best binning method, sequences can also be associated with organisms by comparing the sequence similarity of genes with BLAST searches and by analysing the sequence composition. Codon usage and processes such as restriction-modification systems

and DNA repair mechanisms can generate sequence composition signatures distinct to particular organisms (Deschavanne et al., 1999; Karlin et al., 1997). While the MEGAN software programme can implement binning using homology searches (Huson et al., 2007), both TETRA (Teeling et al., 2004) and Phylophythia (McHardy et al., 2007) use sequence composition for binning. In low complexity environments where a few species dominate the microbial community, reasonably complete draft genomes may be assembled and, therefore, the data analysis will resemble the analysis of isolated genomes. In more complex environments, a gene-centric approach will be more appropriate. In this approach, the community is analysed in aggregate, and the contributions of individual species are ignored. Genes are collected into gene families to highlight functional activities, based on the assumption that high relative abundance is correlated with metabolic and ecological significance (Tringe et al., 2005). Both MEGAN (Huson et al., 2007) and MG-RAST (Meyer et al., 2008) are publically available tools for annotation, binning, phylogenetic classification, gene centric analysis and metagenome comparison.

Applications

Random shotgun sequencing of the total environmental DNA has been used to study both relatively simple and complex environments. The extreme acidic, metal-rich acid mine drainage environment harbours a microbial community dominated by just five types of microorganisms (Tyson et al., 2004). Because of the low number of species, 85% of the 103,462 quality trimmed reads (76.2 million bp) were assembled into contigs with a length greater than 3 kb. Two of the five genomes were nearly completely assembled and the remaining three were partially assembled. This high level of assembly allowed characterisation of the organisms' metabolic pathways, genetic exchange pathways and the genes used for coping with low pH and metal toxicity.

The more complex samples taken from the Sargasso Sea did not lead to any complete genomes. The sample contained at least 1,800 genomic species, including 148 previously unknown bacterial phylotypes and over 1.2 million previously unknown genes (Venter et al., 2004). Notably, 782 new rhodopsin-like photoreceptors were identified. Genes of the proteorhodopsin family were found to be one of the most overrepresented gene populations in ocean surface waters and now seem to be important in oceanic energy cycling (Tringe and Rubin, 2005). Although a follow-up RNA-based study of ocean surface waters confirmed the importance of proteorhodopsins (Frias-Lopez et al., 2008), it found that other genes highly represented in the metagenomics sequencing were not highly expressed as RNA. It therefore may be erroneous to assume that genes abundant in the metagenome are always active in the environment.

The study by Edwards et al. (2006) was one of the first to sequence the total environmental DNA using pyrosequencing. Samples were collected from two adjacent sites deep in a Minnesota mine (Edwards et al., 2006). Each sample produced

over 300,000 sequences and over 70 million base pairs. Despite their close special relationship, a gene-centric analysis using MG-RAST emphasised the unique metabolic potential of each environment. Distinct pathways for carbon utilisation, iron acquisition, nitrogen assimilation and respiratory pathways were used by microbes in the two locations. While one location's population was dominated by *Actinomycetales*, *Chromatiales* were most prominent in the other. Despite the lack of closed genomes, many potential metabolic systems, such as nitrate reduction pathways, were constructed.

Conclusion

Although the sequencing of total environmental DNA is a much more expensive and difficult endeavour than pyrotag sequencing, the amount of information produced is impressive. Knowing the metabolic pathways present in an environment is often more important than determining the species indigenous to the environment, especially if the function of many indigenous species is uncertain. For example, a knowledge of the presence of sulphate-reducing and/or nitrate-reducing genes can play an important role in decisions about corrosion control. However, as was seen in the Sargasso Sea study, the presence of genes in the environment does not necessarily mean that these are also highly expressed as RNA or proteins.

Metatranscriptomics

Although metagenomic sequencing can provide information about the organisms and metabolic processes present in an environment, it does not directly indicate which organisms and processes are currently active. Although an analysis of protein expression would provide the more direct method of determining the biological processes ongoing in an environment, measuring protein expression in environmental samples is still a nascent science (Maron et al., 2007). Instead, although there is not always a one-to-one correlation between messenger RNA (mRNA) and protein expression, mRNA expression is typically used as an indication of protein activity. Also the expression of the rRNA genes can be used as an indication of the microbial activity and provide a method of determining which community members are active and growing. By using reverse transcriptase enzymes to convert the total environmental mRNA to complementary DNA (cDNA), the same sequence-based techniques used to analyse DNA can be used to analyse the transcriptome (all RNA molecules, or 'transcripts,' produced in one or a population of cells). Individual gene expression can be assessed by reverse transcription PCR (RT-PCR). This technique provides a highly accurate and quantitative measure of RNA expression, but it is not a high-throughput technique. By fixing thousands of probes on a glass slide, microarrays can analyse thousands of genes at once, but they can only measure expression for genes that probes were made for and will miss expression of unknown or unexpected gene expression. Using pyrosequencing of

total environmental cDNA (metatranscriptomics) will generate information on all the genes being expressed in the sample. The limitations of metatranscriptomics are the same as those of the metagenomics of DNA sequences and include the inability to obtain truly quantitative results, and costs large sample handling. Obtaining samples for metatranscriptomics is particularly problematic. Not only is a large quantity of RNA required, RNA is easily degraded and has a short half-life. The average half-life of RNA in *E. coli* was measured at less than 3 min and the rate of decay is quite variable, many transcripts were stable for over 20 min, consequently the RNA decay will skew the gene expression measurements (Selinger et al., 2003). If the RNA cannot be rapidly isolated, the captured RNA will no longer represent an accurate reflection of gene expression.

Methodologies

Once the environmental mRNA has been isolated and converted to cDNA the analysis procedure mirrors the gene-centric analysis of environmental DNA. In fact, to generate semi-quantitative results, some studies have extracted the total DNA along with the RNA and have used the extracted DNA for normalisation (Frias-Lopez et al., 2008). The cDNA helps with normalisation because it provides information on the gene copy number of the metagenome. To generate sufficient amounts of cDNA for pyrosequencing (2–5 µg) the extracted RNA must often be amplified. Although several methodological variations of linear RNA amplification are in use, these techniques typically all rely on the T7 RNA polymerase (Friis-Lopez et al., 2008; Moreno-Paz and Parro, 2006). Often the T7 promoter is incorporated into the random primer used for reverse transcription of the mRNA to cDNA, creating cDNA with a T7 promoter that can be amplified. Since amplification is generally not uniform, its use will make attempts at quantification prone to errors. After amplification, pyrosequencing can proceed by using the same methodology for standard DNA samples.

Applications

Frias-Lopez et al. (2008) analysed the community gene expression in ocean surface waters by pyrosequencing amplified cDNA normalised with pyrosequenced total DNA. The cDNA library contained 128,324 reads including 67,859 rRNA reads, while the DNA library possessed 414,323 reads and 5,877 rRNA reads for a total of more than 60 million bases. The RNA amplification was able to generate 2 µg of cDNA from 200 ng of total RNA using the MessageAmp II-bacteria RNA amplification kit (Ambion). Not surprisingly genes related to oxygenic photosynthesis were found to be highly expressed. Surprisingly, the genes for alternative forms of phototrophy, such as the genes from *Roseobacter*-related species for light-harvesting proteins were highly expressed, despite corresponding to low abundances in the DNA libraries (low environmental gene copy). This could indicate

the ecological importance for anoxygenic phototrophy in the aerobic ocean surface layers. Analysis of the phylogenetic information indicated that *Prochlorococcus* and *Pelagibacter* were the most abundant genera in both the cDNA and total DNA libraries.

Since metatranscriptomics does not rely on pre-synthesised probes, it can be used to discover novel RNA species. Many cDNA sequences derived from metatranscriptomics studies share no homology with any known peptide sequences. Although many of these transcripts may represent uncharacterised proteins, some may be small RNAs (sRNAs) that map to intergenic regions of microbial genomes and act as regulatory elements (Shi et al., 2009). Metatranscriptomics may be an ideal technique for the identification of these genetic elements. Analysis of metatranscriptomic data from ocean surface waters not only found a great number of unknown putative sRNAs but also found that their expression could be linked to environmental niches (Shi et al., 2009).

Conclusion

Although the rapid isolation of sufficient quantities of total RNA from the environment represents a significant challenge and the need for amplification can make quantification problematic, metatranscriptomics may be the tool best suited for identifying the organisms and genes that are active in an environment. Although it is important to know all the organisms and genes present in the environment, the environment's genetic potential, it is the active species and genes that will have the greatest immediate impact and should therefore have the greatest impact on resource management decisions. For example, if it can be determined that sulphate-reducing genes are active in an anoxic environment, it can be predicted that the corrosion of any nearby iron will soon follow.

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Chapter 9

How Many Microorganisms Are Present?

Quantitative Reverse Transcription PCR (qRT-PCR)

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Introduction

Quantitative reverse transcription PCR (qRT-PCR) is a variation of conventional quantitative or real-time PCR, whereby mRNA is first converted into the complementary DNA (cDNA) by reverse transcription, the cDNA is then subsequently quantified by qPCR. The use of mRNA as the initial template allows the quantification of gene transcripts, rather than gene copy numbers. mRNA is only produced by actively metabolising cells and is produced by its corresponding gene to provide a ‘blueprint’ in order for a cell to manufacture a specific protein. Conventional qPCR detects not only DNA present in actively metabolising cells but also inactive and dead cells. qRT-PCR has the advantage that only actively metabolising cells are detected, hence provides a more reliable measure of microbial activity in oilfield samples. When qRT-PCR is combined with primers and probes for specific genes, the activity of microbial processes important in the oilfield, such as sulphate reduction, methanogenesis and nitrate reduction can be monitored.

Principles

qRT-PCR differs from conventional qPCR in that the mRNA molecule is first transcribed into its complementary DNA (cDNA) molecule using the enzyme reverse transcriptase. The resultant cDNA is then amplified and quantified by qPCR, using primers specific to a defined nucleotide sequence of the gene of interest. For instance, for detection of the dissimilatory (bi)sulphite reductase gene (*dsrAB*), which is responsible for producing a key enzyme involved in the reduction of sulphate to sulphide by sulphate-reducing bacteria (SRP), primers complementary to a defined region of the *dsrAB* gene sequence would be used depending on the organisms that need to be targeted.

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In conventional PCR, DNA amplification involves three stages. First the double-stranded DNA is denatured by heating in a thermo-cycler to 95°C to produce single-stranded DNA. The reaction mix is then cooled to allow annealing of the primers to each complementary strand of DNA, typically at a temperature of approximately 55°C, but the appropriate temperature will ultimately depend on the length and composition of the primers, hence the primer annealing temperature is always first optimised. The reaction mix is then heated to 72°C to allow the single-stranded DNA molecules to be copied (extended from the primers) to produce two double-stranded DNA molecules using the thermostable enzyme, *Taq* DNA polymerase. The reaction mixture is once again heated to 95°C to denature the double-stranded DNA molecules into single-stranded DNA molecules and the cycle repeated for many cycles (typically 30) to exponentially amplify the amount of DNA present in the original sample. At the end of the cyclic reaction, the DNA can then be visualised by running the DNA on an agarose gel and then staining the DNA with a suitable stain such as ethidium bromide which fluoresces under UV light. Conventional PCR has a major drawback in that the PCR product can only be visualised at the end of amplification and is only semi-quantitative at best.

Quantitative PCR (qPCR) is a method by which DNA amplicons can be visualised at each amplification cycle as the amplification progresses using fluorescent reporter molecules (in ‘real time’). The qPCR thermal-cycler is an instrument capable of collecting the fluorescent data online, in real time with every PCR cycle. The instrument contains an excitation light source that excites the fluorescent reporter molecules to emit fluorescence that can be detected and quantified by the machine. The system is linked to a computer with software to convert the fluorescent signals into DNA amplification curves. The early stages of PCR are characterised by background fluorescence. With each PCR cycle the product amount increases until its fluorescence intensity exceeds the background fluorescence. The PCR cycle at which the product fluorescence rises above the background fluorescence is called the threshold cycle (C_t). An example of a qPCR plot is shown in Fig. 9.1, with the horizontal line representing the limit of the background fluorescence. The exponential phase of PCR begins at this point, and quantification of the PCR product, and later on the initial concentration of the template, is performed at this stage of the reaction. Since the quantity of DNA theoretically doubles every cycle during the exponential phase, relative amounts of DNA can be calculated, e.g. a sample whose C_t appears three cycles earlier than another sample has $2^3 = 8$ times more template. At the end of the PCR cycle the reaction enters a plateau phase characterised by lower product accumulation rates.

In qPCR, there are three main types of fluorescent reporters: non-specific DNA binding molecules such as SYBR green, TaqMan® probes and molecular beacon probes. Non-specific DNA binding molecules such as SYBR green which fluoresce in proportion to the quantity of DNA present offer the cheapest and simplest means of carrying out qPCR; however, any non-specific DNA amplification will also result in fluorescence and hence can give rise to ‘false-positive’ results. TaqMan® probes offer greater specificity since they anneal specifically with a region of DNA amplified by a specific set of primers. As DNA extension progresses, the DNA polymerase will cleave the probe releasing a fluorophore resulting in fluorescence in direct

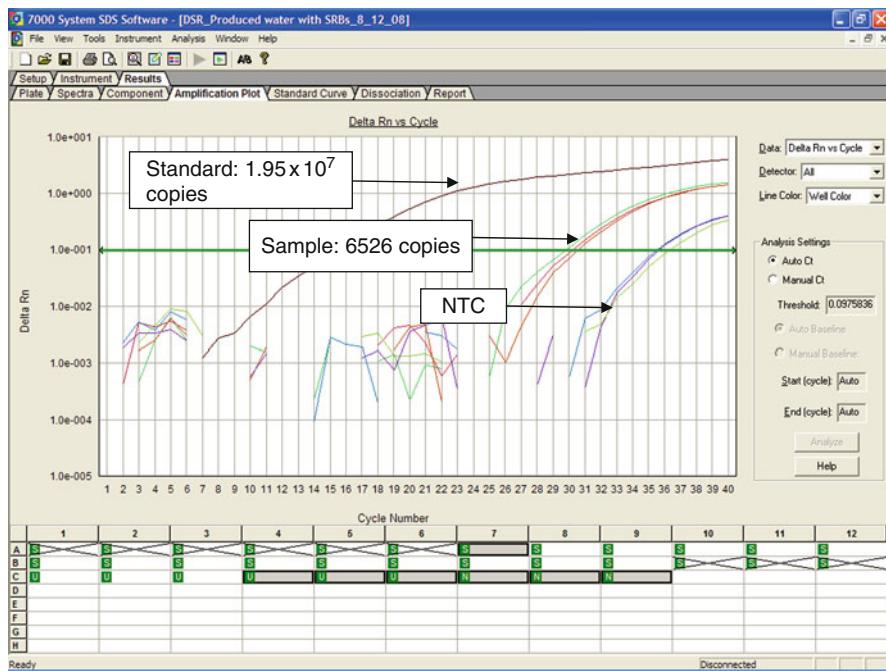


Fig. 9.1 Amplification plot for *dsrA* standard, SRB-spiked water sample and non-template control (NTC)

proportion to the amount of DNA present, allowing quantitative measurements during the exponential stages of PCR. The one disadvantage of TaqMan® fluorescent probes is that a different probe has to be synthesised for each unique target sequence. Molecular beacon probes, in principle, are similar to TaqMan® probes in terms of their specificity but unlike TaqMan® probes, they are designed to remain intact during the amplification reaction and must rebind to the target sequence in every cycle for signal measurement. It has been suggested that molecular beacon probes offer greater sensitivity than TaqMan® probes.

For quantification of the PCR products there are two main types of quantification, absolute quantification and relative quantification:

- Absolute quantification is the most commonly applied technique in microbiology and allows quantification of a single target sequence and express the final result as an absolute value, e.g. *dsrAB* copies per millilitre of sample.
- Relative quantification determines the changes in mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. This reference gene is often a housekeeping gene and can be co-amplified in the same tube in a multiplex assay. Therefore, relative quantification does not require standards with known concentrations and the reference can be any transcript, as long as its sequence is known.

For absolute quantification it is necessary to produce a standard curve with known copy numbers of the gene sequence of interest; environmental samples containing unknown concentrations of the target DNA sequence can then be compared with this standard curve and the copy number of the target sequence determined in the environmental sample, assuming DNA extraction is 100% efficient. In order to construct the standard curve, the purified target sequence should be prepared at a known concentration. qPCR is then performed on a series of dilutions of the standard, and the standard curve obtained by plotting the threshold cycle number (C_t) against the logarithm of the copy number of the standards ($\log C_0$). An example of a qPCR standard curve is given in Fig. 9.2.

To ensure that the amplified product is the target DNA sequence, melting curves should be generated for the PCR product; this is particularly important if a non-specific DNA binding fluorescent reporter dye such as SYBR green is used. A specific PCR product will have a corresponding melting point, which is dependent mainly on the GC (guanine and cytosine) content and length of the amplicon sequence. The melting curve of the known purified standard sequence can then be compared with that for the amplicon from the environmental sample. An example of the melting curve for the *dsrA* gene sub-unit is given in Fig. 9.3. However, to be certain of the identity of the amplified product, DNA sequencing should also be performed on the PCR product.

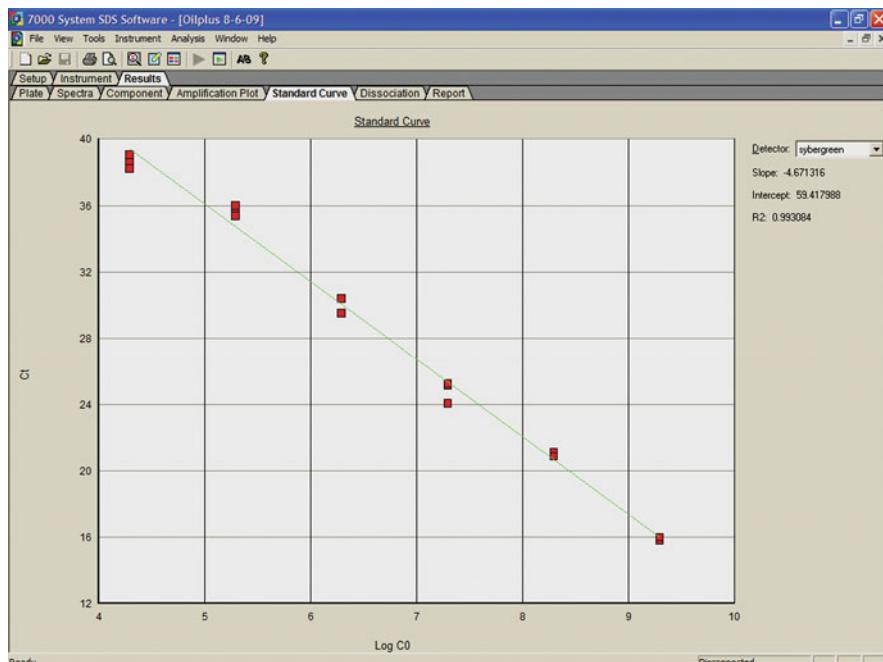


Fig. 9.2 Standard curve for log copy number of *dsrA* gene plotted against C_t

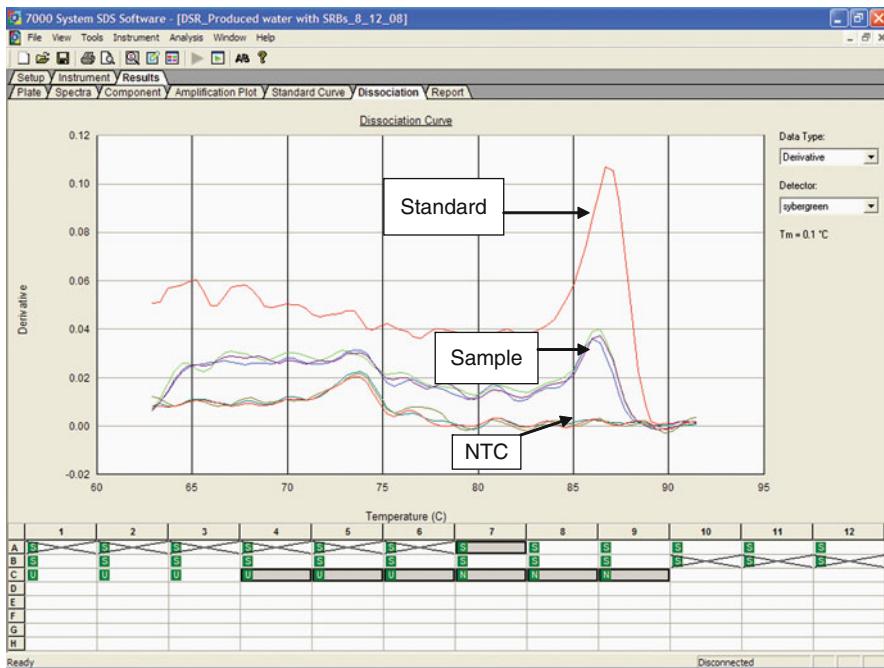


Fig. 9.3 Melting curves for the *dsrA* gene standard, SRB-spiked water sample and non-template control (NTC)

Case Study: Detection of SRP Activity in Produced Water Samples by Quantification of mRNA for the Dissimilatory (Bi)Sulphite Reductase Gene (*dsrA* Sub-unit) by Reverse Transcriptase qPCR

Maersk Oil has been injecting nitrate into the Halfdan Reservoir, a North Sea field in the Danish Sector, for over 8 years to help prevent H₂S production by sulphate-reducing prokaryotes (SRP). The mechanisms by which nitrate mitigate H₂S production are still unclear, but include competitive exclusion of SRP by increasing the growth of NUB, nitrite toxicity and inhibitory effects, change in redox potential and for some SRP a change in metabolism from sulphate reduction to nitrate reduction (Fauque and Ollivier, 2004; Moura et al., 2004; Krekeler and Cypionka, 1994; Dalsgaard and Bak, 1994; Plugge et al., 2002; Mitchell et al., 1986).

Maersk Oil has previously tested and now routinely uses, Fluorescence in situ Hybridisation (FISH) probes for several subphyla within the *Bacteria* and *Archaea* kingdoms. Useful results have been generated for detecting the efficiency of nitrate treatment with respect to the prevention of reservoir souring. Maersk Oil now wishes to not only enumerate these prokaryotic microorganisms but also complete the picture of their metabolic activity as determined

by RNA production. RNA is only produced by actively metabolising microorganisms; hence the activity of different microorganisms can be ascertained by their RNA profile, which is unique to a domain, kingdom, phylum, class, order, family, genus, species, strain or specific genotype of microorganisms. Theoretically, the more actively metabolising the microorganism, the more RNA should be present. Therefore, by measuring the activity of SRP (sulphate-reducing prokaryotes, *Bacteria* and *Archaea*), by determining intracellular RNA levels, it should be possible to understand how nitrate affects SRP activity. Messenger RNA (mRNA) is produced only by active cells to transcribe a specific protein, e.g. the sulphite reductase enzyme involved in sulphate reduction of both *Bacteria* and *Archaea*. mRNA is detected by quantitative reverse transcription PCR (qRT-PCR). qRT-PCR is already widely used in basic research, for biomedical applications and in biotechnology as a routine tool for quantifying cellular mRNA levels. A brief description of the procedure for detecting mRNA for *dsrA* (a sub-unit of *dsrAB*) is as follows:

1. Standard calibration curve for purified *dsrA* gene transcripts produced.
2. Samples collected and preserved chilled with RNAlater® (Ambion), concentrated (by centrifugation or filtration), cells lysed in phenol–chloroform in Lysis Matrix B tubes (Bio 101 System, Q-Biogene®) and bead-beaten, then treated with DNase to remove DNA.
3. Control PCR with primers targeting the *dsrA* gene (to ensure no DNA contamination of the samples).
4. Synthesis of cDNA by reverse transcription of the RNA sample.
5. qRT-PCR with the cDNA as the template.
6. Absolute quantification of *dsrA* mRNA transcripts by comparison with standard calibration curve. The RNA extraction procedure is based on the method published by McKew et al. (2007). The primers and methodology used in the qRT-PCR protocol were based on the procedures published by Chin et al. (2004, 2008).

In this study, mRNA produced by the dissimilatory (bi)sulphite reductase gene (*dsrA*) was extracted and quantified by reverse transcription qPCR to give an indication of SRP activity. The ‘proof of concept’ of quantifying mRNA levels in SRP by qRT-PCR was first demonstrated in the laboratory before taking samples in the oilfield. Two producer wells were sampled on Halfdan: HDA-03XA and HDA-07. There is a direct fracture proceeding from the injection well HDA-12 through to HDA-07 then onto HDA-03XA, leading to seawater, cut at both wells of 80%. The transit time for the water from HDA-07 to HDA-03XA is approximately 1 day, consequently nitrate and nitrite levels are much lower and SRB numbers and sulphide production are much higher at HDA-03XA.

Samples were taken from both producer wells for mRNA analysis, DAPI and qFISH analysis, MPN analysis, H₂S analysis, sulphate analysis, nitrate and nitrite analysis and redox potential (*E_h*). The results of these analyses are shown in

Table 9.1 Results of the analyses conducted on produced water samples from two Halfdan producer wells

Analysis	HDA-07	HDA-03XA
No. of <i>dsrA</i> transcripts (per ml)	Not significant	250,000
Total count (per ml) (DAPI)	107,000	115,000
Viable SRB (per ml) (qFISH)	12,500	62,500
SRB MPN (per ml)	25	140
Sulphate (mg/l)	2,600	1,640
Total H ₂ S (kg/day)	1 (3 ppm gas phase)	16 (38 ppm gas phase)
Nitrate (mg/l)	13.8	1.2
Nitrite (mg/l)	2	0.56
E _h	-328	-337

Table 9.1. It can be seen that for well HDA-07 with significant levels of nitrate present, no mRNA transcripts for the *dsrA* gene could be detected. However, for well HDA-03XA, 250,000 mRNA transcripts for the *dsrA* gene were detected, where only low levels of nitrate were detected. This is mirrored by the H₂S production observed from the wells, 1 kg/day for HDA-07 compared with 16 kg/day for HDA-03XA. Hence, it is clear that nitrate inhibits biogenic sulphidogenesis, although the precise mechanism remains unclear. However, viable SRB numbers as determined by qFISH were 12,500/ml in HDA-07, but only approximately five times greater for HDA-03XA at 62,500/ml, as compared with 16 times greater H₂S production for HDA-03XA, with a corresponding huge increase in the number of *dsrA* gene transcripts. Hence, although there are viable SRB present in HDA-07, they do not appear to be actively metabolising sulphate, as shown by the lack of detectable levels of *dsrA* mRNA transcripts and the low sulphide production. The little H₂S observed from this well is thought to be diffusing from the surrounding reservoir matrix. Since the dominant sulphate-reducing bacteria in this reservoir is *Desulfovibrio* sp., some species of which in the presence of nitrate are known to preferentially use nitrate as their terminal electron acceptor rather than sulphate, could the nitrate in this case, be causing a switch in metabolism from sulphate reduction to nitrate reduction and hence inhibit sulphidogenesis?

In order to draw definitive conclusions from this study, it is planned in the imminent future to take more samples from the Halfdan reservoir. In addition to the aforementioned analyses, the samples will also undergo analysis for the presence of viable nitrate-utilising bacteria (NUB) and mRNA transcripts that indicate the presence of NUB actively metabolising nitrate. One final point is the inadequacy of conventional MPN culture techniques for use in the oil industry. It can be seen in Table 9.1 that the SRB count for HDA-07 is 25/ml compared with a qFISH count of 12,500, similarly the SRB count for HDA-03XA is 140/ml compared with a qFISH count of 62,500. Therefore, only approximately 0.2% of the total SRB are being detected by the MPN culturing technique when compared with the

qFISH method. This compares with previously published work that has shown that only between 0.01 and 10% of the viable microorganisms present in environmental samples are able to grow in culture media in the laboratory, the other 90–99.9% of microorganisms are known as viable but non-culturable organisms (VNCOs) (Amann et al., 1995; Davies and Scott, 2006; Roszak et al., 1984; Turpin et al., 1993; Xu et al., 1982).

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Chapter 10

How Many Microorganisms Are Present?

Techniques for Enumerating Microorganisms in Oilfields

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Introduction

The different techniques that exist for enumerating microorganisms will often yield very different results when applied to oilfield samples. For example, enumeration of sulphate-reducing bacteria (SRB) by cultivation may fail to find any microorganisms in samples for which molecular microbiological methods (MMM) indicate levels of thousands or even millions per millilitre or gram. Therefore, it is important to realise the limitations and advantages of the different techniques available to the industry and to take them into account when interpreting data. In oil systems, the most widely used techniques for quantification are based either on culturing, epifluorescence microscopy, or quantitative PCR. In complex samples in which live, inactive, and dead cells are present together with cell material in various states of decomposition, each of these three methodologies enumerates a different subset of microorganisms (Fig. 10.1).

Methods based on cultivation, such as the widely used most probable number (MPN) technique, enumerate the number of living microorganisms in a sample that will grow in a given enrichment medium. Due to factors such as selectivity of the medium, cell aggregation, and the presence of live, but non-viable microorganisms in the sample, this method tends to underestimate the number of active microorganisms, usually by several orders of magnitude (e.g. Hoffmann et al., 2007; Konuma et al., 2001). DAPI staining and Fluorescence in situ Hybridisation (FISH) are both based on fluorescent labelling of nucleic acids inside the cell and subsequent enumeration by epifluorescence microscopy. DAPI binds to double-stranded DNA and thereby detects all cells irrespective of their metabolic state. In conventional FISH, ribosomal RNA (rRNA) is used as the target molecule and consequently only active microorganisms that contain a certain amount of ribosomes are detected (Amann, 1995). Currently, these methods are routinely applied to samples of, for example, injection water in oilfields. They provide a more exact measure

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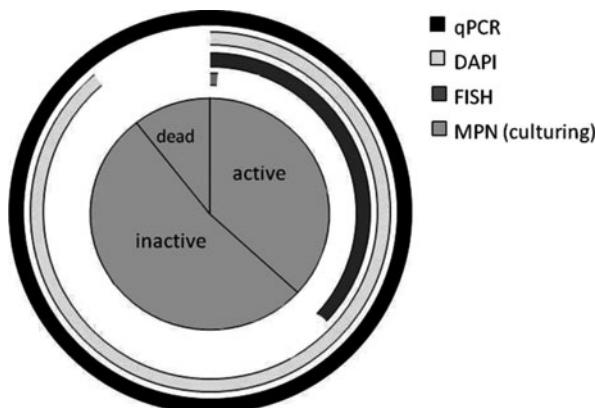


Fig. 10.1 An illustration of the different pools of microorganisms (live, inactive, and dead) typically present in samples from the oil industry, and the portions that are enumerated using different techniques. Each of the methods indicated is discussed further in the text

of the microorganisms present than revealed by culture-based methods. In oil-rich samples such as produced water, epifluorescence microscopy may be difficult to apply because of problems with high background fluorescence from the organic oil compounds. The technique may also be unsuitable for samples in which cells are embedded in a solid matrix such as scale, biofilm, or sediment.

During qPCR, genetic material is extracted from a sample and subsequently the numbers of copies of a specific gene in the extract are quantified. Thus, unlike the methods mentioned above, qPCR does not rely on visualisation, viability, or separation of individual microorganisms, and it is well suited for otherwise difficult samples such as solids, corrosion products, or produced water. This can be a great advantage, for example, in regards to MIC diagnostics, where the relevant microorganisms are associated with biofilms and scale deposits attached to the metal surface rather than the planktonic phase. As illustrated in Fig. 10.1, qPCR does not distinguish between live and dead cells, and it can be problematic to imply activity from the obtained cell numbers. However, as will be illustrated in the case study below, the technique can be modified to overcome at least part of this limitation.

When choosing a technique, for example, surveillance or failure analysis in an installation, it is important to take into account the advantages and disadvantages of each of the techniques mentioned above. The choice of technique should depend on (i) the specific question that needs to be answered, (ii) the nature and state of the sample, (iii) the sampling equipment and facilities available on-site, and (iv) logistics regarding shipment. For example, many microorganisms, including most SRB, will die upon exposure to atmospheric oxygen, with detrimental effects on culturing surveys. Thus, culturing of anaerobic microorganisms should ideally only be applied when sampling and handling can be carried out under strict oxygen-free conditions. On the other hand, samples for molecular analyses like FISH or qPCR need to be fixed on site or at least shipped to the laboratory facility as fast as possible in order

to ensure reliable results. In the following, an example will be given that illustrates the application of qPCR to samples of injection water, produced water, and scale from offshore oilfields in the North Sea, Denmark.

Case Study: The Halfdan Field

The Halfdan and Dan oilfields (Jacobsen et al., 1999; Albrechtsen et al., 2001) are both located in the Danish sector of the North Sea and are operated by Maersk Oil on behalf of the Danish Underground Consortium (DUC). The two fields are adjacent such that the west flank of the Dan field is bordering the south-east flank of the Halfdan field. Nitrate is being added to injection water at the Halfdan field in order to prevent excessive reservoir souring, while no nitrate is added at Dan. The reservoir temperature in both fields is some 80°C. These two fields thus present an ideal opportunity to study the effect of nitrate addition to injection seawater on the microbial communities within the reservoir. The objective of this study was to apply qPCR-based assays to detect and enumerate sulphate-reducing prokaryotes (SRP) in samples of produced water from the two reservoirs, and to reveal whether nitrate addition reduces the number of SRP in the produced water.

Development of qPCR Assay

Prior work in the North Sea oilfields had identified several groups of sulphate-reducing prokaryotes (SRP) to be present in the system, including both sulphate-reducing archaea (SRA) and sulphate-reducing bacteria (SRB) (summarised in Table 10.1). The organisms listed in the table were identified by cloning and sequencing of genes for 16S ribosomal RNA (16S rRNA) and/or dissimilatory sulphite reductase (DSR) from samples of produced water and scale from both fields. They include several different groups of SRB, affiliated with both spore-forming gram-positive *bacteria* (*Desulfotomaculum*) and non-spore-forming *bacteria* (*Deltaproteobacteria*, *Thermodesulfobacteria*, and *Thermodesulfovibrio*). The SRB include potentially mesophilic as well as thermophilic members (Table 10.1). Members of the *Archaeoglobus*, a group of thermophilic SRA, were also previously

Table 10.1 Groups of sulphate-reducing prokaryotes detected in samples from Halfdan and Dan fields

Sulphate-reducing groups	Affiliation	Temperature regime
Genus <i>Desulfotomaculum</i>	SRB	Thermo-/mesophilic
Subphylum <i>Deltaproteobacteria</i>	SRB	Thermo-/mesophilic
Class <i>Thermodesulfobacteria</i>	SRB	Thermo-/mesophilic
Genus <i>Thermodesulfovibrio</i>	SRB	Thermo-/mesophilic
Genus <i>Archaeoglobus</i>	SRA	Thermophilic

detected in samples of produced water and scale material by sequencing genes for both 16S rRNA and DSR.

As described in Chapter 4, a crucial step in the use of PCR based techniques is the choice of PCR primers. These are short pieces of oligonucleotides that are designed to anneal to a specific site in the genome of the target organism. In this case, an initial evaluation of the *dsr* genes of the organisms found in the oilfield revealed that none of the previously published *dsr* gene primers were suitable for qPCR application, and that new primers had to be developed and tested.

Based on the previously obtained DNA sequences of *dsr* genes from the Dan and Halfdan fields as well as related published sequences available at the Genbank web site (www.ncbi.nlm.nih.gov), it was possible to design two specific primer sets for the detection of *dsr* genes from SRB and SRA, respectively. Since the *dsr* gene has so far been found in only one copy per SRB/SRA, the number of *dsr* genes in a sample can be converted directly to a number of cells. These primer sets were tested thoroughly on known positive and negative controls as well as on environmental samples in order to ascertain their specificity. Furthermore, PCR products obtained with each of the primer sets were cloned and sequenced in order to confirm that only *dsr* genes of the respective target organisms were amplified in the reaction. In addition to the *dsr* gene primers, previously published primers for bacterial and archaeal 16S rRNA genes were applied (Amann et al., 1990; Loy et al., 2002; Takai and Horikoshi, 2000; Delong, 1992). This gene is present in all prokaryotes, often in more than one copy per organism. Thus, it is possible to obtain an estimate of the total number of *bacteria* and *archaea* by dividing the measured number of 16S rRNA genes in a sample by an estimated average copy number per organism. In this study assumed that on average three copies of the 16S rRNA genes were present in an organism. The qPCR assays used in this study are summarised in Table 10.2.

Table 10.2 Analysis parameters and methods

Parameter	Unit	Target gene	References	Primer set
Total <i>bacteria</i>	Genes per g/ml	16S rRNA	Amann et al., 1990; Loy et al., 2002	8F/338R
Total <i>archaea</i>	Genes per g/ml	16S rRNA	Delong, 1992; Takai and Horikoshi, 2000	806F/958R
Sulphate-reducing <i>bacteria</i>	Genes per g/ml	<i>dsr</i>	This study	SRB-DSR
Sulphate-reducing <i>archaea</i>	Genes per g/ml	<i>dsr</i>	This study	SRA-DSR

Distribution of SRB and SRA in the Oilfields

The four primer sets were applied on DNA extracts from a collection of samples of scale, injection water, and produced water from Dan and Halfdan fields, thus yielding total counts of genes for 16S rRNA and *dsr* genes from *bacteria* and *archaea*. To make sure that co-extracted PCR inhibitors did not affect the results, we monitored amplification efficiency of positive controls with and without added DNA extract.

Table 10.3 Numbers of total 16S rRNA genes and bacterial/archaeal *dsrAB* genes enumerated with qPCR. Numbers are reported as genes/ml (produced or injection water) or genes/g (scale). The detection limit of the method was 10^3 genes/ml for bacterial and archaeal 16S rRNA genes and 10^2 genes/ml for the *dsr* genes

Sample type	Origin	Total 16S rRNA	<i>dsr</i>	
		Bacteria + Archaea	SRB	SRA
Produced water	Dan field	6.7×10^6	5.3×10^4	1.3×10^6
	Halfdan field	6.5×10^6	4.9×10^4	1.3×10^6
	Halfdan field	6.4×10^6	5.4×10^4	7.3×10^5
Injection water	Halfdan field	2.4×10^5	$<10^2$	$<10^2$
	Halfdan field	2.0×10^8	1.2×10^6	2.8×10^5
	Halfdan field	1.4×10^8	3.2×10^5	1.4×10^4
	Dan field	2.2×10^8	7.3×10^6	1.7×10^5

Furthermore, a dilution series of DNA extracts (1, 10, 100 times diluted) were used as template in qPCR. These experiments indicated that the analysis was not biased by PCR inhibition. The samples and analytical results are summarised in Table 10.3.

The total number of prokaryotes was similar in all the samples of produced water, and the injection water from Halfdan contained fewer microorganisms than the produced water. The solid scale material sampled at Dan and Halfdan X-mas trees had approximately 100 times higher density of microorganisms than the liquid samples of produced water. Figure 10.2, panels (a)–(d) illustrate the abundance of SRA and SRB in samples of produced water and X-mas tree scale from the Dan and Halfdan fields.

As was illustrated in Fig. 10.1, detection by qPCR does not necessarily imply activity, since DNA from both dead and live biomass is extracted during conventional DNA extraction. To investigate whether the detected *dsr* genes originated

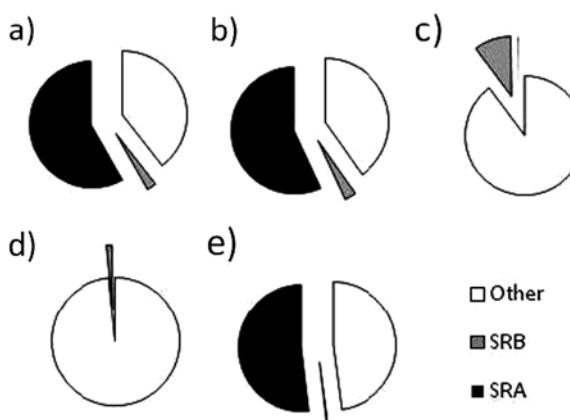


Fig. 10.2 The relative proportions of SRB, SRA, and other microorganisms as indicated by qPCR for 16S rRNA and *dsr* genes. The results are from (a) sample of produced water from Dan field, (b) samples of produced water from Halfdan field (average of 2), (c) sample of X-mas tree scale from Dan, (d) samples of X-mas tree scale from Halfdan (average of 2), and (e) produced water from Dan when excluding DNA from dead cells and debris (see text for details)

from intact cells, a modified DNA extraction was applied on the sample of Dan produced water. This extraction included the addition of propidium monoazide (PMA) to the sample prior to extraction by a specialised procedure (Nocker et al., 2006). PMA penetrates non-intact (i.e. dead or damaged) cells and binds to the DNA inside. The resulting PMA–DNA complex is not retrieved during subsequent DNA extraction. Furthermore, should any PMA-bound DNA enter the DNA extract, the subsequent PCR amplification is strongly inhibited. Thus, by adding PMA to the sample prior to extraction and following the specialised extraction protocol, only DNA from intact (i.e. live) organisms will be included in the qPCR analysis. Interpretation of results obtained with this procedure should take into consideration that cells that are damaged or ruptured during sampling (e.g. due to depressurisation) are not counted, and numbers of active microorganisms may thus be underestimated.

The results of the PMA experiment are summarised (Fig. 10.2). The modified DNA extraction protocol with PMA yielded an overall smaller amount of DNA (about 50%, data not shown), but the relative abundances of the SRB, SRA, and non-SRPs did not change much as a result of PMA addition (Fig. 10.2, compare (e) with (a)). The high degree of similarity between the PMA amended and the normal DNA extraction suggests that the detected microorganisms were intact and alive in the sample.

SRB and SRA were detected in all samples except the nitrate-amended injection water from Halfdan field. It should be noted that several subsequent studies have indicated the presence of SRB, but not SRA, in various levels in injection seawater from the Halfdan and Dan fields (data not shown). Thus, it is likely that SRB were present in the sample, albeit in numbers below the detection limit of 10^2 genes/ml. SRA were numerous in produced water from both the Halfdan and Dan fields. Assuming that the *bacteria* and *archaea* contained on average three 16S rRNA genes and that the SRA contained one *dsr* gene each, the SRA constituted 58% of the total population in the sample from Dan and 34 and 60% in the two samples from Halfdan. In contrast to the produced water, the scale samples contained higher numbers of SRB than SRA.

Conclusions

The results show that produced water from the Halfdan and Dan fields contains high numbers of thermophilic SRA and relatively low numbers of SRB. This relationship is reversed in the scale samples which have higher SRB than SRA numbers. This pattern strongly suggests that SRA are the main group of SRP in the reservoir and that SRB predominantly grow in the surface facilities at lower temperatures. There was no significant difference in the numbers of SRA, SRB, or total microorganisms between the Halfdan and Dan fields, neither in the produced water nor in the X-mas tree scale material. Thus, although the use of nitrate at Halfdan reduces the net sulphide production in the reservoir, it does not appear to reduce the abundance of SRP. This suggests either (i) that the sulphate reducers in the reservoir are able to switch between nitrate and sulphate as alternative electron acceptors or (ii) that

the sulphide produced during sulphate reduction is re-oxidised to sulphate by nitrate reducers.

In summary, this study demonstrates how qPCR can be used as a tool for accurately quantifying sulphate-reducing bacteria and *archaea* in samples of produced water and scale from the DUC fields. The results suggest that qPCR of SRA and SRB can be used as a source tracking tool for increased H₂S production in oilfield technical systems.

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Chapter 11

Which Members of the Microbial Communities Are Active?

Microarrays

Brandon E.L. Morris

Introduction

Here, we introduce the concept of microarrays, discuss the advantages of several different types of arrays and present a case study that illustrates a targeted-profiling approach to bioremediation of a hydrocarbon-contaminated site in an Arctic environment. The majority of microorganisms in the terrestrial subsurface, particularly those involved in ‘heavy oil’ formation, reservoir souring or biofouling remain largely uncharacterised (Handelsman, 2004). There is evidence though that these processes are biologically catalysed, including stable isotopic composition of hydrocarbons in oil formations (Pallasser, 2000; Sun et al., 2005), the absence of biodegraded oil from reservoirs warmer than 80°C (Head et al., 2003) or negligible biofouling in the absence of biofilms (Dobretsov et al., 2009; Lewandowski and Beyenal, 2008), and all clearly suggest an important role for microorganisms in the deep biosphere in general and oilfield systems in particular. While the presence of sulphate-reducing bacteria in oilfields was first observed in the early twentieth century (Bastin, 1926), it was only through careful experiments with isolates from oil systems or contaminated environments that unequivocal evidence for hydrocarbon biodegradation under anaerobic conditions was provided (for a review, see Widdel et al., 2006). Work with pure cultures and microbial enrichments also led to the elucidation of the biochemistry of anaerobic aliphatic and aromatic hydrocarbon degradation and the identification of central metabolites and genes involved in the process, e.g. (Callaghan et al., 2008; Griebler et al., 2003; Kropp et al., 2000). This information could then be extrapolated to the environment to monitor degradation processes and determine if *in situ* microbial populations possessed the potential for contaminant bioremediation, e.g. Parisi et al. (2009). While other methods have also been developed to monitor natural attenuation of hydrocarbons (Meckenstock et al., 2004), we are only at the early stages of understanding the microbial processes that

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occur in petroliferous formations and the surrounding subterranean environment. Important first steps in characterising the microbiology of oilfield systems involve identifying the microbial community structure and determining how population diversity changes are affected by the overall geochemical and biological parameters of the system. This is relatively easy to do today by using general 16S rRNA primers for PCR and building clone libraries. For example, previous studies using molecular methods characterised many dominant prokaryotes in petroleum reservoirs (Orphan et al., 2000) and in two Alaskan North Slope oil facilities (Duncan et al., 2009; Pham et al., 2009). However, the problem is that more traditional molecular biology approaches, such as 16S rRNA gene clone libraries, fail to detect large portions of the community perhaps missing up to half of the biodiversity (see Hong et al., 2009) and require significant laboratory time to construct large libraries necessary to increase the probability of detecting the majority of even bacterial biodiversity. In the energy sector, the overarching desire would be to quickly assess the extent of in situ hydrocarbon biodegradation or to disrupt detrimental processes such as biofouling, and in these cases it may not be necessary to identify specific microbial species. Rather, it would be more critical to evaluate metabolic processes or monitor gene products that are implicated in the specific activity of interest. Research goals such as these are well suited for a tailored application of microarray technology.

Microarray Technology

Gentry et al. (2009) previously presented an in-depth discussion of the different types of arrays and their various uses. Here, we discuss several types of arrays that are pertinent to applications in the oil and gas industry and present a specific case study as an example. Microarray technology was originally developed to combine a more efficient Fluorescence in situ Hybridisation (FISH) technology with DNA blot technique into a smaller and more compact package (Schena et al., 1995; Shalon et al., 1996). In short, a Southern blot-type target probe can be synthesised from DNA (using PCR) or from RNA (using RT-PCR to make cDNA), placed onto a glass slide in a uniform fashion and DNA or cDNA samples are then coupled to a fluorescent dye (Shalon et al., 1996). After hybridisation between the target and sample sequences based on complementary base paring, weak or non-specifically associated probes are washed away and results can be quantified using a fluorescence scanner. With this technology, large numbers of complex DNA mixtures can be screened for known sequences of interest or RNA samples can be analysed to characterise global gene expression patterns (Schulze and Downward, 2001). An array known as the PhyloChip, a phylogenetic oligonucleotide array containing 500,000 16S rRNA gene probes, was first used to characterise population dynamics and species richness for bacterial communities involved in uranium reduction (Brodie et al., 2006) and proved to be as reliable and more reproducible than clone libraries. Following this development, an array named the GeoChip (a functional gene array) was designed to monitor important microbial processes such as carbon fluxes, sulphate reduction, metal reduction, BTEX degradation and many other microbially catalysed activities (He et al., 2007).

Nevertheless, these tools are complicated and require special skills and expensive equipment for analysis. In addition, it may not be important to screen global population changes or gene expression if the intent is to monitor more specific processes such as hydrocarbon biodegradation or microbiologically influenced corrosion (MIC). Therefore, an array containing probes for genes or organisms implicated in anaerobic hydrocarbon degradation or MIC would be a powerful tool for monitoring anaerobic microbial processes important to the oil and gas industry. To our knowledge, such a targeted array is not commercially available but a tool with no more than 100 spots could offer substantial industry-specific information about the microbial status of a particular system. This compact, targeted platform would simplify data analysis and minimise costs, while also providing a simple effective way to replicate experiments throughout a formation or production facility. Also, it would be possible to design this array today using publically available sequence information such as the Ribosomal Database Project (RDP) at Michigan State University (<http://rdp.cme.msu.edu/>), which at the time of writing housed 1,235,044 different 16S rRNA gene sequences (Release 10, Update 16), gene information from PCR probes that were recently designed from known hydrocarbon- or aromatic-degrading organisms (Callaghan et al., 2008; Kuntze et al., 2008), or other publically available sequence databases. The following case study presents a targeted approach to characterising bioremediation in an Arctic system using molecular biology techniques.

Case Study

Increasingly, climate change is opening navigable routes across fragile Arctic ecosystems and increasing the risk of hydrocarbon contamination for these environments. For example, in September 2009 two German vessels made the first successful commercial passage across the entire Arctic Ocean (Holden, 2009). The shipping company, Beluga Shipping, plans to have six vessels operating in the Northwest Passage in 2010. Shipping costs are dramatically reduced when using this route compared to the Suez or Panama Canal. Therefore, economic incentives predict that commercial traffic in this vicinity of the planet will steadily increase. While hydrocarbons are not xenobiotic compounds in the strictest sense, anthropogenic activity can increase the concentrations present in a given area to an inordinate degree. Since biodegradation or enhanced bioremediation often represents the most feasible and cost-efficient strategy for dealing with environmental contaminants, especially in remote regions, it is important to access how different biomes will adapt and respond to the influx of such contaminants and to particular bioremediation strategies (Atlas, 1995). Past environmental contamination events and associated responses have taught many important lessons on how best to deal with a large influx of pollutants (Atlas, 1995; Bragg et al., 1994). The knowledge base must be extended to Arctic environments and assessment made of how such sensitive environments may respond to enhanced bioremediation efforts.

Recently, Yergeau et al. (2009) published a study from two diesel-contaminated sites in the Canadian high-Arctic and characterised microbial community structure

using phylogenetic oligonucleotide arrays and metabolic activity using functional gene microarrays targeted towards aerobic hydrocarbon mineralisation in combination with qRT-PCR methods. This study nicely illustrates the potential for molecular biological methods to be used in a targeted fashion. Here, phylogenetic arrays consisting of bacterial 16S rRNA genes previously associated with polar environments, and functional gene probes consisting of known saturated and aromatic alkane degrading genes involved in aerobic degradation processes were used to compare *ex situ* and *in situ* bioremediation strategies (Yergeau et al., 2009). They were able to demonstrate that bacterial numbers and functional genes involved in hydrocarbon degradation were positively correlated with nutrient amendment at both sites. In addition, it was clear 1 month after the initiation of bioremediation in the *ex situ* treatment that genes important in aerobic hydrocarbon degradation were up-regulated and microbial community structure was significantly altered. However, the expression of these genes related to aerobic hydrocarbon degradation took roughly 1 year for the *in situ* treatment. Statistical analyses also revealed that significant increases in mono- and di-oxygenase genes were correlated with nutrient amendment at both sites. It is clear from this study that remediation treatments can alter prokaryotic communities and selectively increase the transcription levels for genes related to contaminant degradation. The drastic differences between the two sites in this study, however, highlight the fact that treatment effects and nutrient amendments will likely have varying impacts on biodegradation processes. Overall successes of natural attenuation efforts must be tailored towards the specific contaminant, the biogeochemistry of the polluted site and do so within the cost and time limitations of the project.

Concluding Remarks

From this section, we suggest that microarrays are a powerful but underutilised tool for characterising microbial population structure and function. Molecular technologies are evolving constantly and applications of techniques for specific industries, as evidenced by the publication of this book, are increasingly becoming available. For example, the application of 16S rRNA gene oligonucleotide arrays to monitor sulphate-reducing populations (Caffrey et al., 2008) in a oil production facility was recently carried out successfully. The most significant limitations to more broad-scale applications of microarray technologies lies with the dependence upon sequence information and the difficulty in extracting viable nucleic acids from oil or contaminated environments and these issues need to be addressed, especially for samples with low biomass and a high presence of compounds that may interfere with downstream analysis such as metals and BTEX. It is known that the stringency of sequences detected on an array can vary depending on the hybridisation conditions and temperature used during this step of the analysis. For example, Zhou and Thompson (2002) constructed a model SSU rRNA array and showed that signal intensity decreased in a predictable manner with increasing mismatches, if they did not occur in the critical terminal or sub-terminal positions. The hybridisation

specificity for DNA-based functional gene arrays is much less than for oligonucleotide systems, although genus-level information can be obtained on a reliable basis. Critical genes involved in important cellular processes such as carbon mineralisation or sulphate reduction are highly conserved in nature, and it is difficult to assess the overall diversity of these genes in the environment. Therefore, microarrays need to be carefully replicated and analysed with regard to the interpretation of hybridisation specificity, and results should be validated with more specific and quantitative methods of functional gene detection such as qPCR. It is also difficult to compare microarray experiments among different labs or even different experiments. Biomedical research fields combat this limitation by maintaining large microarray databases, such as the Stanford Microarray Database (Demeter et al., 2007), where researchers can compare experiments and identify common ‘contaminating’ sequences, and by purchasing commercially produced arrays (such as those from AffymetrixTM). The creation of such tools for environmental systems would be a valuable addition to the diagnosis of microbial processes in oil formations and facilities. The question about the exact quantitative nature of microarrays still remains and, to fully address microbial problems associated with subterranean systems, it is important to know if increased signal intensity arises from growing populations or sequence heterogeneity. Despite the disadvantages mentioned, the benefit of microarray technology comes in the ability to screen a large number of samples in a relatively small amount of time. With future advances, it may be possible to have microarrays deployed in the field to garner real-time information about microbial population changes (Liu and Zhu, 2005). However, with the development of new extraction methods (DNA, RNA, proteins and metabolites) for environmental samples, design of more sensitive instrumentation and advancements in microarray (Stoevesandt et al., 2009) or metagenomic technology, it will be possible to characterise uncultured microbes from the subsurface with greater ease and efficiency. Ultimately though the major advances in subsurface microbiology will come from the marriage of classic microbiology techniques including cultivation with novel high-throughput molecular approaches.

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Part III

Problems Caused by Microbes to the Oil Industry and Treatment Strategies

Chapter 12

Problems Caused by Microbes and Treatment Strategies

Monitoring and Preventing Reservoir Souring Using Molecular Microbiological Methods (MMM)

Antje Gittel

Introduction

The injection of seawater during the process of secondary oil recovery in offshore oilfields supplies huge amounts of sulphate to the prokaryotic reservoir communities. Together with the presence of oil organics and their degradation products as electron donors, this facilitates the enrichment and growth of sulphate-reducing prokaryotes (SRP) in the reservoir, as well as in pipings and top-side installations (Sunde and Torsvik, 2005; Vance and Thrasher, 2005). The activity of SRP causes severe economic problems due to the reactivity and toxicity of the produced hydrogen sulphide (H_2S), one of the major problems being reservoir souring. Besides the use of broad-spectrum biocides or inhibitors for sulphate reduction, the addition of nitrate effectively decreased the net production of H_2S in model column studies (Myhr et al., 2002; Hubert et al., 2005; Dunsmore et al., 2006) and field trials (Telang et al., 1997; Bødtker et al., 2008). The mechanisms by which nitrate addition might affect souring control are (i) the stimulation of heterotrophic nitrate-reducing bacteria (hNRB) that outcompete SRP for electron donors, (ii) the activity of nitrate-reducing, sulphide-oxidising bacteria (NR-SOB), and (iii) the inhibition of SRP by the production of nitrite and nitrous oxides (Sunde and Torsvik, 2005; Hubert and Voordouw, 2007).

Identification and quantification of reservoir microorganisms, including hNRB, NR-SOB, and SRP, were so far most frequently assessed with cultivation-dependent methods, i.e. the most probable number (MPN) technique (Telang et al., 1997; Davidova et al., 2001; Bødtker et al., 2008). Cultivation-independent methods have only recently been introduced into the field of reservoir microbiology

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This chapter focuses on the microbiology of oil reservoirs with respect to the key microbial communities that are responsible for causing many reservoir problems.

(Grabowski et al., 2005; Larsen et al., 2005; Li et al., 2007; Dahle et al., 2008). Considering the small number of cultivation-independent studies, information currently available on the microbial communities and especially on the abundance of hNRB, NR-SOB, and SRP present in oil reservoirs and production systems is sparse and, most notably, not quantitative.

In a recent case study on two adjacent hot oil reservoirs in the North Sea, we studied the prokaryotic community structure (targeting *bacteria*, *archaea*, and specifically SRP) and the activity of SRP in production water (PW) of a nitrate-treated reservoir (Halfdan) versus an untreated oil reservoir (Dan) (Gittel et al., 2009). The two oil reservoirs share similar physicochemical characteristics with regards to injection water composition and reservoir conditions, but nitrate injection has only been applied at Halfdan since the start of production. It is hypothesised that the addition of nitrate to the injection water favoured the growth of hNRB and/or NR-SOB, thereby inhibiting the activity of SRP and reducing the concentration of H₂S, and is consequently reflected in a lower abundance of SRP and a more specialised prokaryotic community.

Case Study

To assess the prokaryotic community structure and diversity at Dan and Halfdan, clone libraries for bacterial and archaeal 16S rRNA genes were generated (Fig. 12.1). In total, nine bacterial (*Firmicutes*, *Alpha-*, *Gamma-*, *Delta-*, and *Epsilonproteobacteria*, *Deferribacterales*, *Synergistes*, *Thermotogales*, Candidate division OP9) and three archaeal phyla (*Thermococcales*, *Methanothermococcales*, *Archaeoglobales*) were recovered. Bacterial communities at Dan and Halfdan were significantly different, with a higher diversity and species richness in samples from Halfdan. The major group of bacterial 16S rRNA sequences were affiliated with members of the *Firmicutes* (70 and 62%, Fig. 12.1). They were assigned to clone sequences or isolates previously obtained from other high-temperature oil production systems (Rees et al., 1995; Dahle et al., 2008). Bacterial SRP were the second-most abundant group of phylotypes. For Dan PW, the majority of these sequences were assigned to delta-proteobacterial sulphate reducers (10%, Fig. 12.1) affiliated with the genera *Desulfacinum* and *Desulfovibrio*. Only a minor fraction of the sequences (<1%) were assigned to *Desulfotomaculum geothermicum*. In contrast, *Desulfotomaculum*-related SRB appeared to be more abundant at Halfdan (13%, Fig. 12.1). Clone sequences representing members of the nitrate-reducing, sulphide-oxidising *Epsilonproteobacteria* were recovered from both Dan and Halfdan PW in very low numbers, but showed a slightly higher relative abundance in Halfdan PW. Remarkably, members of the *Deferribacterales* were only found at Halfdan and constituted a large fraction in the clone library (11%, Fig. 12.1). Several of the described species within this phylum utilise nitrate as an electron acceptor and have been found to play a major role in nitrate-dependent souring control of oilfield systems (Greene et al., 1997; Hubert and Voordouw, 2007). Archaeal communities were not significantly different at Dan and Halfdan and were dominated by members of the *Thermococcales* (Fig. 12.1).

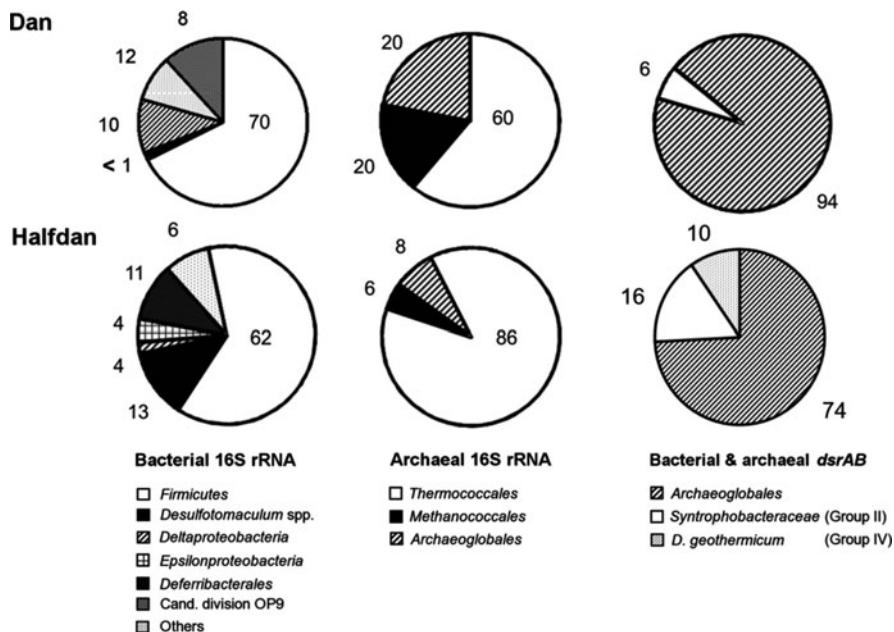


Fig. 12.1 Community composition of *bacteria* and *archaea* from 16S rRNA gene libraries and *dsrAB* gene libraries in PW from Dan and Halfdan. Numbers indicate the percentage of clones from each group. The *dsrAB* gene group names refer to those used by Kaneko et al. (2007)

To gain a more detailed picture of the sulphate-reducing community, *dsrAB* gene clone libraries were constructed and confirmed the results obtained from the 16S rRNA-based approach. Both *dsrAB* gene clone libraries were dominated by sequences closely affiliated with *Archaeoglobus fulgidus*. In addition, representatives of the Deltaproteobacteria (*Syntrophobacteraceae*, e.g. *Desulfacinum*; and *Desulfotomaculum geothermicum*) were recovered from Halfdan PW and accounted for approx. 25% of all *dsrAB* sequences in this library (Fig. 12.1). At Dan, deltaproteobacterial SRP represented only a minor fraction of the clone library (6%, Fig. 12.1) and were exclusively affiliated with the *Syntrophobacteraceae*.

In situ abundances of *bacteria*, *archaea*, and sulphate reducers were determined with specific quantitative PCR (qPCR) assays. *Archaea* slightly dominated the prokaryotic community in Dan PW (57.6% of all prokaryotes), whereas *bacteria* clearly dominated the prokaryotic community in PW from Halfdan (75% of all prokaryotes). In addition, about five times higher bacterial and archaeal *dsrAB* gene copy numbers were detected in Halfdan PW than in Dan PW. The qPCR results corroborated the cloning results as archaeal *dsrB* (i.e. *Archaeoglobus* relatives) were highly abundant and dominated the *dsrAB* gene-carrying community. Relative to the sum of prokaryotic 16S rRNA gene copies, SRP in total accounted for 14.5% of the total prokaryotic community at Dan, but only for 3.2% at Halfdan. The contribution of archaeal SRP to the total prokaryotic community was much lower at Halfdan

(2.9%) than at Dan (14.1%), as already indicated from the archaeal 16S rRNA gene-based clone libraries. Deltaproteobacterial sulphate reducers occurred at low abundance (0.3–0.4%) in both PW samples.

Conclusions

Microbial molecular methods were successfully applied to two high-temperature oil reservoirs and used to assess the effect of nitrate addition in souring control. In contrast to previous molecular studies on seawater-flooded, high-temperature oil reservoirs (Orphan et al., 2000; Li et al., 2007; Bødtker et al., 2009), bacterial and archaeal SRP were identified to form a major fraction of the prokaryotic communities at Dan and Halfdan. Differences in the SRP community composition, a lower relative abundance, and the absence of detectable activity at Halfdan (not discussed here) most likely resulted from the addition of nitrate. The lower abundance of SRP relative to the total prokaryotic community at Halfdan was mainly due to a smaller fraction of archaeal SRP, consistently inferred from clone libraries and *dsrAB* quantification. Apparently, the addition of nitrate primarily affected the archaeal SRP in the reservoir, but had an only minor effect on the abundance of bacterial SRP. The importance of archaeal SRP in high-temperature reservoirs was most likely overlooked in previous studies, as their abundance and activity have not been specifically targeted. SRP in oil production systems were routinely monitored applying the most probable number technique (MPN) that requires cultivation and cannot mirror *in situ* conditions and the particular needs of different physiological groups of SRP (e.g. temperature, nutrients, syntrophic interactions). Cultivation therefore often favoured fast-growing, moderate SRP, e.g. *Desulfovibrio* spp. (Bødtker et al., 2008), that were not necessarily the numerically most abundant or *in situ* active SRP. To assess differences between fields and to target the effect of nitrate systematically, archaeal SRP need to be detected more specifically, e.g. by the amplification and quantification of archaeal *dsrAB* genes.

Although the activity of hNRB and NR-SOB was not specifically assessed, the recovery of sequences affiliated with representatives of both types of nitrate reducers, including members of the *Epsilonproteobacteria* (*Sulfurospirillum* spp., *Arcobacter* spp.) and the *Deferribacterales* (*Deferribacter* spp.), is promising for future studies. Most interestingly, signatures of the *Deferribacterales* were exclusively recovered from Halfdan PW indicating that their growth was favoured due to the addition of nitrate.

In conclusion, our comparative analyses showed that the addition of nitrate resulted in the inhibition of SRP activity, a decrease in the relative abundance of SRP, and a general shift of the microbial community including the appearance of putative hNRB of the *Deferribacterales*. However, the increase in total microbial biomass and absolute numbers of SRP may aggravate plugging problems and cause resumption of sulphide if nitrate is omitted from the injection water. Further understanding of microbial populations and mechanisms is therefore needed before finally assessing the prospects in nitrate addition in souring control.

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Chapter 13

Problems Caused by Microbes and Treatment Strategies

The Effect of Nitrate Injection in Oil Reservoirs – Experience with Nitrate Injection in the Halfdan Oilfield

Jan Larsen and Torben Lund Skovhus

Introduction

This chapter deals with the use of nitrate injection for reservoir souring mitigation in an oilfield with seawater injection in the Danish sector of the North Sea. Nitrate impacts on the activity of sulphate-reducing bacteria (SRB) and biofilm redox potential (Larsen et al., 2007), as a result of which corrosion due to SRB activity will be reduced, souring inhibited and previously formed sulphide removed (Larsen, 2002). One important aspect is that the microbiological reduction of nitrate provides approximately three times more energy to SRB than the reduction of sulphate. Therefore, when both nitrate and sulphate are present, nitrate becomes the preferred electron acceptor and SRB capable of growing on nitrate will dominate. Nitrate provides a competitive advantage to nitrate-utilising bacteria (indicated by the general acronym NUB) during competition for available carbon sources as the NUB are capable of much faster growth than SRB.

A second mechanism has been found from laboratory studies on strains of SRB recovered during backflows of Halfdan water injectors (Larsen et al., 2004). The 16S rRNA gene sequencing identified the dominant SRB strain as being *Desulfovibrio gracilis*. This strain can preferentially use nitrate in the presence of high sulphate concentrations. As a result, H₂S is no longer produced while the SRB remain active and nitrate achieves the desired effect. A further mechanism that suppresses SRB activity is the inhibitory action of nitrite, formed by the activity of nitrate-utilising bacteria (NUB) (Reinsel et al., 1996). In addition, nitrite can act as a chemical H₂S scavenger, thereby contributing to H₂S removal. This results in an increase in the redox potential, which inhibits SRB activity (Larsen et al., 2007). A final mechanism that has been found to play an important role is through the action of nitrate-reducing sulphide-oxidising bacteria (NRSOB) (Voordouw et al., 2002). This special group

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of NUB are capable of reducing nitrate, while simultaneously oxidising sulphide, thereby providing a curative process for H₂S already present in the system.

Case Study: The Halfdan Oilfield

The Halfdan oilfield is located in the Danish sector of the North Sea and is operated by Maersk Oil on behalf of the Danish Underground Consortium (DUC). The Halfdan oil and gas accumulation was discovered in December 1998 by a 30,000-ft-long horizontal well drilled from the Dan field. Production of oil and gas started in February 1999. Four wellhead platforms are now installed in the Halfdan field. Three-phase separation of oil, gas and water is carried out on Halfdan, and the stabilised oil is exported to the Gorm-C platform. The gas is exported to the Dan-FF and Tyra West Platforms.

The Halfdan field is laterally extensive and comprises a high porosity (25–30%), low permeability (0.5–2 mD) chalk reservoir located outside structural closure (Albrechtsen et al., 2001). The producing horizon is the Maastrichtian Age Chalk with a reservoir temperature and pressure of typically 80°C and 4,200 psi, respectively. The field is developed with long horizontal wells of 10,000–15,000-ft reservoir sections drilled in a dense parallel pattern of alternate producers and injectors with well spacings of 600 ft. At present (July 2009), 46 producers and 25 water injectors have been drilled.

Water injection for reservoir pressure maintenance and line sweep commenced in January 2001 from one injector, HDA-04. Injection seawater is supplied from the Dan-FF platform, via a 9-km long sub-sea pipeline made of carbon steel. The Halfdan water injection rate has currently reached a level of 200,000 bbls/day (July 2009). Nitrate has been injected continuously into the Halfdan water injection pipeline from the start of seawater injection in January 2001 in order to mitigate reservoir souring. The injection rate is 135 ppm (ml/m³) calcium nitrate product (45% w/w), which corresponds to 65 mg nitrate ion/l.

Small amounts of H₂S are often measured in the produced gas when new wells are brought on-stream. Levels typically vary between 0.2 and 20 ppm in the gas phase. The origin of the H₂S is microbiological as sulphur isotope ratio ($\delta^{34}\text{S}$) determinations have shown these to fall within the known boundaries of biologically derived $\delta^{34}\text{S}$ signatures (Herbert et al., 1985). The H₂S is believed to originate from SRB introduced by the use of seawater-based fluids during drilling, completion and stimulation of the Halfdan wells.

Monitoring of Halfdan Producing Wells with Seawater Breakthrough

The term ‘seawater breakthrough’ is used for reservoir conditions under which a water blend, which was injected to maintain reservoir pressure via injection wells, breaks through to one or more of the producing wells.

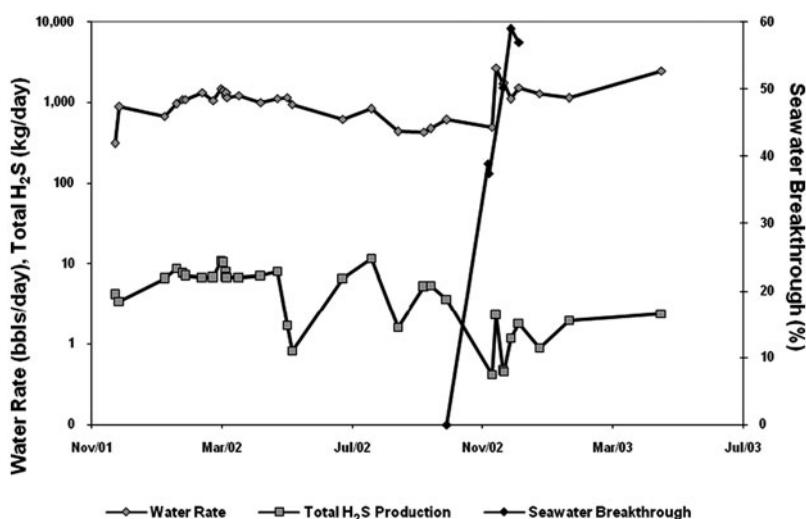
Table 13.1 Seawater breakthrough data from Halfdan oilfield

Parameters	Well HDA-07	Well HDA-03XA
Occurrence of seawater breakthrough	September 2002	October 2002
Percentage seawater breakthrough	~80	40–60
Nitrate concentration range, mg/l	1.3	0.3–5.6
Nitrite concentration range, mg/l	0	0.2–0.7

Seawater breakthrough through natural or induced fractures in the Halfdan reservoir has occurred in four producing wells (HBA-05, HBA-07, HDA-03XA and HDA-07). For HDA-03XA and HDA-07 (Larsen et al., 2004) partial seawater breakthrough occurred over a short time period during late 2002 due to a path of communication between the wells. The seawater breakthrough occurred from the water injector HDA-12 through the fractures. Details on seawater breakthrough and nitrate and nitrite residuals in the produced water from HDA-03XA and HDA-07 are given in Table 13.1.

Nitrate was detected in both wells after seawater breakthrough. Nitrite was also found to be present in HDA-03XA, confirming the activity of NUB.

As seawater broke through, the water production from HDA-03XA and HDA-07 increased significantly. For HDA-03XA, the water production rate increased from an average of 950–2,500 bbls/day. For HDA-07, the water production increased from an average of 600–3,300 bbls/day. For both wells, the total amount of H₂S produced showed a significant decrease after seawater breakthrough (Larsen et al., 2004). For HDA-03XA, the average H₂S production was reduced from 6.5 to 1.4 kg/day. For HDA-07, the average H₂S production was reduced from some 3.0 to 0.7 kg/day (Figs. 13.1 and 13.2).

**Fig. 13.1** HDA-03XA H₂S production and seawater breakthrough

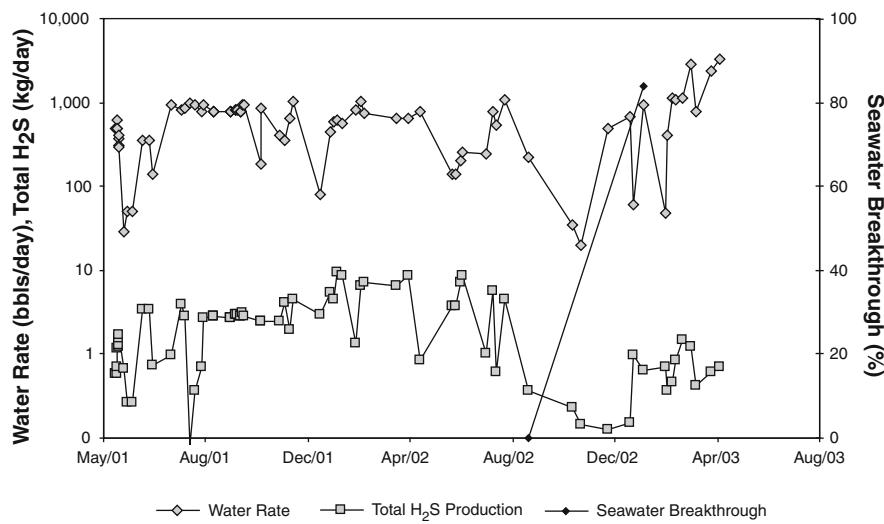


Fig. 13.2 HDA-07 H₂S production and seawater breakthrough

Total Bacterial Abundance and Enumeration of Specific Bacterial Groups

During early 2005 the total bacterial abundance in HDA-03XA and HDA-07 produced waters was determined with DAPI-stained cells (Larsen et al., 2006). The total bacterial counts were in the range of 6.2×10^5 – 1.8×10^6 cells/ml. Determination of active bacteria was performed by the FISH technique, which is based on gene probes targeting ribosomal RNA (16S rRNA) in bacterial cells. Only living and active cells contain sufficient ribosomes that can be detected by FISH. The FISH probes EUB338(I+II+III) were used for quantification of all living bacteria. The FISH probes SRB385 and SRB385Db were used for quantification of *Delta*proteobacteria as most SRB belong to this phylogenetic group (see Chapter 7 by Holmkvist et al., this volume).

The ratio between active cells (FISH) and total cells (DAPI) increased from 20% in HDA-07 to 82% in HDA-03XA (Skovhus et al., 2009). This shows that the high level of bacteria in HDA-07 consisted mainly of inactive cells. The SRB in HDA-03XA made up a significant fraction of 10% of all living bacteria. For comparison, the SRB made up only 2.0% of all living bacteria in HDA-07. The results are shown in Table 13.2.

A path of direct communication exists between the water injector HDA-12 and the producer HDA-07. Furthermore, an indirect path of communication exists between HDA-12 and the producer HDA-03XA through HDA-07. As nitrate-treated injection seawater passes through HDA-07 to HDA-03XA, as a result of the indirect communication path between the two wells, both nitrate and nitrite became depleted. Nitrate became depleted from 12 mg/l in HDA-07 to <1 mg/l

Table 13.2 Bacterial numbers using FISH/DAPI (January 2005)

Parameters	Well HDA-07	Well HDA-03XA
Total bacterial counts by DAPI, cells/ml	1.8×10^6	6.2×10^5
Viable bacteria (FISH) (%)	20	82
Viable SRB (FISH) (%)	2.0	10
H ₂ S production, kg/day	4	25
Nitrate concentration, mg/l	12	0.6
Nitrite concentration, mg/l	9	0.3

in HDA-03XA. Similarly, nitrite became depleted from 9 mg/l in HDA-07 to <1 mg/l in HDA-03XA (Table 13.2). As a consequence, the H₂S production from HDA-03XA is higher compared to HDA-07. The data show that there exists a good correlation between SRB numbers determined by the FISH technique, nitrate/nitrite concentrations and the H₂S production in these wells with seawater breakthrough.

Determination of the Activity of SRB from the Halfdan Oilfield

Since 2003 molecular microbiological methods (MMM) have routinely been used in the Halfdan field in order to identify and quantify microorganisms involved in souring and MIC. In order to obtain a more complete understanding of the effects of nitrate injection on the activity of SRB, the mRNA for the gene responsible for sulphate reduction in SRB (the *dsrA* gene) present in produced waters from HDA-03XA and HDA-07 was quantified by reverse transcription quantitative PCR (RT-qPCR). The mRNA for the *dsrA* gene is only produced by SRB actively reducing sulphate. Therefore, the RT-qPCR technique provides a rapid monitoring tool for SRB activity (see Chapter 4 by Sørensen and Chapter 9 by Price about abundance and activity, this volume).

During May 2009 the total bacterial abundance in HDA-03XA and HDA-07 produced waters was again determined with DAPI-stained cells (Price et al., 2010). The total bacterial counts were in the range of 1.1×10^5 – 1.2×10^5 cells/ml.

Determination of active bacteria was performed by the FISH technique. The FISH probes EUB338(I+II+III) were used for quantification of all living bacteria. The FISH probes SRB385 and SRB385Db were used for quantification of SRB.

The fraction of viable bacteria in HDA-03XA and HDA-07 produced waters had increased compared to the monitoring conducted in 2005. The percentage of viable bacteria was in the range of 97–100%. Also, the fraction of viable SRB had increased; the SRB in HDA-07 and HDA-03XA now made up 12 and 56% of all living bacteria. The data is shown in Table 13.3.

The absence of detectable expression of the *dsrA* gene in the produced water sample from HDA-07, and significant expression in the sample from HDA-03XA, shows sulphate-reducing bacterial activity only in HDA-03XA. Since nitrate/nitrite

Table 13.3 Bacterial numbers using FISH/DAPI and activity of SRB (May 2009)

Parameters	Well HDA-07	Well HDA-03XA
Total bacterial counts by DAPI, cells/ml	1.1×10^5	1.2×10^5
Viable Bacteria (FISH) (%)	100	97
Viable SRB (FISH) (%)	12	56
No. of <i>dsrA</i> mRNA transcripts/SRB cell	0	4.4
H ₂ S production, kg/day	1	16
Nitrate concentration, mg/l	14	1
Nitrite concentration, mg/l	2	0.6

levels (Table 13.3) were much higher in the sample from HDA-07 (14 mg nitrate/l and 2 mg nitrite/l), compared to HDA-03XA (1 mg nitrate/l and < 1 mg nitrite/l), it is likely that the nitrate treatment has significantly reduced the sulphate reduction activity of the SRB, thereby explaining the absence of significant expression for the *dsrA* gene in HDA-07.

Five times as many SRB are present in HDA-03XA than in HDA-07 as given in Table 13.3. However, HDA-03XA is producing 16 times more H₂S than HDA-07 even though the well is assumed to contain only approximately five times as many viable SRB. Therefore, it can be concluded that the SRB in HDA-03XA are actively reducing sulphate and are extremely active, especially as each gene (one per SRB) is producing multiple mRNA transcripts (4.4) of the *dsrA* gene. However, although SRB were detected in HDA-07, no significant levels of mRNA for the *dsrA* gene were detected and it can be concluded that the SRB in HDA-07 are either not reducing sulphate or that sulphate reduction is occurring at a very low level. This suggests that the nitrate present in HDA-07 may be switching the metabolism of SRB from sulphate reduction to nitrate reduction. It should be noted that some dominant SRB species which can switch their metabolism from using sulphate as a terminal electron acceptor to using nitrate as a terminal electron acceptor under certain conditions are present in the Halfdan process system (Krekeler and Cypionka, 1994; Larsen et al., 2004).

Conclusion

This study has shown the useful application of molecular microbiological methods in monitoring the effect of adding nitrate to a water-flooded oil reservoir for mitigation of souring. Future work will focus on source tracking microorganisms that produce sulphide in individual wells and the whole oilfield reservoir for an early warning of reservoir souring.

Acknowledgements Laboratory experiments and field monitoring were sponsored by DUC Partners (A.P. Møller-Mærsk, Shell and Chevron).

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Chapter 14

Problems Caused by Microbes and Treatment Strategies

Monitoring Microbial Responses to Biocides; Bioassays – A Concept to Test the Effect of Biocides on both *Archaea* and *Bacteria* in Oilfield Systems

**Lars Holmkvist, Uffe Sognstrup Thomsen, Jan Larsen, Michael Jensen,
and Torben Lund Skovhus**

Introduction

The oil and gas industry seeks to reduce the costs of oil and gas production and to minimise the risks of the operation, i.e. to have a high degree of safety for the personnel and protection of the environment. This imposes considerable demands on corrosion inhibition technologies and chemical management. The oil industry has traditionally used cultivation-based methods for microbiological surveillance of oil production facilities to monitor microbiologically influenced corrosion (MIC) risk (Sooknah et al., 2007). However, studies show that it is only possible to cultivate less than 10% of all viable microorganisms and that the population characteristics in a sample may change during the cultivation steps (Maxwell et al., 2004). Therefore, it is obvious that alternative methods are needed that can detect all the microorganisms related to MIC in a sample independent of the cultivation method. Recently, a number of studies have applied molecular microbiological methods (MMM), such as 4'-6-diamidino-2-phenylindole (DAPI), Fluorescence in situ Hybridisation (FISH) and quantitative polymerase chain reaction (qPCR), with the purpose of detecting and monitoring MIC-related microorganisms in offshore oil facilities in the Danish sector of the North Sea (Skovhus et al., 2009; Larsen et al., 2008, 2009 and Chapter 10 by Sørensen et al., this volume). They have provided new and important insights into the distribution and occurrence of troublesome microorganisms in oilfield facilities and demonstrated that MMM can be a relevant approach for the detection of microorganisms in fluid or solid samples from oilfields. For instance, a recent molecular study by Larsen et al. (2008) showed that sulphate-reducing microorganisms are present in both reservoirs and topside debris, which suggest that these microorganisms are potential contributors to both souring (i.e.

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high production of sulphide) and MIC. Likewise, methane-producing prokaryotes (methanogens) that are suspected to play an important role in MIC particularly on metal surfaces (Davies and Scott, 2006) have also been found in high numbers in solid samples within topside oilfield installations (Larsen et al., 2008).

The presence of both sulphate-reducers and methanogens in topside oilfield facilities suggests that these microorganisms may be pulled up from the deep sub-surface oil reservoirs during oil production along with the produced water. For instance, the sulphate-reducing archaea (SRA), *Archaeoglobus fulgidus*, has been isolated from hot produced water from a North Sea oilfield (Beeder et al., 1994). This microorganism which was first isolated from a hydrothermal vent close to Vulcano Island (Italy) is involved in reservoir souring (Stetter et al., 1987).

Biocide treatment is a common practice to mitigate the generation of sulphide by inhibiting the sulphate-reducing microorganisms and thus minimise souring and MIC within oilfield facilities (Larsen et al., 2000). The types of biocides that have been used historically in oilfield systems include TetrakisHydroxyMethylPhosphonium Sulphate (THPS), glutaraldehyde, formaldehyde, 2-propenal and anthraquinone and these have been used with all oilfield systems with varying degrees of success (Oates et al., 2006). With a new understanding of the specific microorganisms involved in MIC from MMM, it has become highly relevant to test the effect of specific biocides against known strains of troublesome microorganisms. For example, biocide testing should be performed in the laboratory on selected pure cultures as well as in water samples collected from offshore facilities. Such tests would provide a valuable insight into the effect of the biocides against specific groups and strains of troublesome microorganisms and finally lead to a more cost-efficient biocide strategy for oil companies.

The work discussed here deals with some recent biocide studies, one from the laboratory and another from the field. Both studies aimed to use bioassays to test the efficacy of biocides against microorganisms. The laboratory study presents results from biocide assays on pure cultures grown in a pressure chamber, whereas the field study demonstrates testing of biocides on microbial communities from produced water samples collected from an oil platform in the Danish sector of the North Sea.

Case Study 1: The Effect of Biocide Treatment on Pure Cultures of Sulphate-Reducing Microorganisms and Methanogens

In the following, a laboratory study will be described where pure cultures of *Archaeoglobus fulgidus* (an SRA), *Thermodesulfobacterium commune* (an SRB) and *Methanothermococcus thermolithotrophicus* (a methanogen) were tested for their tolerance against a biocide. The particular cultures were selected for the study because they have all been isolated from either deep sub-surface sediments or oil reservoirs (Beeder et al., 1994, Zeikus et al., 1983 and Nilsen and Torsvik, 1996). Consequently, these microorganisms are relevant in the context of MIC and reservoir souring. The cultures were grown in a pressure chamber under 150 bars and 60°C for 14 days in order to simulate reservoir and pipeline conditions. Microorganisms

that thrive under these conditions (e.g. the selected cultures described above) are, therefore, thermophilic, i.e. adapted to high temperatures.

qPCR was used in this study to estimate the number of cells in the pure cultures with and without the addition of biocide (Chapter 10 by Sørensen et al., this volume). This enabled us to follow the increases or decreases in numbers of cells over time. The qPCR results are all presented in Figs. 14.1, 14.2 and 14.3, and the changes in cell numbers from the graphs will be discussed in calculated \log_{10} units to exemplify growth or inhibition during incubation. The biocide tests on the three pure cultures showed that when the biocide was not added, *A. fulgidus* (SRA) and

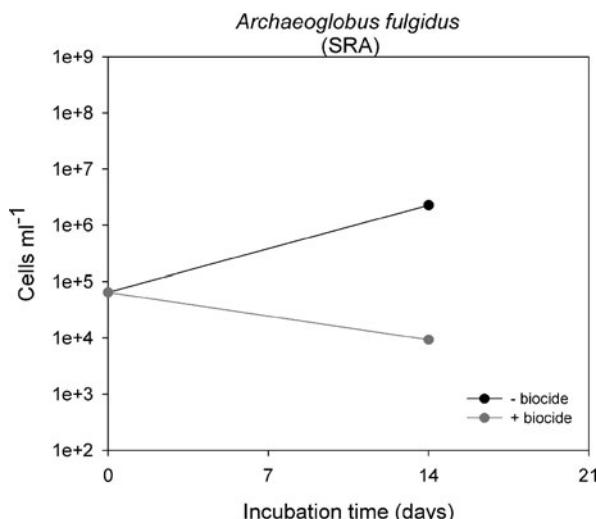


Fig. 14.1 Graph showing numbers of cells per millilitre of *A. fulgidus* obtained with qPCR

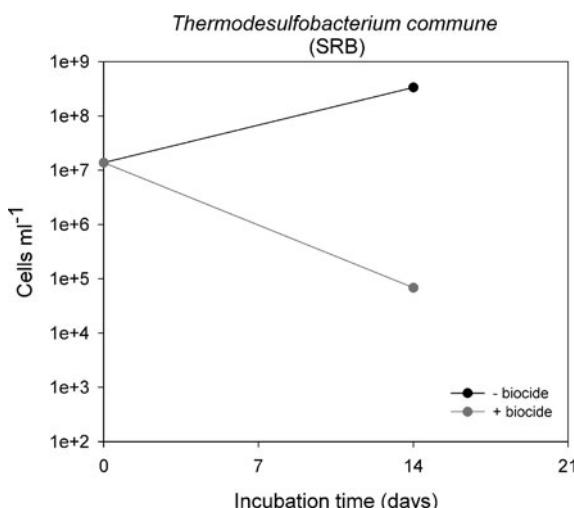


Fig. 14.2 Graph showing numbers of cells per millilitre of *T. commune* obtained with qPCR

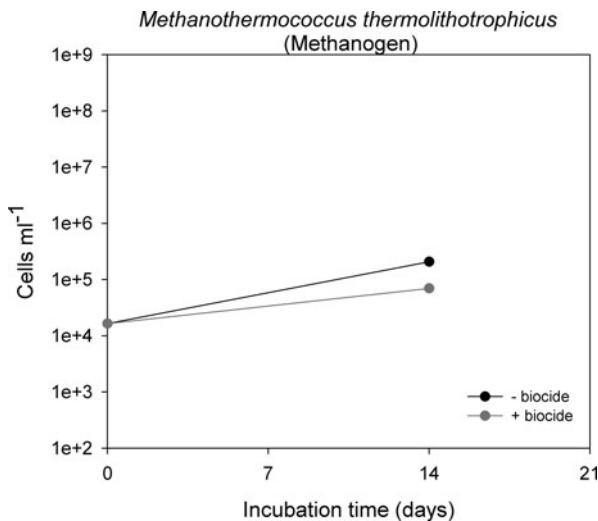


Fig. 14.3 Graph showing numbers of cells per millilitre of *M. thermolithotrophicus* obtained with qPCR

T. commune (SRB) were actively growing in the pressure chamber and exhibited an increase in their respective cell number of $1.4 \log_{10}$ unit after 14 days of incubation (Figs. 14.1 and 14.2). However, once the tested biocide was added to the cultures, it had an immediate negative effect on the growth of both *A. fulgidus* and *T. commune*, as the cell numbers after 14 days of incubation were observed to decrease $2 \log_{10}$ unit and $2.3 \log_{10}$ unit, respectively (Figs. 14.1 and 14.2).

Results showed that the tested biocide successfully inhibited *A. fulgidus* and *T. commune* within a relatively short period of time. Thus, by adding this biocide into production facilities it would most likely inhibit these troublesome microorganisms effectively. The tested biocide did not inhibit growth of *M. thermolithotrophicus*, since there was no decrease in the number of cells after 14 days of incubation (Fig. 14.3). This was, however, not surprising because large morphological differences exist among strains of methanogens and bacteria (e.g. cell membrane and wall structure and thickness) (Brock et al., 1994), and the biocide tolerance of the cells may vary accordingly.

Case Study 2: Biocide Testing on Produced Water Samples from a North Sea Oil Platform Using Bioassays

We describe here a field study where bioassay experiments were carried out on produced water (PW) samples with and without the addition of another tested biocide. The aim of this study was to evaluate the effect of the tested biocide on the activity of the microorganisms that were present in water samples from an oilfield platform.

In general, water samples collected from oilfield systems contain mixed cultures of different troublesome microorganisms. In many cases, it is difficult to accurately test the effect of specific biocides against groups of *bacteria* or *archaea*, and the number of studies that have aimed to test the effect of biocides on specific microbes is few.

In Fig. 14.4, a general concept is presented that demonstrates how bioassays are applied on water samples containing mixed cultures of MIC-related microorganisms. The figure exemplifies that it is necessary to combine several methods in order to be able to fully evaluate the effect of biocides on the major groups of troublesome microorganisms. More specifically, the concept in Fig. 14.4 highlights that when bioassay studies are performed, it is central to combine measures on changes in numbers of cells during incubation (with DAPI or qPCR), numbers of active microorganisms (using FISH), as well as measuring the concentrations of chemical metabolites (H_2S , TCO_2 , CH_4 , etc.) in the water sample. Only when these parameters are performed and afterwards compared, the immediate effect of adding biocides against microorganisms such as sulphate-reducing microorganisms and methanogens can be assessed.

The two PW samples that were used in the field experiment were from an offshore platform in the Danish sector of the North Sea (Hansen et al., 2009). The tested biocide was added *in situ* to one of the PW samples, with the purpose of studying the biocidal effects on the measured parameters in this sample. Besides, we wanted to compare the results from this sample with another PW sample where biocide was not supplied offshore. Before and after 20 days of incubation in closed anaerobic bottles, sub-samples of PW were removed from the anaerobic bottles and analysed for total cell number (DAPI), numbers of active SRB and *archaea* (FISH) as well as changes in the concentrations of H_2S , CH_4 and TCO_2 .

Biocide dosing is expected to temporarily lower the presence of microorganisms, including the SRB and *archaea*. Yet the results obtained from the field study showed that growth and activity of microorganisms were not inhibited after 20 days of incubation in the PW sample where biocide was supplemented (Table 14.1). The maximum growth rate in the PW with biocide was found to be within the same range (~0.5 per day, Table 14.1) as the PW sample where no biocide was supplied. Furthermore, numbers of SRB and *archaea* in the PW sample with biocide (from FISH) were found to increase 3 \log_{10} units and 2 \log_{10} units (Table 14.1), respectively, indicating that these microorganisms were stimulated by the presence of the

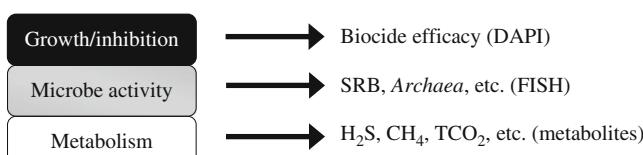


Fig. 14.4 Bioassay: combination of three important methods that should be applied in a biocide assay experiment to obtain knowledge on the activity of microorganisms in a sample either from the laboratory or from the oilfield

Table 14.1 Microbiological and chemical data from PW with and without biocide of a North Sea oil-producing platform

Biocide	No	Yes
Max. growth rate (from DAPI)	0.46 per day	0.49 per day
SRB growth (FISH)	1 log ₁₀ units	3 log ₁₀ units
Archaea growth (FISH)	0.5 log ₁₀ units	2 log ₁₀ units
H ₂ S production	9.8 mg/l/day	0.9 mg/l/day
CH ₄ production	0 mg/l/day	0 mg/l/day
TCO ₂ production	11 mg/l/day	0 mg/l/day
Evaluation	Growth occurred	Stimulated growth of SRB and archaea

Data from Hansen et al. (2009)

tested biocide. Possibly, the PW samples of this study contained communities of microorganisms that were able to degrade complex carbon chains present in the crude oil. Hence, the possibility exists that these microorganisms also had the ability to degrade and use the added biocide for growth. This finding is supported from other studies that have demonstrated some microorganisms to have the metabolic ability to detoxify biocides (e.g. formaldehyde) or harmful compounds in general (Vorholt et al., 2000).

The concentrations of the metabolites, H₂S and TCO₂, in the PW sample with biocide had decreased by a factor of more than 10 after the 20 days of incubation (Table 14.1). These results are not directly in agreement with the observed increase in activity of the SRB and *archaea* that was observed from the FISH results. However, it is likely that the sulphide produced from sulphate reduction reacted chemically with iron compounds (e.g. Fe²⁺) during incubation. We observed that the PW became blackish in colour, indicating that precipitation of iron sulphide minerals occurred (i.e. FeS). Production of sulphide from sulphate reduction was therefore hidden. The lowered concentration of TCO₂ in the PW sample after incubation could not be ascribed to methanogenesis, since no CH₄ was observed to accumulate after incubation (Table 14.1). However, an explanation for the lowered concentration of TCO₂ in this PW sample could be a reduced activity of CO₂-producing microorganisms due to the presence of biocide.

Conclusions

Overall, the two cases presented here demonstrate that biocide tests are relatively straightforward to apply when bioassays are taken into use. The results obtained from the two studies could potentially be used for a more efficient mitigation strategy, resulting in a cost-efficient strategy for the oil industry. Both studies showed that the outcome of adding biocides to microorganisms that are typically found in PW is difficult to predict, and further experiments are needed in order to optimise the use of biocides offshore to inhibit MIC-related microorganisms. The laboratory

study showed that pure cultures of the methanogenic *archaea*, *M. thermolithotrophicus* were not negatively affected by the addition of biocide. In comparison, the field study showed that the SRB and *archaea* in the PW samples were also not inhibited after addition of the tested biocide. Instead, the SRB and *archaea* of this study were stimulated by the addition of the tested biocide. Hence, the results suggest that they might be resistant to the biocide or perhaps use the biocide as a carbon source for growth.

Perspectives

Further studies are obviously needed in the future to investigate the efficiency of biocides against MIC-related microorganisms in more detail. Such investigations should aim to obtain more knowledge on how biocides are most effectively applied within offshore facilities. In the second case study described above, where growth of sulphate reducers was stimulated after incubation with biocide (Table 14.1), it could be interesting not only to measure the increase in the number of cells but also to include direct measures of sulphate reduction rates (SRR). Determination of the sulphate reduction rates (SRR) can be applied using a carrier-free radio-labelled $^{35}\text{SO}_4^{2-}$ (Jørgensen, 1978; Fossing and Jørgensen, 1989). Sulphate reduction rate experiments together with the molecular techniques would undoubtedly be a very strong and precise tool for future ‘state of the art’ assessment of the activity of sulphate-reducing communities in a sample before and after the supplement of biocides.

Likewise, it may also be relevant to conduct studies that focus on reducing the activity of microorganisms in biofilms in oilfield facilities. Biofilms such as those situated on the inner surfaces of pipelines are the location where most microbial activity is expected and thus the source of many MIC problems within oilfield facilities. Biocide testing directly on biofilm material can be done using biostuds, coupons or probes from side stream systems or corrosion monitoring units. They are all used as practical devices for studying biofilm development over time or to obtain biofilm material for further tests with biocides. When such devices are in place more advanced and dynamic testing of contact time and dosing regimens can be investigated.

Acknowledgements Laboratory experiments and field monitoring were sponsored by DUC Partners (A.P. Møller-Mærsk, Shell and Chevron).

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Chapter 15

Problems Caused by Microbes and Treatment Strategies

Identification of H₂S-Producing Bacteria in Corrosion Product of a Gas Pipeline

Márcia T.S. Lutterbach and Luciana S. Contador

Introduction

Microbiologically influenced corrosion (MIC) is characterised by material degradation through the action of microorganisms that can act as accelerators or inducers of the reactions of the electrochemical corrosion process. The corrosion study occurred in the past under an essentially abiotic approach, involving chemical and electrochemical experiments. This approach prevented for many years the proper interpretation of MIC phenomena and our understanding of these processes (Videla, 1991). Today, this type of corrosion receives more attention, arousing great industrial interest, since few industries are free of the occurrence of MIC in their systems. The costs of electrochemical corrosion in the US may reach 3.1% of gross domestic product (Koch et al., 2001). According to some estimates, MIC is responsible for around 20–30% of total corrosion costs (Javaherdashti, 1999).

MIC or biocorrosion is directly related to the adhesion of microorganisms on the surface of materials, and its subsequent colonisation. These microbial structures, known as biofilms are composed of cellular aggregates, extracellular polymeric material (EPS) resulting from microbial metabolism, inorganic and organic matter, and, mainly water (Davey and O'tolle, 2000) (Fig. 15.1). Biofilms occur both in natural and industrial environments, especially when there is a limitation of nutrients in the aqueous phase. Microbial adsorption to solid surfaces is a known strategy used by microorganisms in oligotrophic environments in order to benefit from the accumulation of nutrients on the surfaces. Another great advantage of this mode of life is the development of stable interactions, and increased resistance to biocides when compared to the single cell mode of life. The resulting biofilm structure is highly heterogeneous and dynamic (Flemming, 2009).

The introduction of methods such as fluorescence microscopy and confocal laser scanning microscopy, and, molecular microbiological methods (MMM) has

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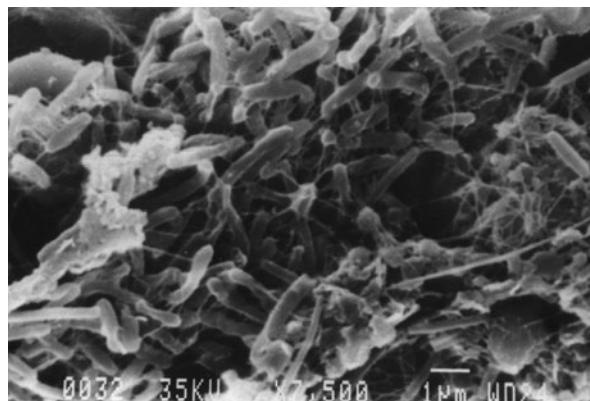


Fig. 15.1 SEM microphotograph showing biofilm of the stainless steel surface after 30 days of in situ exposure to cooling water (bar = 1 μm) (Lutterbach and de França, 1996)

allowed a better understanding of the complex structure of biofilms. These techniques have facilitated the study of microbial diversity in this micro-environment. MIC is rarely associated to one single mechanism or to one single species of microorganism (Videla et al., 2005). Some of the main bacterial groups implicated in MIC are sulphur-oxidising bacteria, methanogens, iron-oxidising/reducing bacteria, manganese-oxidising bacteria, and bacteria secreting organic acids and slime (Beech and Coutinho, 2003).

The sulphate-reducing bacteria (SRB) are traditionally identified as the major contributors to MIC due to the great practical incidence and economical reflex linked to their presence in the corrosive process (Iverson, 1987; Ford and Mitchell, 1990; Beech and Gaylard, 1999; Hamilton, 2003). The general concern about SRB is mostly due to their capacity to produce hydrogen sulphide (H_2S), which directly attacks metal surfaces leading to anaerobic corrosion and pit formation (Hamilton, 1985). However, according to Javaherdashti (2008), one of the ‘myths’ of MIC is the importance of SRB. Little and Wagner (1997) also affirmed that the role of SRB’s has been exaggerated. There are other microorganisms in addition to SRB which are also important in corrosion, that are still not well studied, such as iron-reducing bacteria.

Various culture media for the enumeration of SRB support the growth of facultative sulphide producers: e.g. *Shewanella putrefaciens* and *Aeromonas veronii*. These facultative H_2S -producing bacteria may contribute considerably towards MIC (Dawood and Brözel, 1998). *Shewanella* are facultative anaerobic iron-reducing bacteria that are well known for their versatile metabolism (Tiedje, 2002; Serres and Riley, 2006). Due to their ability to reduce ferric iron and sulphite, oxidise hydrogen gas, and produce sulphide, these bacteria may be involved in biocorrosion (Videla et al., 2008). Some studies, although, suggest that iron-reducing bacteria may have an inhibitory effect towards corrosion (Dubiel et al., 2002; Lee et al., 2006). It has, therefore, been suggested that a microbial monitoring programme should quantify not only the true SRB but also all H_2S -producing bacteria (Cloete et al., 1998; Iverson, 1987).

One of the various methods employed for the quantification of microorganisms in different kinds of samples is the most probable number (MPN) technique, which is based on microbial cultivation. In the specific case of sulphate-reducing bacteria, Postgate in his book *The sulphate-reducing bacteria* (Postgate, 1984) proposes many culture media to cultivate SRB. One of the media recommended for the enumeration of these bacteria is the Postgate E, which due to the presence of iron salts leads to the formation of FeS when sulphide is produced. When the medium turns black, the sample is considered positive for SRB presence. Nevertheless, there are other bacteria capable of producing H₂S as previously mentioned. The growth of these bacteria in Postgate E medium would give a false positive for SRB presence. An example of this situation is given by *Shewanella*. These bacteria are able to produce H₂S but not through sulphate reduction as a true SRB.

This work shows an example of the use of MMM in the identification of microorganisms related to MIC. Corrosion residues of gas pipelines, known as black powder, are mixtures of iron sulphide or iron oxides with contaminants, mainly water, liquid hydrocarbons, salts, sand, among others. According to some researchers, the appearance of black powder in gas pipelines, besides its chemical corrosion origin, may be directly related to sulphate-reducing bacteria metabolism in this environment (Baldwings, 1997).

The Laboratory of Biocorrosion and Biodegradation – LABIO has performed the monitoring of SRB in residues from gas pipelines for some time (Lutterbach et al., 2003, 2004; Pimenta and Lutterbach, 2005). A positive culture from a black powder sample in Postgate E medium was kept in the laboratory for 4 years. A bacterial strain was isolated from this positive culture and identified through MMM. The microorganism was preserved cryogenically for further studies (Table 15.1).

The DNA sequence of the bacterial strain isolated from the black powder sample had 99% similarity to *Shewanella putrefaciens* from the Genbank database. Even though the strain has been isolated from a positive culture in Postgate E medium, which is primarily used for SRB quantification, it has been identified as an iron-reducing bacteria (IRB). *Shewanella* are capable of H₂S production, but not through sulphate reduction, as a true SRB.

The SRB are widely studied and usually considered the foremost responsible for MIC process. However, recent studies revealed that *Shewanella* may have a key role in MIC. Most SRB are strictly anaerobes and have a slow growth rate. The

Table 15.1 Morphological observation of colonies on selective agar media (CETESB L5-207) and non-selective agar media (plate count agar OXOID CM0325) and optical microscopic view of Gram stain

Strain	Sample	Bacterial colonies (selective agar media)	Bacterial colonies (non-selective agar media)	Gram	Identification
0033	Black powder				<i>Shewanella putrefaciens</i> 99%

Shewanella are able to grow under both aerobic and anaerobic conditions, which give it an advantage at the oxic–anoxic interfaces within a biofilm.

In aerobic conditions *Shewanella* uses oxygen as the final electron acceptor. During anaerobic growth, *Shewanella* can use a wide range of terminal electron acceptors such as manganese, nitrate, nitrite, thiosulphate, elemental sulphur, ferric iron, sulphite (Lovley et al., 1989; Myers and Myers, 1994; Moser and Nealson, 1996). *Shewanella putrefaciens* can use hydrogen and diverse carbon compounds as electron donors (Dawood and Brözel, 1998). Due to its versatile metabolism, the bacteria from the genus *Shewanella* are strong competitors within the biofilm community.

Some studies have shown a competitive behaviour between SRB and IRB. The SRB growth inhibition in the presence of *Shewanella* has been observed in previous studies (McLeod et al., 2002; Lee et al., 2006; Lutterbach et al., 2009). Another work indicates that SRB are able to out-compete IRB (Roden et al., 2000). Nevertheless, the competition between SRB and IRB do not avoid the coexistence and activity of these bacteria under anaerobic conditions (Blodau et al., 1998; Cummings et al., 2000; Wielinga et al., 1999).

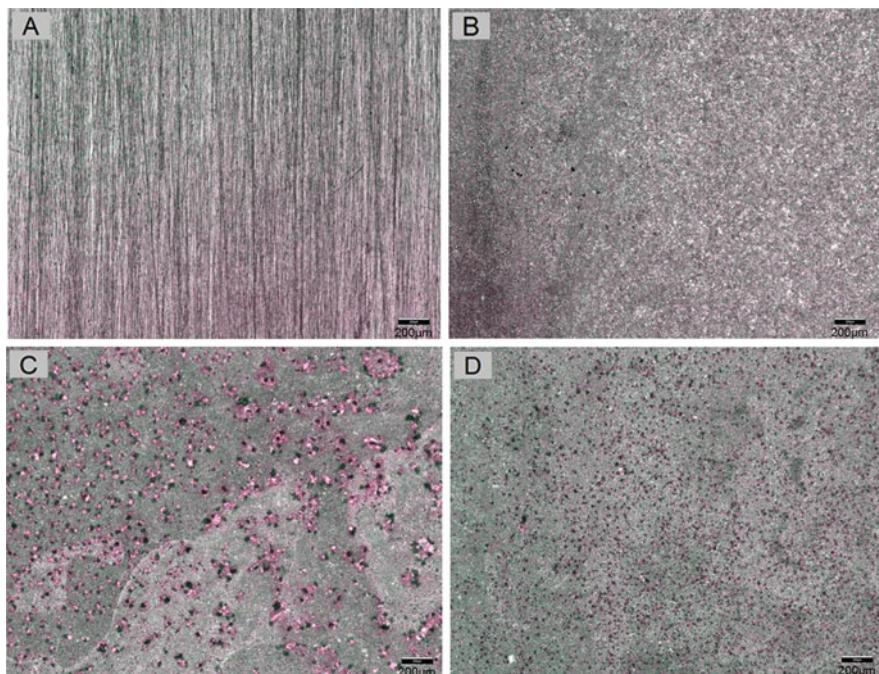


Fig. 15.2 Microscopic examination using the Alicona infinite focus microscope. (a) Coupon exposed to sterile medium (control). (b) Coupon exposed only to *Shewanella putrefaciens*; (c) coupon exposed only to SRB; (d) coupon exposed to a mixture of SRB and *S. putrefaciens*

The *Shewanella* isolated from the black powder sample was used in corrosion experiments (Lutterbach et al., 2009) with carbon steel coupons in a batch culture for 30 days. The coupons were inoculated with *Desulfovibrio desulfuricans* and *Shewanella putrefaciens*, either alone or mixed together. Determination of corrosion rates were observed by infinite focus microscopy (IFM, Alicona) at 50 \times magnification with polarised light; the microscopic examination indicates that the coupon exposed to sterile medium (control) presented no localised corrosion (Fig. 15.2a). The coupons exposed only to *Shewanella putrefaciens* exhibited less corrosion pits (Fig. 15.2b) in comparison to those exposed to *Desulfovibrio desulfuricans* alone (Fig. 15.2c) or in the mixture with *S. putrefaciens* (Fig. 15.2d). Larger pits were observed on the surface of coupons exposed only to *D. desulfuricans* (Fig. 15.2c).

Conclusion

SRB are not the sole microorganisms responsible for MIC. Other bacteria and their interactions are also involved in the corrosion process. The iron-reducing and facultative H₂S-producing bacteria isolated from a black powder sample and identified through MMM as *Shewanella putrefaciens* revealed the ability to corrode carbon steel coupons and may contribute considerably towards MIC. Bacterial identification through 16S rDNA sequencing proved to be a good tool in MIC study and the study of microbial communities involved in MIC processes.

Prevention and mitigation actions towards/against MIC and/or biological H₂S production should take into account not only the SRB but also all the H₂S-producing bacteria. Some studies suggest that iron-reducing bacteria, like *Shewanella*, can outcompete SRB and may have an inhibitory effect towards corrosion. Nevertheless, these bacteria have a versatile metabolism and may be capable of producing H₂S and therefore cause souring and MIC. It is important to remember that aerobic conditions that are sometimes used to prevent SRB growing are ineffective against *Shewanella*.

Acknowledgements We thank Petrobras for funding this work.

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Chapter 16

Problems Caused by Microbes and Treatment Strategies

Rapid Diagnostics of Microbiologically Influenced Corrosion (MIC) in Oilfield Systems with a DNA-Based Test Kit

Torben Lund Skovhus, Ketil Bernt Sørensen, and Jan Larsen

Introduction

In the past, many operators have encountered failures due to MIC in pipelines and topside facilities contaminated with sulphate-reducing bacteria (SRB). In some cases, severe pitting has resulted in flow lines being either abandoned or replaced (Davies and Scott, 2006). However, there are reports of little or no significant MIC in some systems, despite an apparent significant contamination with SRB (Maxwell, 2006). As most bacterial counts were conducted using serial dilution techniques such as the most probable number (MPN) technique selective enumeration of SRB strains (depending on the type of growth medium used) will inevitably be conducted. Therefore, high bacterial numbers derived from cultivation-based techniques do not necessarily correlate to high SRB numbers causing MIC in the production system (Larsen et al., 2005). In addition, MIC can be caused by other microbes such as sulphate-reducing archaea (SRA), methanogens and fermentative microbes (Larsen et al., 2008, 2009). Also most samples taken by the oil industry are water samples. However, the majority of microbial activity takes place in biofilms that attach to pipeline walls, well tubing and on the inside of topside facilities.

With the introduction of molecular microbiological methods (MMM) based on DNA and RNA we now have the tool box in place to properly monitor microbial numbers and microbial activity in both water and solid samples from pipelines and process streams (Larsen et al., 2006; Skovhus et al., 2009; Larsen et al., 2008, 2009, 2010). The application of MMM allows for improved optimisation of MIC mitigation strategies (e.g. biocide treatments, pigging operations and material selection) as well as improved MIC monitoring in oilfields and petroleum systems (Hansen et al., 2009).

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Recent work on corrosion scales from the Halfdan and Dan fields in the Danish sector of the North Sea has now facilitated a robust and rapid tool based on MMM for MIC diagnostics in solid corrosion scales. Overcoming the earlier barriers with the FISH technique of separating bacterial cells from crude oil components and solid materials has brought the microbiological analyses in oil production to a new level.

In this chapter, an example from the Danish sector of the North Sea shows the large diversity of sulphide-producing microorganisms in corrosion deposits recovered from the severely corroded water outlet pipe from a Halfdan HP separator. Both bacterial and archaeal sulphide-producing microorganisms were found in large numbers by applying qPCR protocols for SRB, SRA and methanogens (Larsen et al., 2009, 2010). If only cultivation-based techniques had been applied, this understanding of the corrosion mechanism would not have been achieved, and most importantly, taking correct mitigating action towards troublesome MIC microbes would not have been possible.

Key Organisms Causing MIC in Oilfields

Traditional ways of verifying the presence of MIC have been based on visual observations of pits, the measurement of SRB and their byproducts (e.g. FeS, pyrite). Several reports have been made of failures where SRB have been detected (Davis et al., 2006). However, assuming that measuring SRB with the MPN method gives good control of asset integrity in respect of MIC is a serious mistake. This is true for several reasons. First, the MPN technique will only measure a minor portion of the SRB present. Second, and more importantly, SRB are just one out of several key players among microbes that have influence on corrosion processes in oilfields (Crolet, 2005).

It has become clear that sulphide is not only produced by SRB. H₂S is also produced by sulphate-reducing archaea, archaeal methanogens and fermentative microorganisms in oilfield systems (Woese et al., 1990; Hugenholtz, 2002; Magot et al., 2000). Previously published results obtained with MMM on produced water and scale samples recovered from the Halfdan and Dan production systems showed that both archaeal and bacterial sulphate-reducing microorganisms were present as detected with clone libraries and DGGE (Larsen et al., 2006, 2008, 2009; Skovhus et al., 2009; Gittel et al., 2009). In order to measure the entire spectrum of sulphate-reducing prokaryotes (SRP) in oilfield systems it was demonstrated that SRB as well as SRA must be taken into account, especially in connection with monitoring well and pipeline corrosion. In addition, results of the above studies showed that H₂S can be generated in oilfield systems during respiratory sulphate reduction not only by SRP but also by the release of organically bound, reduced sulphur during breakdown of dissolved oil constituents. In particular, microorganisms affiliated with fermentation (e.g. Gram-positive *Clostridia*) or archaeal methanogens can generate H₂S in this manner (Magot et al., 2000). Although the underlying mechanisms for MIC are not clear, both methanogens and SRP have been the key suspects with respect to accelerated corrosion rates (Zhu et al., 2003; Larsen et al., 2009, 2010).

Case: Diagnostics of MIC in High Pressure Separator at Halfdan Oilfield

Accelerated corrosion rates have been observed in a piece of piping (made of carbon steel) from the water outlet of a Halfdan HP separator. The temperature of the produced water in the piping has typically been in the range 40–45°C. It is estimated that 7% of the Halfdan produced water separated in the HP separator is injected sea-water. Ultrasonic inspections carried out from 2004 to 2008 showed corrosion in the water discharge piping from the Halfdan HP separator. Corrosion rates across the system were measured from 0.3 to 1.2 mm/yr. Ultrasonic measurements conducted during 2008 showed high corrosion rates in the order of 0.8 mm/yr. In particular, one location showed in 2008 a very high corrosion rate of 2.6 mm/yr and here the wall thickness was close to the minimum allowable wall thickness. Therefore, the piping was dismounted in January 2009 and a piece of the piping was cut out and examined by chemical and microbiological analytical tools aiming at identifying possible indications of MIC (Fig. 16.1). Samples for microbiological investigation were removed by sterile tools in the laboratory.



Fig. 16.1 Water outlet from Halfdan HP separator onshore (a), panel cut out for investigation (b), close-up of scale/metal interface with pits (c) and acid-rinsed metal surface from sub-part of panel (d)

Microbiological Investigation

The newly developed quantitative polymerase chain reaction (qPCR) assays for quantification of troublesome microbes such as SRP and methanogens were applied (Larsen et al., 2009, 2010). The protocol uses amplification of DNA extracted from solid samples as the basis for quantification of different target organisms involved in MIC. The method has been described elsewhere (Larsen et al., 2009, 2010). Figure 16.2 shows the results of the qPCR analyses on biofilm from the water outlet of the Halfdan separator V-3402.

Analyses with qPCR assays showed very high levels of microorganisms in the scale. Figure 16.2 shows the total number of prokaryotes and the fractions of SRB, SRA and methanogens. The total number of prokaryotes was highest at the scale/water interface (2.4×10^{10} cells/g versus 1.0×10^8 cells/g at the scale/metal interface).

SRB dominated at the top layer (3.4×10^9 cells/g corresponding to 14% of the total population of prokaryotes) compared to the pipe surface (5.9×10^5 cells/g corresponding to 0.6% of the total population of prokaryotes). Such a SRB profile could be expected due to the highest sulphate concentration found at the scale/water interface.

High levels of SRA were found at the top layer (1.6×10^7 cells/g corresponding to 0.1% of the total population of prokaryotes) and at the pipe surface (1.8×10^5 cells/g corresponding to 0.2% of the total population of prokaryotes). Representatives of *Archaeoglobus* have been revealed in Halfdan produced water using MMM (Larsen et al., 2008, 2009). These indigenous archaea are thermophilic and are, most likely involved in reservoir souring, but can also be engaged in MIC in the long horizontal reservoir sections of the individual Halfdan producers with a typical downhole temperature of 80°C. However, these microbes might not be active in the separator, due to the lower temperature as compared to the reservoir.

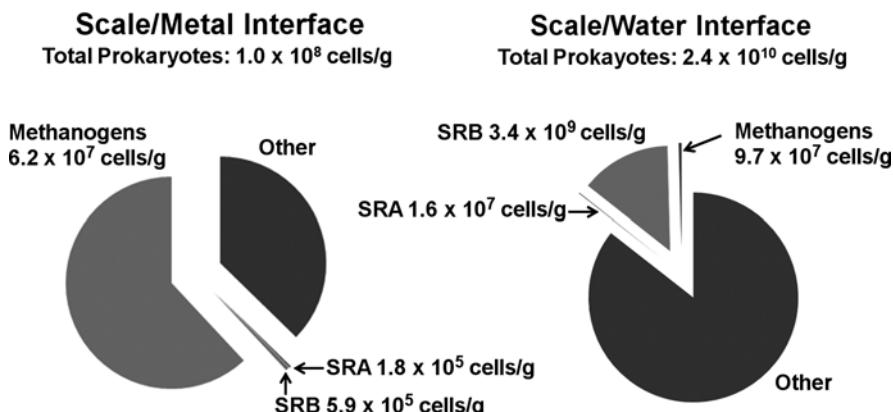


Fig. 16.2 Microbiology of scale from the water outlet of the separator V-3402 at Halfdan

Methanogens constituted the majority of microorganisms close to the metal/scale interface with 6.2×10^7 cells/g (corresponding to 62% of the total population of prokaryotes) versus 9.7×10^7 cells/g (corresponding to 0.4% of the total population of prokaryotes) at the top layer. It is evident that the abundance of methanogens is not correlated to the abundance of SRP. The methanogens at the metal surface were affiliated with the genera *Methanothermococcus* and *Methanocaldococcus* which are specialised in utilising hydrogen as the electron donor. In addition to these two groups, the methanogens at the top layer included members of the genera *Methanosarcinales*, which have a much more diverse range of metabolites (e.g. volatile fatty acids) available in the produced water for their metabolism.

Chemical and Surface Characterisation

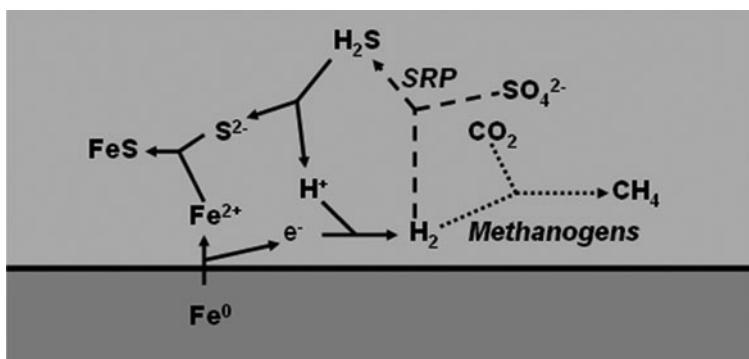
The surface layer of the deposit was red in colour showing that the surface layer has been oxidised due to contact with the atmosphere after being taken down offshore. A closer examination showed that the deeper layers had remained black and thus protected from oxygen intrusion (Fig. 16.1c). When the scale layer was rinsed off with acid, distinct pits were visible, which could be a potential indication of microbial activity (Fig. 16.1d).

Quantitative X-ray fluorescence analysis of the solids showed a high content of iron compounds varying from 36% w/w at the top layer to 54% w/w towards the pipe surface. Also, a significant level of sulphur was present varying from 13% w/w at the top layer to 7.5% w/w at the pipe surface. The red colour in the surface layer indicates that iron is present as Fe_2O_3 , the black-coloured deposits close to the pipe surface indicate that iron is present as FeS and Fe_3O_4 .

Discussion

The applied qPCR protocol for potential troublesome microbes includes SRP and methanogens. At the scale/water interface these microorganisms represented 14.5% of the total prokaryotic population. At the metal surface the same microorganisms constituted 62.8%. Both methanogens and SRP are known to consume hydrogen which by some authors have been suggested as a mechanism that will speed up the corrosion rates at the metal surface (by cathodic depolarisation) and thereby causing MIC (Zhu et al., 2003). Based on the qPCR results for SRP and methanogens a MIC model driven by these microorganisms utilising the protective hydrogen layer during dissolution of iron at the metal surface has been proposed for the Halfdan HP separator water outlet (Fig. 16.3).

In many environments methanogens are as common as SRB and are often found together with SRB (Roberge, 1999; Boopathy and Daniels, 1991). The reason that methanogens in the oilfield operation have not been recognised for being implicated in MIC to the same degree as SRB is most likely that the methanogens in their



Net Reactions:

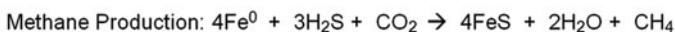
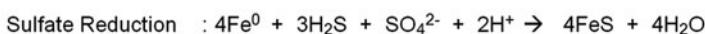


Fig. 16.3 MIC model (driven by SRP and methanogens) for corroded piping from the water outlet of the Halfdan separator V-3402

metabolism do not produce distinctive and solid byproducts such as FeS (Roberge, 1999). To summarise the findings in this case study it can be concluded that

1. A high number of troublesome microorganisms are present in the analysed deposit, including sulphate-reducing prokaryotes and methanogens.
2. Methanogens were particularly abundant close to the metal/scale interface corresponding to 62% of the total population of prokaryotes. It was detected that the abundance of methanogens is not correlated to the abundance of sulphate-reducing prokaryotes.
3. SRP produce H₂S, and both SRP and methanogens consume hydrogen and are, therefore, involved in MIC.
4. From both visual inspection of the corroded pipe, calculations of chemical parameters from the process waters and from microbiological analysis on solid samples it was concluded that the corrosion taking place in the water outlet of the V-3402 separator is most likely MIC.

Novel DNA-Based Approach to MIC Diagnostics and Mitigation

Characterising corrosion as being influenced by some culturable mesophilic sulphate-reducing bacteria only is a gross simplification of the actual processes leading to MIC. Such an inadequate microbiological approach will eventually lead to misinterpretations in risk assessment models and in a lack of understanding when prevention of MIC is the task. The above results clearly show that both SRP and methanogens must be taken into account, especially when investigating corrosion

and MIC in wells, pipelines and topside facilities in oilfield systems. This study has shown that it is possible to obtain comprehensive and detailed information regarding the abundance and distribution of MIC-promoting microorganisms in scale samples. The combination of molecular microbiological methods (MMM) and chemical characterisation has provided a strong tool for failure analysis of the collected piping. It is suggested by the authors, that SRP and methanogens should be included in MIC monitoring programmes and risk assessment models in the oil and gas industry. These programmes should be based on MMM such as the qPCR technique used in the Halfdan case described in this chapter and at best be combined with physical/chemical measures for identification of corrosion products by, e.g. X-ray fluorescence. It is also suggested that the effect of any chemical treatment in the oil and petroleum industry against microbes involved in MIC should be evaluated with the molecular microbiological approach described in this case study.

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Chapter 17

Problems Caused by Microbes and Treatment Strategies

Anaerobic Hydrocarbon Biodegradation and Biocorrosion: A Case Study

Joseph M. Sufita and Kathleen E. Duncan

Introduction

The anaerobic biodegradation of petroleum hydrocarbons is important for the intrinsic remediation of spilt fuels (Gieg and Sufita, 2005), for the conversion of hydrocarbons to clean burning natural gas (Gieg et al., 2008; Jones et al., 2008) and for the fundamental cycling of carbon on the planet (Caldwell et al., 2008). However, the same process has also been implicated in a host of difficult problems including reservoir souring (Jack and Westlake, 1995), oil viscosity alteration (Head et al., 2003), compromised equipment performance and microbiologically influenced corrosion (Duncan et al., 2009). Herein, we will focus on the role of anaerobic microbial communities in catalysing biocorrosion activities in oilfield facilities. Biocorrosion is a costly problem that remains relatively poorly understood. Understanding of the underlying mechanisms requires reliable information on the carbon and energy sources supporting biofilm microorganisms capable of catalysing such activities.

Many important reviews (e.g. Widdel et al., 2006; Heider, 2007) summarise the multiple biochemical mechanisms that anaerobes have evolved to metabolise hydrocarbons. In one of the more common bioconversions, alkylbenzenes, *n*-alkanes, cyclic alkanes and polynuclear aromatic hydrocarbons can be activated by addition to the double bond of fumarate to form substituted succinic acid derivatives. The free radical nature of such bioconversions can be correlated with the extent of substrate transformation making reliable predictions of anaerobic fate processes possible (Beasley et al., 2009). While the mechanistic details associated with such bioconversions are progressing, it is clear that such reactions can be coupled with a variety of terminal electron acceptors to result in the production of various intermediates and endproducts that are important for the corrosion of metal surfaces.

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Hydrocarbon biodegradation was recently implicated in biocorrosion processes in oilfield facilities on the North Slope of Alaska (Duncan et al., 2009). Signature metabolites associated with the anaerobic biodegradation of C₁–C₄ *n*-alkanes were detected in multiple wellheads and pipeline fluids. This finding suggested that the suite of low molecular weight hydrocarbons, routinely produced and reinjected into reservoirs for oil recovery purposes, provided biocorrosive microbial communities with an important source of carbon and energy.

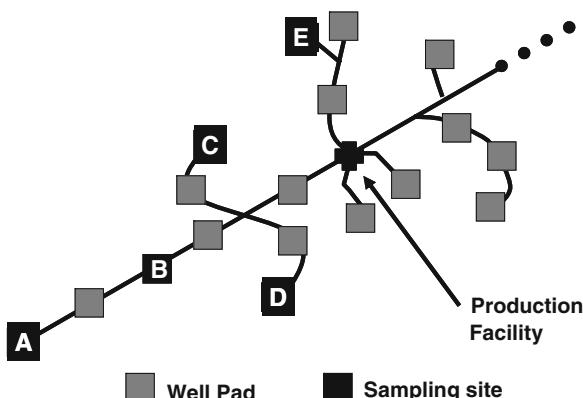
Importance of Biofilms

The biocorrosion of metallic oilfield equipment is most often associated with the development of biofilms or mixed microbial assemblages enclosed by a matrix of extracellular polymeric substance and innervated by a network of channels. It is the community metabolism of such biofilms that ultimately results in the proliferation of cells to the point where the failure of colonised structures occurs. In pipelines, anaerobic conditions prevail and biofilm communities routinely develop. Control measures (e.g. pigging, biocide treatments, corrosion inhibitors) are designed to remove and/or inhibit corrosive biofilms. However, the carbon and energy sources supporting the proliferation of biofilm communities remain somewhat enigmatic. Herein, we suggest that selected hydrocarbons can represent the primary carbon sources supporting the growth and activity of biofilm communities. It remains to be determined if the hydrocarbonolytic activity results from reservoir organisms, or in pipeline and associated infrastructure biofilm communities, or both. In many of the North Slope oilfield facilities, the reservoirs are hot (average temperature 69°C, Masterson et al., 2001), but compatible with the known requirements for microbial growth (e.g. Stetter et al., 1993). The above ground infrastructure is insulated to minimise potential impacts on the arctic tundra and much of the heat of the reservoirs is retained throughout the oil processing system. The partially oxidised hydrocarbon metabolites are more water soluble and may easily travel with production fluids and support biofilm growth on above ground metallic surfaces. Alternately, it is conceivable that the requisite organisms preferentially reside in the pipeline infrastructure. In either case, the biofilm microflora would be exposed to a variety of important potential electron acceptors for anaerobic respiratory processes. While the origin of the requisite communities is not known with certainty, what is known is that various groups of bacteria can specifically congregate, form metabolic consortia and produce corrosive agents, including CO₂, various organic acids, hydrogen sulphide, mineral acids or other materials. Our studies implicate the importance of anaerobic hydrocarbon activity and provide some insight on the dominant microbial assemblages.

Metabolite Profiling

A schematic representation of a portion of the oil facility on the Alaskan North Slope is depicted in Fig. 17.1. A series of well pads, each harbouring multiple oil wells servicing multiple oil reservoirs, are connected by aboveground pipelines to

Fig. 17.1 A schematic representation of a portion of the oil production facility sampled during this study



a central processing facility. The distance between the production facility and the most distal well sampled was more than 60 km.

In the latter facility, oil is separated from production waters and light molecular weight hydrocarbons and eventually enters the trans-Alaska pipeline system.

The production water separated in the processing facility gets reinjected in the reservoirs to maintain formation pressures and to increase oil recovery. Similarly, the light molecular weight hydrocarbons are separated, dried, compressed and also reinjected into the formations to help maintain reservoir pressures. Production fluids ($\geq 50^\circ\text{C}$) were obtained from individual wells on the well pads indicated in Fig. 17.1 or from the processing facility and analysed for metabolites associated with anaerobic hydrocarbon metabolism.

Instead of intermediates associated with the more water-soluble oil components (benzene, toluene, ethylbenzene and the xylene isomers) we found low molecular weight alkylsuccinate metabolites associated with the anaerobic biodegradation of $\text{C}_1\text{--}\text{C}_4$ *n*-alkanes (Table 17.1).

The fumarate addition intermediates associated with anaerobic low molecular weight hydrocarbon metabolism were found facility wide (Table 17.1). Methyl-, ethyl- and propylsuccinate were the most common putative metabolites detected in this survey as they were found in five of the six sampling locations (Table 17.1). Butylsuccinate proved more ephemeral in that it was only measured in wells A and D. The identity of the metabolites was confirmed by comparison of the profiles with authentic standards derivatised and analysed in the same manner by GC-MS (Duncan et al., 2009) The fumarate addition metabolites associated with $\text{C}_1\text{--}\text{C}_4$ *n*-alkanes suggested that the low molecular weight hydrocarbons, routinely reinjected for about 30 years of oil recovery operations, were being oxidised by organisms likely to be selectively enriched over this period.

In addition to the fumarate addition metabolites, we also detected an important suite of downstream branched and straight-chain alkanoic acid metabolites nearly facility wide (Table 17.1). Of course, these downstream intermediates are predicted based on the assumption that the low molecular hydrocarbons are metabolised in

Table 17.1 The fumarate addition and downstream branched and straight chain alkanoic acid metabolites detected facility wide on the North Slope of Alaska. The well designations and processing facility correspond to the locations schematically illustrated in Fig. 17.1

Parent hydrocarbon	Fumarate Addition and [alkanoic acid metabolites]	Well A	Well B	Well C	Well D	Well E	Processing facility
Methane	Methylsuccinate	✓	✓		✓	✓	✓
	[butanoate]	✓	✓	✓	✓	✓	✓
Ethane	Ethylsuccinate	✓	✓	✓	✓		✓
	[pentanoate]	✓	✓	✓		✓	✓
Propane	Propylsuccinate	✓	✓	✓	✓		✓
	[4-methylpentanoate]	✓	✓	✓	✓	✓	✓
	hexanoate]		✓		✓	✓	✓
Butane	Butylsuccinate	✓			✓		
	[4-methylhexanoate]	✓	✓		✓	✓	✓
	heptanoate]	✓	✓		✓	✓	✓

analogous fashion to their higher molecular weight counterparts. That is, these metabolites can be formed as a result of the carbon skeleton rearrangement and subsequent decarboxylation of the alkylsuccinate intermediates (Wilkes et al., 2002; Davidova et al., 2005; Grossi et al., 2008). The presence of these intermediates is consistent with the hypothesis that the oxidation of the low molecular weight hydrocarbons may help supply the carbon and energy needs for biocorrosive microbial communities.

Microbial Profiling

Cultivation-independent techniques were used to determine the types of microorganisms existing in the oilfield facilities. DNA was extracted from production fluids collected from wells A and B and from two sites in the oil processing facility for the creation of 16S rRNA and *dsrAB* gene sequence libraries. The sequences we obtained were similar to those of *bacteria* (Fig. 17.2) and *archaea* (Fig. 17.3) typical of those previously detected from oil reservoirs and facilities maintained at high ($\geq 50^{\circ}\text{C}$) temperatures (Magot, 2005; Dahle et al., 2008). Highly similar 16S rRNA gene sequences to particular taxa were repeatedly encountered, notably *Thermovirga* ('Synergistetes'), *Desulfomicrobium*, *Pelobacter*, *Desulfacinum* (all Deltaproteobacteria), *Thermosiphlo* (Thermotogae), *Thermoacetogenium*, *Thermoanaerobacter* (both 'Clostridia') and *Thermodesulfobacterium* (Thermodesulfobacteria) among the *bacteria*. *Archaeoglobus* (Archaeoglobi), *Thermococcus* (Thermococci) and *Methanothermobacter* (Methanobacteria) represented the majority of the *archaea*. In addition, there was a cluster of sequences distantly related to *Moorella* ('Clostridia'), while well A was dominated (90%) by sequences similar to those of *Petrobacter* (Betaproteobacteria), although this sequence type was not detected in the other samples (see Duncan et al., 2009 for

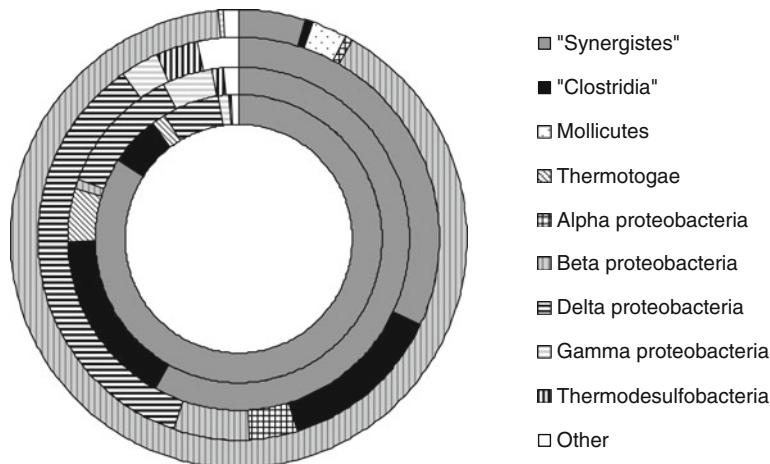


Fig. 17.2 Relative proportion of higher order bacterial taxa from 16S rRNA gene sequence clone libraries constructed from DNA extracted from production fluids collected from (inner to outer ring, # sequences per library in parentheses): sampling site B (648), two sites in the oil processing facility (544, 329) and sampling site A (363). Sampling locations are as named in Fig. 17.1

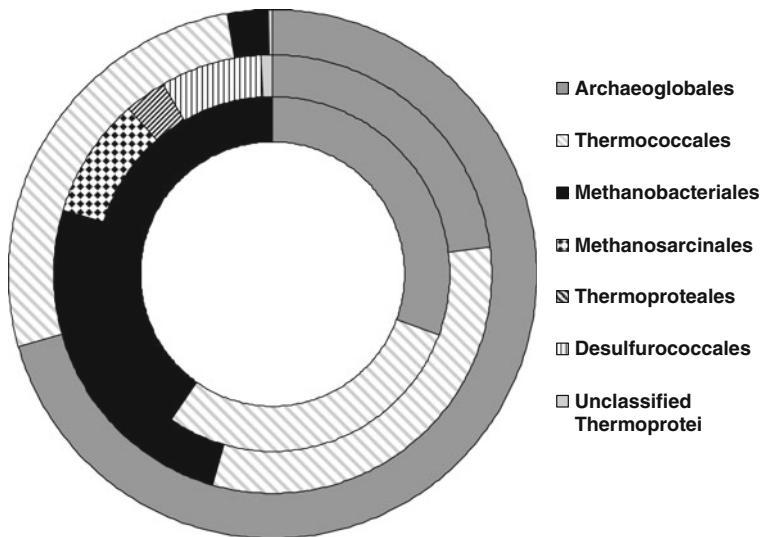


Fig. 17.3 Relative proportion of higher order archaeal taxa from 16S rRNA gene sequence clone libraries constructed from DNA extracted from production fluids collected from (inner to outer ring, # sequences per library in parentheses): sampling site A (226), two sites in the oil processing facility (252, 240). Sampling locations are as named in Fig. 17.1

a complete summary of the clone libraries). The *dsrAB* libraries from A and the two oil processing facility samples were dominated (60–99%) by gene sequences similar to *Archaeoglobus fulgidus*, a thermophilic sulphate-reducing archaeon, rather than by bacterial *dsrAB* gene sequences (Fig. 17.4).

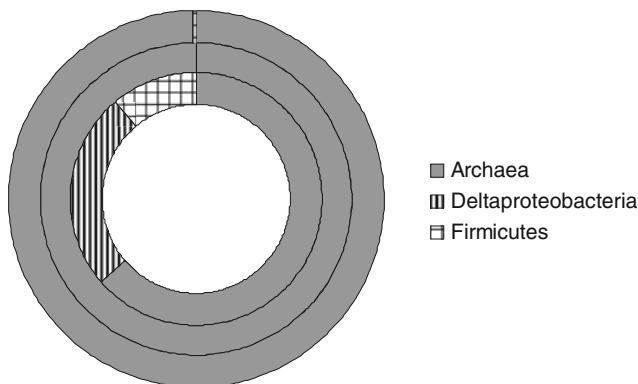


Fig. 17.4 Relative proportion of sulphate-reducing archaea and bacteria from *dsrAB* gene sequence clone libraries constructed from DNA extracted from production fluids collected from (inner to outer ring, # sequences per library in parentheses): sampling site A (342), two sites in the oil processing facility (342, 361). Sampling locations are as named in Fig. 17.1

Importance and Implications

The microbial profiles associated with an oilfield on the North Slope of Alaska suggest that the microbial diversity is not particularly high. This has important implications for the types of organisms that might be targeted as biocorrosion monitoring efforts get considered. For instance, oil industries have for decades monitored sulphate-reducing bacteria as indicators of pipeline corrosion. However, our findings suggest that sulphate-reducing archaea are likely more important in this regard (see also Gittel et al., 2009). It clearly remains to be determined how generalising such observations may prove to be.

While it is always tenuous to infer microbial physiology information based on 16S rRNA gene sequences, organisms with sequences most similar to the ones detected on the North Slope include typical inhabitants of thermophilic microbial communities associated within oil reservoirs and petroleum facilities, e.g. hydrogen-using methanogens, syntrophic bacteria, peptide- and amino acid-fermenting bacteria, iron reducers, sulphur/thiosulphate-reducing bacteria and sulphate-reducing archaea. Indeed, thermophilic members of most of the aforementioned genera have been isolated from environments long exposed to crude oil (L'Haridon et al., 1995; Fardeau et al., 1993; Stetter et al., 1993). However, the niches of the organisms in the North Slope oilfield community and their specific role in biocorrosion have yet to be determined.

A major implication of the current study is that the resident microflora can metabolise light molecular weight hydrocarbon components that have been recycled for decades through the reservoirs for oil recovery purposes. This observation is reminiscent of small *n*-alkane metabolism and alkylsuccinate formation by sulphate-reducing bacteria isolated from marine hydrocarbon seeps (Kniemeyer et al., 2007). While the metabolic profiling effort forces us to the conclusion that such processes

are important, we cannot dismiss the prospect that other oil components may be similarly metabolised.

However, the ability of thermophilic enrichments or cultures to metabolise oil components of any kind is far from clear (Orphan et al., 2000; Stetter et al., 1993; Mardanov et al., 2009), and this ability is only rarely assessed in formal taxonomic descriptions of the relatively few isolates that seem to be truly indigenous to petroliferous deposits (Beeder et al., 1995; Fardeau et al., 2000; Dahle and Birkeland, 2006, etc.). Further, there is a discrepancy between the increasing number of alkane and aromatic hydrocarbon-degrading microbes isolated from mesophilic environments and the relative scarcity of hydrocarbonoclastic thermophiles. In particular, strains of sulphate-reducing, iron-reducing and denitrifying mesophilic Deltaproteobacteria and Betaproteobacteria are known to degrade hydrocarbons (Widdel and Rabus, 2001; Van Hamme et al., 2003). Although both Deltaproteobacteria and Betaproteobacteria sequences were found in our samples, no members of the genera we detected (*Desulfomicrobium*, *Pelobacter*, *Desulfacinum*, *Petrobacter*) have been verified to be hydrocarbon degraders.

So what organisms or processes might be involved in the production of the putative metabolites? Is there some abiotic process under the pressure and temperature regimes in oil reservoirs that could account for the oxidation of the C₁–C₄ *n*-alkanes? Such answers await further experimentation, but we are unaware of chemical processes that could explain the differential oxidation of the lower molecular weight hydrocarbons and produce an observed suite of succinate derivatives. Regardless of the initial oxidation mechanism, the detection of the expected downstream metabolites is at least consistent with known anaerobic hydrocarbon biodegradation pathways (Wilkes et al., 2002; Davidova et al., 2005; Grossi et al., 2008). Therefore, microbial conversion processes would likely be involved at some stage, but exactly where the requisite organisms specifically reside and catalyse such reactions are enigmatic.

One possibility is that the actual hydrocarbon-degrading bacteria are confined to the reservoir per se and thus not captured in our small survey of oilfield samples from well head production fluids or from an oil separation processing facility. Another possibility is that thermophilic strains related to known mesophilic hydrocarbon degraders are the active catalysts, but they are too rare to be detected in clone libraries consisting of only a few hundred sequences. A third possibility is that hydrocarbon degradation is more widespread than previously thought and new taxa may yet be discovered with this capability. The latter possibility can be readily examined by testing representative thermophilic strains from oil environments for their ability to grow at the expense of various crude oil constituents and also catalyse corrosion.

As a complement and guide to selection of relevant isolates, molecular protocols can be used to probe via PCR amplification and cloning for gene sequences coding for enzymes in anaerobic hydrocarbon biodegradation pathways (Callaghan et al., 2008). This can certainly be successful for organisms closely related to known hydrocarbon degraders, as their gene sequences will undoubtedly be similar. However, metagenomic analysis of microbial communities in tandem with

metabolite profiling can uncover candidate sequences of homologous enzymes derived from more distantly related microbes and allow for a more complete assessment of hydrocarbon metabolic potential (Maron et al., 2007). Pyrosequencing, because of its ability to screen vast numbers of sequences, should prove useful to assay for rare sequences with high similarity to those of known mesophilic hydrocarbon degraders as well (Kowalchuk et al., 2007).

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Chapter 18

Problems Caused by Microbes and Treatment Strategies

Health and Safety Issues from the Production of Hydrogen Sulphide

Nicole Williamson

Introduction

Hydrogen sulphide (H_2S) is a colourless, transparent gas that is heavier than air ($\text{SG} = 1.18$). It is extremely flammable and is explosive across a very wide range of concentrations 4.3–46% by volume in air (in comparison, methane is explosive at 5–15% volume in air). The boiling point of hydrogen sulphide is -60°C and so it exists as a gas at standard conditions; it burns with a blue flame to produce water and sulphur dioxide, which is also a very toxic gas. At low concentrations hydrogen sulphide has a very pungent smell, typically described as ‘rotten eggs’; at higher concentrations it can become sickly sweet. Hydrogen sulphide can be easily identified by its smell at very low concentrations, detectable down to 0.0047 ppm (HSE, 2009). However, smell alone cannot be relied upon to detect the continued presence of hydrogen sulphide. At high concentrations, or after sustained exposure to lower concentrations, hydrogen sulphide becomes undetectable by smell as it rapidly paralyses the olfactory nerve.

The health and safety concerns generated by the presence of hydrogen sulphide are twofold: first, poisoning from the direct inhalation of hydrogen sulphide gas and, second, the risk posed to the integrity of equipment due to the reaction of hydrogen sulphide with metals. The potential for sudden failure of metalwork as a result of sulphide stress corrosion cracking is a serious safety risk to personnel and also an environmental hazard. The solubility of hydrogen sulphide in water is approximately 4 g/l at 20°C and so it is soluble in the bloodstream and can be quickly transported around and react with the body after inhalation. Its solubility in water also enhances its corrosive potential as it will readily partition into any small volume of water present in a system. At typical produced water pHs of 6–7 the speciation of sulphide in water is such that more than 50% of it is present in the form of H_2S (the remainder being HS^-), this drops to 10% as the pH rises to 8.

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Since hydrogen sulphide is heavier than air, the risks to people are often greatest in enclosed, poorly ventilated areas, particularly in the bottom of tanks and in sewers, but even in out-door low-lying ground, e.g. in swamps (hence it has been called ‘swamp gas’) or around oil/gas wells in well-head sumps, there is a risk.

Sources of Hydrogen Sulphide

There are two main sources of hydrogen sulphide in the oil and gas industry, it can be either biogenic or geochemical in origin and can occur during drilling, in produced fluids or in enclosed spaces on drilling or production platforms. The principal source of hydrogen sulphide is from produced fluids (water and crude oil) and gas from sour reservoirs. Hydrogen sulphide may be present in reservoirs naturally, as a consequence of thermal degradation of organic material and associated bacterial activity during the deposition and subsequent maturation of the reservoir in the geological past. In particularly hot ($>100^{\circ}\text{C}$) reservoirs thermochemical reduction of sulphate is also thought to have generated sulphide (Killops and Killops, 2005); it may be present at concentrations of anything from a few ppm to several thousand ppm. If this is the case then the risk is initially posed during the exploration and development drilling of the reservoir. The presence of hydrogen sulphide will become apparent as gas is circulated out of the hole as it is drilled, but also if any inflow of formation fluids into the wellbore occurs; upon reaching the surface the gas will break out of the fluid into the atmosphere. This is of particular concern during drill stem testing, coring (formation gases may become trapped in the core barrel) and during well logging.

Hydrogen sulphide may also develop in reservoirs as a consequence of bacterial activity after oil production begins and is usually called ‘reservoir souring’ (Vance and Thrasher, 2005). As a reservoir matures, water injection may be required to boost reservoir pressure and maintain oil production; seawater is commonly used as the source water. The sulphate present in the seawater, together with the carbon source (carboxylic acid anions) present in the reservoir, allows sulphate-reducing bacteria (SRB) to proliferate and generate hydrogen sulphide. Bacterial activity occurs either around the near wellbore area or infiltrates deeper into the reservoir as the flood front of injection water progresses. In both cases the produced hydrogen sulphide will be carried with the injection water and partition between it and the crude oil and will ultimately be produced to the surface. Recent work has shown that hydrogen sulphide is produced not only by SRB but also by archaea (SRA) and collectively these are described as SRP, sulphate-reducing prokaryotes (Birkeland, 2005).

Souring of a reservoir may also occur directly around the production well, by contamination of the near wellbore area during drilling and production. SRP which are able to colonise this area or migrate further into the reservoir can subsequently produce hydrogen sulphide by utilising any sulphate present in the formation water, and this will then be produced to the surface facility.

SRP-generated hydrogen sulphide can also occur in the ‘topside’ areas of a production platform/facility; wherever water is present without adequate microbiological control then bacteria may proliferate. Both production process systems and water injection networks are susceptible to SRP activity; pipelines, crude oil storage, water injection plant, production separators all have the potential to host SRP and become sources of hydrogen sulphide. Confined spaces and areas of stagnant water are high-risk areas for hydrogen sulphide generation and accumulation; this includes the legs of drilling rigs, drains and slop tanks. Mixing of water or oil from drains back into the main system should generally be avoided as this is an easy way to introduce SRP into a previously clean system.

Physiological Effects of Hydrogen Sulphide

Exposure in the majority of cases, to hydrogen sulphide gas, is by direct inhalation or by reaction with the mucous membranes; there is little absorption through the skin (OSHA, 2005). However, where hydrogen sulphide needs to be stored, it is held in a liquefied state, and if this liquid comes into contact with the skin it can result in frostbite.

Hydrogen sulphide is able to poison multiple body systems, with the most seriously affected being the respiratory and the central nervous systems. The effects of exposure increase with the intensity and duration of exposure, hydrogen sulphide also has a sensitising effect; repeated exposure can result in an increasingly severe response to the same intensity of exposure.

Hydrogen sulphide acts by the disruption of oxidative metabolism. It inhibits the cytochrome oxidase enzyme by bonding with iron, and so prevents oxygen being used by cells, and forcing anaerobic respiration. The subsequent accumulation of lactic acid causes an acid–base imbalance. No carcinogenic effects have been associated with exposure to hydrogen sulphide. There is no specific antidote to hydrogen sulphide poisoning; the only treatment is exposure to fresh air, with administration of oxygen where available.

Hydrogen sulphide is detectable by the human nose at 0.0047 ppm; this is much lower than the concentration at which legal working limits become applicable (HSE, 2009). Above 20 ppm eye irritation will begin, followed by minor irritation to the respiratory system. While hydrogen sulphide does not accumulate in the body, repeated or prolonged exposure may cause headache, nausea, irritability and insomnia. Chronic exposure has also been linked with neurological symptoms and psychological disorders, eye inflammation and chronic cough.

With increasing concentrations, these effects become more pronounced: at 150–200 ppm the olfactory nerve is paralysed within minutes and all sense of smell disappears. Breathing may initially be excited, but subsequently more difficult as fluid accumulates in the lungs, together with dizziness and staggering. At 320–530 ppm pulmonary oedema occurs and may lead to death (HSE, 2009). At this point immediate removal of fresh air is required to avoid respiratory paralysis; however, it may be up to 72 h before all effects become apparent.

At very high concentrations, 530–1,000 ppm, the central nervous system is rapidly stimulated causing rapid breathing which will very quickly (after maybe only a few breaths) lead to loss of consciousness, coma and death. Exposure to concentrations of 1,000 ppm or more may cause immediate collapse after only one breath, with permanent brain damage and death if not removed to a safe atmosphere immediately. In humans, the LC₅₀ for a 5 min exposure to hydrogen sulphide is accepted to be 800 ppm (HSE, 2009). All the above concentrations are in air, and, unless otherwise stated, the physiological reactions are as described by the Agency for toxic substances and disease registry (ATSDR, 2005). Hydrogen sulphide is equally toxic to other animals as it is to humans and is very toxic to aquatic organisms.

Reaction with Materials

As steel corrodes, hydrogen atoms and ions are generated at its surface (the cathode); the presence of sulphide ions in a system inhibits the formation of molecular hydrogen resulting in an accumulation of hydrogen ions at the metal surface (Byars, 1999). This accumulation of hydrogen ions can significantly damage steel in three ways:

1. *Hydrogen blistering* is a result of hydrogen atoms at the steel surface diffusing into the steel through the grain boundaries, they can accumulate within the metallic structure in defective regions such as voids, micro-cracks, laminations or discontinuities about inclusions. At this point the hydrogen atoms combine to form molecular hydrogen gas which exerts a pressure causing the metal to blister. Hydrogen blistering occurs more readily in lower strength steel grades, with a Rockwell hardness rating lower than 22. In higher strength or hardened steels, the metal is more likely to crack than deform (Byars, 1999).
2. *Hydrogen-induced cracking* (HIC) occurs when multiple elongated defects are present parallel to the metal surface; hydrogen accumulates in these defects and causes deformation such as a blister or a crack. At the edge of these deformations, cracks propagate and tend to join up with adjacent cracks and blisters creating a layer of deformation parallel to the pipe or wall surface. This will effectively reduce the wall thickness and consequently its strength.
3. *Sulphide stress cracking* (SSC or sulphide stress corrosion cracking, SSCC) is also a consequence of atomic hydrogen diffusing into steel and causing embrittlement. The exact mechanism by which metal loses its ductility in these circumstances is not fully explained at present. There are four conditions which must be met for it to occur: (1) hydrogen sulphide in system; (2) water, even trace amounts are sufficient; (3) high strength steel and (4) a tensile stress or loading (residual or applied) to the steel. If these conditions are met then at some point following exposure to the hydrogen sulphide failure may occur in the steel. The length of time between exposure and failure will depend on a combination of factors: (1) concentration of hydrogen sulphide; (2) pH of the solution; (3) stress applied to the metal; (4) system temperature and (5) yield strength of steel.

These mechanisms can generate sudden, catastrophic failures in pipe work or vessel walls. This may cause immediate danger to personnel, loss in control to operations or environmental damage from a leaking pipeline. As well as catastrophic failures in metals, hydrogen sulphide can also attack certain rubber seals and fittings. This is of particular relevance in selecting appropriate safety equipment, such as face masks and door seals.

Microbiologically Influenced Corrosion (MIC)

The comments above, on reactions of hydrogen sulphide with materials, do not really include the more direct effects of bacteria and particularly of SRB/SRP in causing pitting corrosion. The mechanisms involved in MIC are still a subject of great debate, and on-going research, and the uncertainty is indicated in the commonly used terminology of microbiologically ‘influenced’ corrosion, which may involve consortia of many microbes and layering of several different organisms to create under-deposit corrosion. There is no doubt that hydrogen sulphide is involved in pitting corrosion and also 6 o’clock ‘groove’ corrosion which has been seen in many carbon steel pipelines, with very high corrosion rates in several cases, even exceeding 25 mm/yr. This is believed to be associated with a semi-protective iron sulphide precipitate/corrosion product forming a (large) cathode, while an adjacent area of bare carbon steel forms a rapidly corroding (small) anode, generating a pit. Perforation of pipe and vessel walls within a few months has been seen, with loss of containment leading to oil spills in several cases, e.g. the well-publicised BP Alaska pipeline incident in 2006. A further problem associated with MIC and the presence of hydrogen sulphide in general, is from the generation of suspended solids, often containing biofilms, corrosion products and scale, which may cause blockages and process upsets, possibly with HSE impacts.

Workplace Regulations

In the UK safe working limits for exposure to hazardous substances in air are defined by the Health and Safety Executive (HSE), document EH40/2005. This defines a series of workplace exposure limits (WEL) for the protection of workers who may be exposed to hazardous substances. These limits are defined by a time-weighted average (TWA) of the concentration of a substance in the air. Two limits are set, one designed for long-term exposure based on an 8 h working day and another for short-term exposure. The short-term exposure limit (STEL) is 15 min and is intended to avoid occurrence of acute effects which may develop rapidly. For hydrogen sulphide, the 8 h TWA limit is 5 ppm (or 7 mg/m³) and the STEL is 10 ppm (or 14 mg/m³) (HSE, 2005).

In the USA, the Occupational Safety and Health Administration (OSHA) and the National Institute for Occupational Safety and Health (NIOSH) also sets equivalent limits, though in a slightly different manner. A permissible exposure limit (PEL)

is defined for general industry, and this limit constitutes a ceiling gas concentration which may not be exceeded irrespective of duration. The PEL for hydrogen sulphide is 20 ppm. There is an allowance for a single exposure to up to 50 ppm hydrogen sulphide, but this may be for a maximum duration of 10 min and only if no other measurable exposure occurs within the 8 h working period. For specific industries (maritime, construction) a TWA PEL is set at 10 ppm. OSHA also gives a concentration of a gas which is considered immediately dangerous to life or health (IDLH), and for hydrogen sulphide this is listed as 100 ppm (NIOSH, 2005).

Appropriate materials selection is required to protect workers and the environment from the risks of hydrogen sulphide-induced metal failure. The various system parameters must be detailed and then matched to the appropriate materials standard described for them by NACE (National Association of Corrosion Engineers). This should be done with consideration for the future as hydrogen sulphide production typically increases with the operating life of an oilfield. As well as material selection, the hazards posed by hydrogen sulphide can also be mitigated by use of appropriate chemicals; sulphide scavengers can be introduced into drilling mud and also to produced gas and fluid streams.

As well as these measures which aim to avoid exposure of people to hydrogen sulphide, a detection system and appropriate safety equipment should also be present. The nature of this detection system is dictated by the likelihood of hydrogen sulphide being encountered. The UK HSE recommends the following system: category 0, hydrogen sulphide is assumed to be present at high concentrations. No fixed detection, with entry only using breathing equipment; category 1, hydrogen sulphide may be present under normal conditions, and so entry should be controlled, and use of portable monitoring equipment is mandatory; category 2, hydrogen sulphide should not be present under normal conditions, but there is the possibility of a leak. A fixed detection system should operate in this environment (HSE, 2009).

The presence of hydrogen sulphide in flare gas should also be managed to avoid the production sulphur dioxide during combustion. However, cold-venting of hydrogen sulphide in hydrocarbon gas should be avoided in case the cold gas plume falls to ground and creates a toxic layer there. Plume dispersion calculations for hot and cold vents must be carefully calculated to ensure such hazards do not arise in any circumstances.

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Chapter 19

Problems Caused by Microbes and Treatment Strategies

Downstream Petroleum Microbiology – An Industry Perspective

Elaine McFarlane

Introduction

In the mid-1800's it was discovered that crude oil could be extracted and exploited to produce energy. However, it was the invention of the first four-stroke internal combustion engine in 1876 that transformed the petroleum industry from a localised to a global business (Dell and Rand, 2004). Crude oil is made into useable products at the refinery via separation, conversion and treatment processes. Separation starts with distillation where the crude is evaporated and condensed into fractions based on their boiling ranges (Fig. 19.1). As well as carbon and hydrogen, the fractions consist of sulphur, nitrogen and oxygen (present in low concentrations) and metals like copper and iron (in trace amounts). After separation, heavy fractions are converted into lighter ones using intense heat, pressure and a catalyst to speed up chemical reactions. Molecules like sulphur can then be stripped out by heat treatment under pressure with hydrogen. Injection of refinery additives makes a finished fuel. For example, static dissipator is added to automotive gas oil (AGO) to reduce the risk of spark and explosion during fuel movements; middle distillate flow improver to improve low-temperature operability and lubricity improver to lubricate engine components. Finally, fuel quality measurements are made to ensure that the finished fuel meets the relevant specification.

During this processing, fuel sterility¹ is assured due to the high temperatures involved. However, transfer into bulk refinery storage presents the first real opportunity for microbial problems to develop. As the fuel cools, some free water can settle at the bottom of the tank, some remains free in suspension and the rest stays dissolved within the fuel. These three water phases are in dynamic equilibrium and are affected by diurnal fuel temperature changes, relative humidity of the air in contact with the fuel and degree of turbulence due to mechanical fuel movements. Wherever free water is present there is potential for unacceptable levels of microbial growth to exist.

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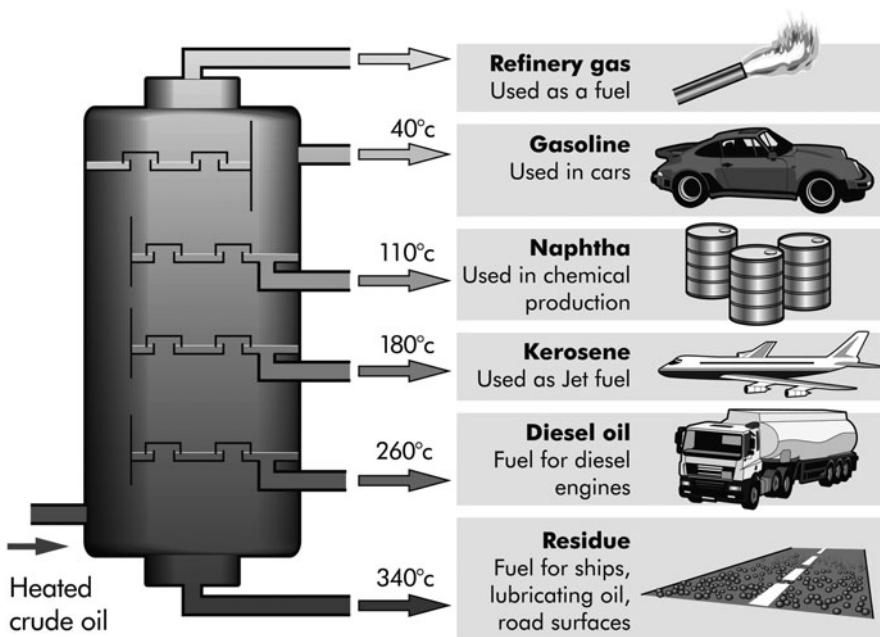


Fig. 19.1 Crude oil distillation

Fuel is uploaded directly from bulk refinery storage onto road tanker, ship/barge, rail or pipeline and can also be made into branded products by addition of performance additive packages before distribution. Fuel transportation often involves several stages (Fig. 19.2), which increases the potential for contamination with solid particulate matter, water and microbes.

To ensure that fuel is delivered clean, dry and free from contamination to the customer, a range of measures are taken:

- Sequencing and interface management with multi-product pipelines.
- Allowance of sufficient settling time for contaminants to sink to the bottom of storage tanks.
- Where practical, tanks are engineered with low points or sumps to facilitate drainage of water and contaminants.
- Dedicated compartments (ships, road and rail) to avoid grade contamination.
- Filtration.
- Sampling and testing for visual appearance, density, etc.

However, given that all living things require free water, an energy source (carbon from the fuel) and a balanced diet (inorganic nutrients from trace elements in the fuel), it is easy to see that all the requirements for microbial growth are satisfied during fuel storage and transportation. Operation of a completely sterile fuel supply

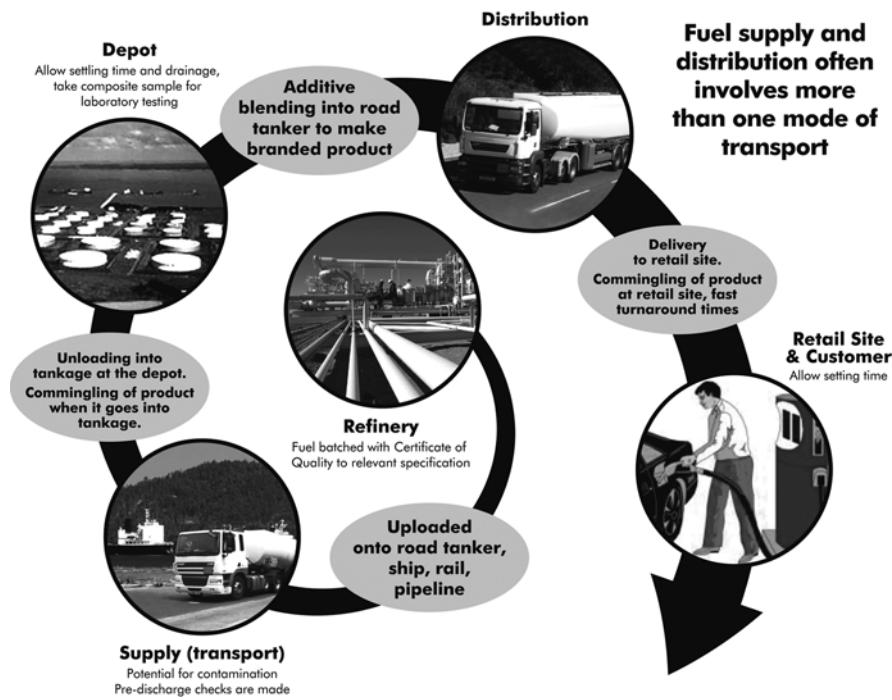


Fig. 19.2 Example supply and distribution chain

and distribution system is therefore unattainable. Growth rates are also dependent on environmental factors like the presence of oxygen and optimum temperatures, typically 15–35°C (Fig. 19.3).

The Nature of Hydrocarbon-Utilising Microbes

Among the hydrocarbon-utilising microbes are gram-negative² bacteria and several genera of yeasts and filamentous fungi (moulds) (Gaylarde et al., 1999).

Bacterial cell walls consist of a thin layer of peptidoglycan, sandwiched between an outer membrane of lipoproteins and lipopolysaccharides and the cell membrane. Outside the cell wall is another layer: the glycocalyx, which may be closely attached to the cell wall as a capsule or more loosely as a slime layer. Yeast cell walls are made up of glucan and/or mannan. Some yeasts can produce extracellular capsules consisting of mucopolysaccharide that helps to protect them against chemical attack. Mould cells are bounded by a well-defined, multilayered cell wall composed of chitin. They have a typically branched growth or mycelium made up of individual filaments called hypha, which forms microbial mats at the fuel/water interface.

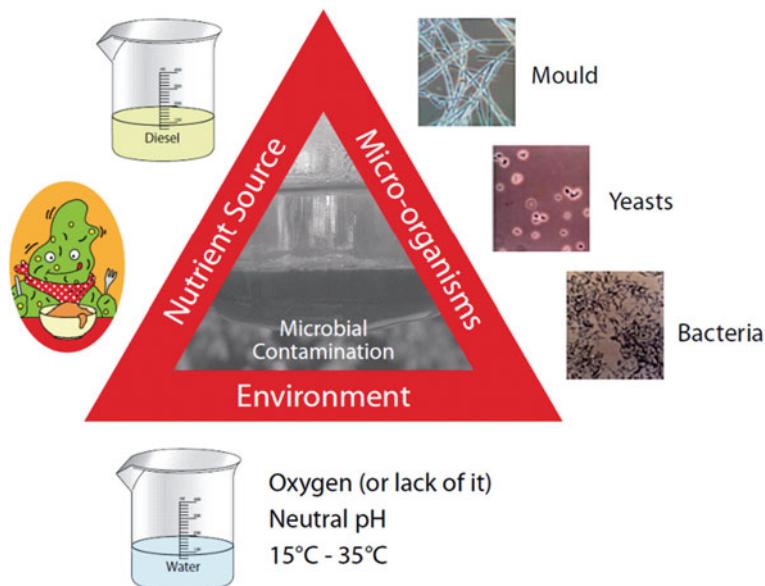


Fig. 19.3 Fuel supply and distribution provides all the requirements for microbial growth

Operational Issues

It is these slimes, mucopolysaccharides and mycelia that can lead to operational problems like filter blocking. In addition, biosurfactants³ produced as metabolic byproducts can cause hazy fuel and disarm coalescer filters⁴. Most microbes also produce acids, which can directly cause microbiologically influenced corrosion



Fig. 19.4 Biofilm formation in a retail tank angle check valve

(MIC) of metal. It is common for aerobes⁵ (mainly bacteria) to adhere irreversibly to a surface (e.g. fuel tank sidewalls and bottoms) to form a biofilm⁶ (Fig. 19.4). Organic acids are produced by this consortium⁷ and oxygen supplies are depleted allowing anodic corrosion pits to form. Anaerobic⁸ sulphate-reducing bacteria (SRB) can then add to this corrosion by producing H₂S, HS⁻ and S²⁻, all of which are very aggressive to steel.

Prevention and Control

Physical control methods such as good housekeeping⁹, settling, filtration, centrifugation and heat treatment can be used to minimise microbial growth in fuel systems. In-line heat treatment can be used during transfers to sterilise fuel, but it is often impractical to heat treat bulk fuel in situ. In addition, chemicals (biocides¹⁰) are used in conjunction with physical methods such as filtration to remove dead organisms following treatment.

Historical Perspective

As early as 1932 there were reports of SRB in gasoline (Hill and Hill, 1993). However, incidents have been sporadic due to the toxicity of tetra-ethyl lead (TEL)¹¹, the ability of gasoline to easily shed water (there is a large density difference between water and gasoline) and the solvent effect exerted on microbial cell membranes, which causes disruption of the osmotic balance within the cell (Gaylarde et al., 1999). However, there was a rare, but interesting contamination incident that occurred about 7 years ago in the USA.

Case Study 1

Unusually heavy corrosion and rust particles were observed in gasoline underground storage tanks and steel tank risers at four retail sites. These deposits and particles caused dispenser failures, plugged filters, failed leak detectors and clogged dispenser hose breakaway valves. Fuel samples for chemical and microbiological analyses were taken and microstructural examinations of the riser tube corrosion pits were made. MIC was confirmed from (a) the formation of tubercles on the pipe surfaces (Fig. 19.5), (b) the presence of acetate in storage tank water bottoms and (c) corrosion pit morphology. The exact causes of the problem remain unknown, but the incident reinforced the need for good housekeeping and changes in tank design to allow effective water draining.

It is the middle distillate¹² fuels that have historically been most at risk to microbial attack. In the late 1950's, a United States Air Force Boeing B-52 bomber was starved of fuel due to blocked filters, with disastrous consequences. Further problems with wing fuel tank corrosion and fuel tank gauge malfunctions were identified

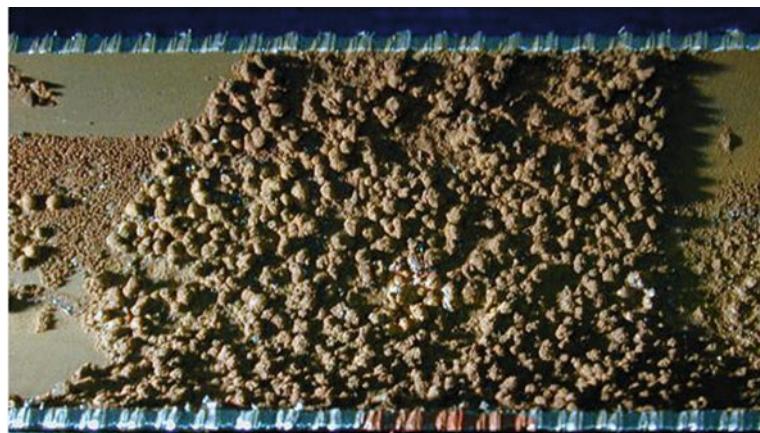


Fig. 19.5 Corrosion product tubercles on a steel tank riser

2 years later in civil aircraft; a global survey of jet fuel supply and distribution systems was initiated to understand the root cause of the epidemic. Using traditional test methods (agar slopes and plates), it was found that a mould, *Cladosporium resinae* (now *Hormoconis resinae*), was prevalent in 90% of the systems (Graef, 2003). Good housekeeping was immediately implemented and three classes of biocides were in use by the 1970's.

With jet fuel problems now relatively infrequent due to the rigorous control measures applied, the spotlight shifted to diesel fuel, particularly marine and AGO. By the 1980's problems with diesel vehicles were on the increase. The reasons cited for the increasing incidences changed over time. Initially, reduced refinery storage tank settling times, fewer refinery staff available to control housekeeping and changes in the contaminating population of microbes from moulds to yeasts and bacteria were implicated. Later, factors such as biocide resistance, increased use of additives (particularly performance additives) and changes in vehicle technology were cited. Now, the focus is on lower sulphur fuels¹³ and increasing use of biofuels¹⁴. In all this flux, the one constant is housekeeping. Practices within the diesel supply and distribution systems remained the same with no response to these changing conditions, but perhaps the advent of biofuels will provide the catalyst for change in operational practice. Increasing the frequency of water drainage is a consideration.

Biofuels

Biofuels (see also Chapters 26 and 27) have been in use since the inception of the internal combustion engine. Nikolaus Otto ran his first engine on ethanol, and peanut oil was the fuel of choice for Rudolf Diesel, inventor of the first diesel engine. However, the low cost of crude throughout most of the twentieth century guaranteed their limited demand. This demand changed in response to concerns around the effects of greenhouse gas emissions from burning fossil fuels on global warming. The European Union signed the Kyoto protocol in 1997, agreeing to reduce EU

annual greenhouse gas emissions by 8% from 1990 to 2010 (Dell and Rand, 2004). This has ensured that biofuels will continue to play an increasing role in the global transport fuel mix.

Biofuels are produced from recently living organisms (usually plants) and their byproducts. The most widespread is ethanol, made from fermentation by yeast of crops like sugar cane, corn or wheat and subsequent distillation. It has emerged as a renewable component for gasoline, with Brazil and the USA among the first countries to extensively exploit it as an energy source. Brazil introduced hydrated ethanol/gasoline blends in 1979 and now uniquely uses 100% hydrated ethanol (~6% water) as an automotive fuel. However, the most common blends are E5 to E24 (5–24% ethanol, 95–76% gasoline) and E85 (85% ethanol, 15% gasoline).

Biofuel made up from oils extracted from plants like rape, palm or soya is also in common use. Produced by a process known as transesterification, these oils are reacted with an alcohol (e.g. methanol), in the presence of an alkaline catalyst (e.g. caustic soda), to convert the oil into biodiesel¹⁵ also known as fatty acid methyl ester (FAME), which is then separated from the reaction mixture and purified. FAMEs are broadly compatible with AGO, but display different properties dependent on the parent oil source. They fall into two main types: saturated (no double bonds) and unsaturated (with double bonds). FAME in its pure form is often referred to by the abbreviation B100. For blends with AGO, the general term BX is used, where X refers to percentage of biodiesel in the blend. So, B5 is 5% biodiesel and 95% diesel.

Impact of Biodiesel on Microbial Activity

It has been postulated that biodiesel will be more susceptible to microbial spoilage than conventional hydrocarbon fuels as it is structurally much simpler (FAME is a simple straight carbon chain with two oxygens, whereas conventional hydrocarbon fuels are a complex mixture of aromatic, straight, branched and cyclic aliphatic and heterocyclic forms). Biodiesel is also hygroscopic, dissolving up to 20 times more water than conventional diesel (Price, 2008). However, in a recent literature review only six, and these being somewhat contradictory reports, were found pertaining to biodiesel and its blends (Price, 2008). The microbiological mechanisms underpinning the degradation of biodiesel remain poorly understood, but a recent study suggests that a more diverse group of microorganisms are involved (see Chapter 26). Field evidence is also mounting to support the view that biodiesel is more prone to microbial attack. This is illustrated in the case studies below.

Case Study 2

A European refinery experienced reduced flow rates and subsequent filter blockages in the B5 arms of the loading gantry. Blocked filters were also reported at retail sites. Field and laboratory microbiological testing of water bottom samples from

refinery and retail tanks confirmed microbiological infection. A biocide treatment programme was initiated and remedial action taken, which included

- Implementation of improved maintenance, training, housekeeping and treatment procedures.
- Increase in the frequency of checks for microbial contamination.

Case Study 3

Filter blocking caused by microbial infection of B5 was observed at a few unmanned commercial and retail sites in northern Europe (Fig. 19.6). Plate count testing confirmed microbiological infection together with FAME, rust and dirt on the filters. The entire FAME pool at the supplying depot was also found to be heavily contaminated. Remedial action included

- Shock treatment of affected systems with biocide.
- Implementation of improved housekeeping and a microbiological testing programme.



Fig. 19.6 Example contaminated suction line

Conclusion

Undoubtedly microbial growth in fuels has historically caused difficulties for fuel suppliers and looking to the future, problems will continue to occur as fuel quality aligns with environmental and vehicle technology requirements. Preventative rather than simply control strategies will become of principal importance, with

effective fuel handling (particularly control of water contamination) throughout a well-designed and maintained supply and distribution. This is the real key to controlling microbial growth.

Notes

1. Temperatures in excess of 70°C pasteurise fuels.
2. Gram stain is a technique for categorising types of bacteria into either Gram-negative or Gram-positive by their ability to absorb certain biological stains.
3. A compound produced by living organisms that helps solubilise compounds by reducing surface tension between the compound and liquid.
4. Water separation devices that are rendered useless by the presence of surfactant molecules in either the fuel or the water phase.
5. Microbes can be classified as *aerobic* if their metabolic processes require oxygen.
6. Attachment of high numbers of microbes to a surface.
7. A group of organisms that occupy the same habitat and interact with each other.
8. Microbes can be classified as *anaerobic* if their metabolic processes do not require oxygen.
9. Frequent and effective drainage of free water from tanks.
10. Active substances and preparations containing one or more active substances, in the form in which they are supplied to the user, which are intended to destroy, deter, render harmless, prevent the action of or otherwise exert a controlling effect on any harmful organism by chemical or biological means (as defined in the Biocidal Products Directive, 98/8/EC).
11. An organometallic compound once commonly used as an antiknock additive in gasoline. It is still used in aviation fuel for piston engine-powered aircraft.
12. Crude oil fractions with a boiling range of approximately 150–400°C.
13. The 2009 Euro 5 standard ensures that diesel sulphur levels are now 10 ppm.
14. Neat components derived from a source of biomass.
15. Neat bio-component that can be used in a diesel engine.

Acknowledgements The retail tank angle check valve and contaminated suction line images were supplied by Eurotank Environmental Ltd. All the other images are the author's own.

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Part IV

**How Specific Microbial Communities
Benefit the Oil Industry**

Chapter 20

How Specific Microbial Communities Benefit the Oil Industry

Biorefining and Bioprocessing for Upgrading Petroleum Oil

Ajay Singh

Introduction

Recent advances in molecular biology of microbes have made possible in exploring and engineering improved biocatalysts (microbes and enzymes) suitable for the oil biorefining and recovery processes (Monticello, 2000; Van Hamme et al., 2003; Kilbane, 2006). Crude oil contains about 0.05–5% sulphur, 0.5–2.1% nitrogen and heavy metals such as nickel and vanadium associated with the asphaltene fraction. High temperature- and pressure-requiring expensive hydrotreatment processes are generally used to remove sulphur and nitrogen compounds from petroleum. Biorefining processes to improve oil quality have gained lots of interest and made a significant progress in the last two decades (Le Borgne and Quintero, 2003) and is the focus of this chapter.

Biodesulphurisation

Most of the sulphur in crude oil is contained in condensed thiophenes. Thus, the removal of sulphur from crude oil requires costly and extreme conditions using processes such as hydrodesulphurisation (HDS). Microbial cultures known for desulphurisation activity include *Rhodococcus*, *Nocardia*, *Agrobacterium*, *Mycobacterium*, *Gordona*, *Klebsiella*, *Xanthomonas* and *Paenibacillus* that are capable of selective desulphurisation of organic sulphur (Table 20.1). The sequence of catabolism of dibenzothiophene (DBT) by *Rhodococcus* is mediated by two monooxygenases and a desulphinase and results in successive production of dibenzothiophene-5-oxide (DBTO), dibenzene-5,5-dioxide (DBTO₂), 2-(2-hydroxybiphenyl)-benzenesulphinate (HPBS) and 2-hydroxybiphenyl (HBP) with associated release of inorganic sulphur (Monticello, 2000; Gupta et al., 2005).

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Table 20.1 Potential biocatalysts and their role in biorefining and bioprocessing

Biorefining method	Microorganism/enzyme	Process
Biodesulphurisation	<i>Agrobacterium</i> MC501, <i>Arthrobacter</i> sp., <i>Corynebacterium</i> sp. SY1, <i>Gordona</i> CYKS1, <i>Nocardia</i> sp., <i>Rhodococcus erythropolis</i> H2, <i>Rhodococcus</i> sp. IGTS8	Biotransformation of organic sulphur compounds, selective removal of sulphur from crude oil or refined petroleum products
Biodenitrogenation	<i>Comamonas acidovorans</i> , <i>Nocardiooides</i> sp., <i>Pseudomonas aeruginosa</i> , <i>Pseudomonas ayucida</i> , <i>Rhodococcus</i> sp.	Biotransformation of organic nitrogen compounds in crude oil, nitrogen removal
Biodemettalation	<i>Bacillus megaterium</i> , <i>Caldariomyces fumago</i> , <i>Escherichia coli</i>	Enzymatic removal of Ni and V from crude oil using microbial enzymes chloroperoxidase, cytochrome C reductase and heme oxygenase
Biocatalysis	Microbial enzymes, cytochrome p450-dependent-monoxygenases, dioxygenases, lipoxygenases, peroxidases	Bioconversion of petroleum fractions and pure hydrocarbons to produce fine chemicals
Biotransformation	<i>Thiobacillus</i> , <i>Achromobacter</i> , <i>Pseudomonas</i> , <i>Sulphobolbus</i>	Biotransformation of heavy crude into light crude
Biodemulsification	<i>Acinetobacter calcoaceticus</i> <i>Bacillus subtilis</i> , <i>Corynebacterium petrophilum</i> , <i>Nocardia amarae</i> , <i>Pseudomonas aeruginosa</i> , <i>Rhodococcus globerulus</i>	De-emulsification of oil emulsions, oil solubilisation, viscosity reduction, wetting

Desulphurisation genes have been manipulated by gene shuffling and directed evolution to broaden substrate specificity, and improved biocatalysts have been engineered (Gray et al., 1996; Rambosek et al., 1999). Genetic manipulation to improve the *Rhodococcus erythropolis* IGTS8 strain leads to a 200-fold increase in DBT desulphurising activity allowing shorter residence times compatible with a commercial application (Pacheco et al., 1999). The manipulation consisted of increasing *dsz* genes copy number, eliminating the sulphate repression by promoter change and deleting the last gene of the metabolic pathway (*dszB*), to eliminate the slowest step of the Dsz pathway and create opportunities to use these biocatalysts for production of potentially valuable sulphur-containing metabolic intermediates as products (Monticello, 2000). The recombinant *Rhodococcus* sp. T09, constructed using a *Rhodococcus-Escherichia coli* shuttle vector utilised both DBT and benzothiophene (BT) as the sole sulphur source (Matsui et al., 2001).

Biodesulphurisation carried out in two-phase aqueous–alkane solvent systems exhibited increased sulphur removal rates as compared with aqueous systems (Monticello, 2000; Caro et al., 2008). The key techno-economic challenge to the

viability of biodesulphurisation processes is to establish a cost-effective means of implementing the two-phase bioreactor system and de-emulsification steps as well as the product recovery step (Kaufman et al., 1998; Pacheco et al., 1999; McFarland, 1999). Use of multiple-stage air-lift reactors can reduce mixing costs, and centrifugation approaches facilitated de-emulsification, desulphurised oil recovery and recycling of the cells (Ohshiro et al., 1996). Extent of biodesulphurisation varies dramatically with the nature of the oil feedstock range from 20 to 60%, 20 to 60%, 30 to 70%, 40 to 90%, 65 to 70% and 75 to 90% for crude oil, light gas oil, middle distillates, diesel, hydrotreated diesel and cracked stocks, respectively. Lower sulphur standards are being enforced in some jurisdictions and future values for diesel fuel are expected to be <10 ppm in Europe. Since conventional technologies cannot achieve the target cost effectively, a combination of biodesulphurisation and HDS technology has the potential to achieve the future goals.

Biodenitrogenation

Nitrogenous compounds in crude oil consist of pyrroles, indoles and carbazole. Carbazole is not only a toxic and mutagenic compound, but also a potent inhibitor of hydrodesulphurisation process, can denature petroleum-cracking catalysts and has deleterious environmental impacts by contributing to formation of air polluting NO_x. Some species of *Alcaligenes*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Comamonas*, *Mycobacterium*, *Pseudomonas*, *Serratia* and *Xanthomonas* can utilise indole, pyridine, quinoline and carbazole compounds (Table 20.1).

Pyrrole and indole are easily degradable, but carbazole is relatively resistant to microbial attack (Fetzner, 1998; Kilbane et al., 2000). Biofilm-immobilised *Burkholderia* sp. IMP5GC in a packed reactor has demonstrated good activity for the semi-continuous degradation of carbazole present in a mixture of gas oil and light cycle oil (Castorena et al., 2008). The genes responsible for carbazole degradation by *Pseudomonas* sp. CA10 have been identified and cloned to generate recombinant strains that were able to transform a wide range of aromatic compounds including carbazole, *N*-methylcarbazole, *N*-ethylcarbazole, dibenzofuran, dibenzothiophene, dibenzo-*p*-dioxin, fluorene, naphthalene, phenanthrene, anthracene, and fluoranthene (Sato et al., 1997). A potential pathway for the selective removal of nitrogen from carbazole is also shown, which could be created using metabolic engineering to combine the CarA enzyme from carbazole degraders such as *Sphingomonas* sp. GTIN11 with a suitable deaminase (Kilbane et al., 2002).

From a practical perspective denitrogenation and desulphurisation processes need to be integrated. An effective biodenitrogenation and biodesulphurisation process requires removal of sulphur and nitrogen through specific enzymatic attack of the C–N and C–S bonds, respectively, but without C–C bond attack, thereby preserving the fuel value of the residual products. *Gordonia* sp. strain F.5.25.8 was the first reported strain that has the ability to simultaneously metabolise DBT and carbazole (Santos et al., 2005).

Biodemettallation

Asphaltenes are high molecular weight compounds containing aromatic and aliphatic constituents, hetero-atoms (S, O and N) and heavy metals (Ni and V). Although microorganisms have been shown to associate with bitumen and asphaltenes, only some fractions are susceptible to enzymatic or microbial attack.

Some haeme proteins such as chloroperoxidase, cytochrome c peroxidase, cytochrome C reductase and lignin peroxidase from *Caldariomyces fumago*, *Bacillus megaterium*, *Catharanthus roseus* and *E. coli* can perform biocatalytic modifications of the asphaltene fraction to Ni and V from petroporphyrins and asphaltenes (Mogollon et al., 1998; Garcia-Arellano et al., 2004). The enzymatic treatment with chloroperoxidase has been demonstrated to remove up to 93% of Ni from nickel octaethylporphine, 53% of V from vanadyl octaethylporphine and 20% of the total Ni and V from the asphaltene fraction of heavy oil (Fedorak et al., 1993). While cytochrome C reductase and chloroperoxidase enzymes have potential applications in metal removal from petroleum, further investigations on the biochemical mechanisms and bioprocessing aspects are required for development of a commercially feasible biodemettallation process.

Biocatalysis for Novel Compounds

The unique regio- and stereo-specificity properties of enzymes and their capacities to catalyse reactions in non-aqueous media can be exploited in systems for biotransformation of petroleum compounds into novel high-value chemicals (Holland, 2000). Enantiospecific conversions of petrochemical substrates and their derivatives can be achieved by stereoselective biocatalytic hydroxylation reactions using cytochrome p450-dependent monooxygenases, dioxygenases, lipoxygenases and peroxidases (Kikuchi et al., 1999). A range of attractive diol precursors for chemical synthesis can be produced by naphthalene dioxygenase (NDO) which also catalyses a variety of other reactions including monohydroxylation, desaturation, O- and N-dealkylation and sulphoxidation (Resnick et al., 1996). These approaches may be extended to create powerful biocatalysts with applications for specific transformations for upgrading of petroleum fractions through applications of molecular/protein engineering methods in expanding substrate specificity, enhancing enzyme stability in non-aqueous media and manipulating reaction rates (Cherry, 2000). Cytochrome p450_{cam} monooxygenase from *Pseudomonas putida* has been successfully evolved to function more efficiently in the hydroxylation of naphthalene and dioxygenases with improved thermostability and substrate specificity (Furukawa, 2000).

Biotransformation of Heavy Crudes into Lighter Crudes

The progressive depletion of high-quality light crudes has led to investigate biochemical conversion of heavy crudes into lighter crudes utilising extremophilic bacterial species of *Thiobacillus*, *Achromobacter*, *Pseudomonas* and *Sulpholobus*

(Premuzic and Lin, 1999). The strategy used was to adapt these bacteria to resist high temperatures, pressures and salt and hydrocarbon concentrations, followed by introducing them into the crudes in aqueous solution and incubating at temperatures of 50–65°C. Generally, the treated crudes became lighter and contained about 24–40% less sulphur, nitrogen, oxygen and heavy metals. Although exact mechanism was not known, the microorganisms might have specifically directed their action to the hetero-atoms and organo-metallic structures involving oxidations and degradation of higher hydrocarbons and depolymerisation of asphaltenes allowing the liberation of smaller molecules. The increase in the concentration of saturated chains from C8 to C26 indicated the degradation of high molecular weight hydrocarbons, probably alkanes. More research is needed to elucidate the metabolic pathways responsible for these transformations at molecular level and to further improve the activity of these strains (Le Borgne and Quintero, 2003).

Biodemulsification

Oilfield emulsions, both oil-in-water and water-in-oil, are formed at various stages of exploration, production, oil recovery and processing and represent a major problem for the petroleum industry. Traditional de-emulsification methods to recover oil include centrifugation, heat treatment, electrical treatment and chemicals containing soap, fatty acids and long-chain alcohols. However, physico-chemical de-emulsification processes are capital intensive and emulsions often generated at the wellhead have to be transported to central processing facilities. A major disadvantage with the chemical de-emulsification method is the disposal of the chemical de-emulsifier in the aqueous phase and removal of the de-emulsifier from the oil phase.

Since biological processes can be carried out at non-extreme conditions, an effective microbial de-emulsification process could be used directly to treat emulsions at the wellhead, thus saving on transport and high capital equipment costs. Several microorganisms are known to possess de-emulsification properties (Table 20.1), e.g. *Nocardia amarae*, *Corynebacterium petrophilum*, *Rhodococcus aurantiacus*, *Bacillus subtilis*, *Micrococcus* sp., *Torulopsis bombicola*, *Acinetobacter calcoaceticus*, species of *Alteromonas*, *Rhodococcus*, *Aeromonas* and mixed bacterial cultures (Kosaric, 1996; Das, 2001; Nadarajah et al., 2002; Huang et al., 2009).

Microbes exploit the dual hydrophobic/hydrophilic nature of biosurfactants or hydrophobic cell surfaces to displace the emulsifiers that are present at the oil–water interface (Kosaric, 1996). Some biologically produced agents such as acetoin, polysaccharides, glycolipids, glycoproteins, phospholipids and rhamnolipids exhibit de-emulsification properties (Das, 2001). The ability of most of the bacterial cultures to break emulsions is not significantly affected by lyophilisation or freezing and thawing, but can be completely destroyed by autoclaving or alkaline methanolysis (Kosaric, 1996). Washing of cells with any lipid-solubilising solvent such as *n*-pentane, *n*-hexane, kerosene or chloroform–methanol decreases de-emulsification capability for water-in-oil (w/o) emulsions, whereas the de-emulsification rates for oil-in-water (o/w) emulsions increases with *n*-pentane- and kerosene-washed cells.

Due to variability in the properties of crude oil emulsions, inconsistencies are experienced in performance of the different de-emulsification processes. Further research on microbial de-emulsification processes with field emulsions needs to be aimed at development of more reliable and universally effective systems. Genetic manipulations on biodemulsifying organisms have not been attempted so far.

Conclusions

Recent advance in molecular techniques and protein engineering developments will undoubtedly result in creation of powerful biocatalysts with applications for upgrading of crude oil, petroleum fractions and specific transformations of pure hydrocarbon compounds. It is also expected that future practical biocatalysts with the capability of simultaneously removing sulphur, nitrogen and metals from petroleum could be developed.

Glossary

Biocatalysis The use of *enzymes* to perform chemical transformations on *organic compounds*.

Biodegradation The process whereby a *compound* is decomposed by natural biological activity.

Biodemettalation Use of microorganisms or enzymes to remove metals from petroleum products.

Biodemulsification Use of microorganisms or biologically produced chemicals for breaking of oil–water emulsions to separate oil, water and solids phases.

Biodenitrogenation Use of microorganisms or enzymes to remove nitrogen atoms from petroleum products.

Biodesulphurisation Use of microorganisms or enzymes to remove sulphur atoms from petroleum products.

Bioprocessing The use of *microbial, plant or animal cells* for the *production of chemical compounds*.

Biorefining A process of *purification* of natural *substances* (e.g. oil, cellulosic materials, metals) for more usable form using biological means.

Biosurfactant Surface-active substances synthesised by living cells, having the properties of reducing *surface tension*, stabilising *emulsions* and promoting foaming.

Biotransformation The chemical modification made by an organism on a chemical compound.

Genetic engineering Genetic engineering, *recombinant DNA* technology and genetic modification are terms that apply to the direct manipulation of structure and characteristics of *genes*.

Hydrodesulphurisation A catalytic chemical process widely used to remove sulphur (S) from petroleum products.

Protein engineering Protein engineering is the process of developing useful or valuable *proteins* using either detailed knowledge of the structure and function of the protein or random mutagenesis and DNA shuffling.

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Chapter 21

How Specific Microbial Communities Benefit the Oil Industry

Microbial-Enhanced Oil Recovery (MEOR)

Svetlana Rudyk and Erik Søgaard

Introduction

Microbial-enhanced oil recovery (MEOR) involves injecting into the oil-saturated layer microbes and/nutrients to create the *in situ* production of metabolic products or nutrients which stimulate indigenous microbes. The purposes of MEOR are to increase oil production, decrease the water cut and prolong the productive life of the oilfield. The most probable targets of MEOR are reservoirs that have reached the limits of oil production by injection of water to displace oil (Donaldson and Obeida, 1991). MEOR is the cheapest approach of oil recovery after water flooding. MEOR investigations have been conducted all over the world and resulted in many successful field applications in the USA, UK, China, Russia, Malaysia, Germany, Romania, Poland and others.

The spectrum of MEOR applications is broad (Jenneman, 1989). They can be applied to viscous oil to reduce oil viscosity and for paraffin removal from well tubing; for oil recovery enhancement by microbes producing gases pressurising oil reservoir and other products of metabolism; to depleted fields by injecting biopolymer-generating bacteria and nutrients to block high-permeability beds; to sour fields to reduce sulphur corrosion. Two main techniques are applied when injecting microorganisms and nutrients: field flooding and single-well treatment (huff-and-puff technique). Field flooding implies water injection together with bacteria and nutrients according to the scheme of injection that was created from the observations of the microbial behaviour during laboratory experiments.

Another more effective procedure may be when the bacteria and nutrients are injected in the single well; the well would stay shut-in from several weeks to several months. This would allow better interaction between metabolic products and oil and dispersion of the bacteria deep into the reservoir. The longer time of shut-in period will let bacteria create colonies and penetrate into the distant parts of the oil layer.

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Selection of Microbes for MEOR Purposes

It was previously believed that petroleum reservoirs were biologically sterile but this idea was negated when oil producers met the problem of corrosion caused by microbes (biocorrosion). The theory was that the microbes were introduced during water injection and other field operations. Detailed studies showed that petroleum reservoirs are populated by different coexisting bacteria (Rosnes et al., 1991). The suggestion that bacteria could be used for oil recovery was made by Beckmann (1926) and ZoBell (1947). The choice of MEOR candidates can be made between bacteria isolated from oil reservoirs (keeping in mind that they are already tolerant to their reservoir conditions like temperature, pressure and salinity). However, this could take a long time period before noticeable effects could be seen. Another option is to inject exterior bacteria which have been previously selected and grown in the laboratory. However, caution must be taken in this approach as the metabolic abilities of the injected microbes may be seriously restricted by reservoir stress conditions.

Depending on the purpose of the MEOR treatment, microorganisms producing different by-products can be analysed (Hitzman, 1991). For example, gases such as CO₂, H₂ and CH₄ produced by specific microbes would serve as a mechanism for increasing reservoir pressures. Gases which are miscible with oil will reduce oil viscosity. The alcohols will act as a solvent to cause oil swelling and also reduce oil viscosity. The acids would dissolve carbonates creating higher permeability. The biosurfactants reduce interfacial tension. The biopolymers improve sweep efficiency by increasing displacing fluid viscosity and adsorption on the rock. Gels are also used for plugging channels swept after water injection to redirect water flows and recover hydrocarbons from unswept areas.

Sub-surface Environment

To be viable and actively growing, microbes need specific optimal conditions. Going out of the limits of those conditions can be extreme and crucial for most of the bacteria. To imagine the conditions where MEOR microbes are supposed to live and proliferate needs better and deeper studying of the entire reservoir environment.

Salinity: General concentrations of oilfield produced by brines range from less than 100 mg/l to more than 300 g/l and can widely differ in the range of the same formation or geological area. Compared to the composition of modern seawater, the sub-surface waters have undergone considerable change. Some waters were diluted by meteoric water, the composition of others were changed by the fluids expelled from shales, or by solution of ions from rocks (Donaldson et al., 1989). When microbes are exposed to high salinities, they shrink releasing water into the surrounding space. To overcome this osmotic stress, microorganisms use different mechanisms. Many microorganisms respond to an increase in osmolarity by accumulating osmotica in their cytosol, which protects them from cytoplasmic

dehydration and desiccation (Csonka, 1989). Compatible solutes such as K⁺, glutamate, proline, glycine, betaine, sucrose and trehalose accumulate away from proteins, forcing water nearby and thus stabilising them, and possibly stabilising dry membranes (Marquis, 1991).

Pressure and temperature: Pressure and temperature increase with depth, and both affect microbial growth. There are specific ranges of temperatures where bacteria exist but the majority of them cannot tolerate temperatures higher than 37°C. Most microorganisms die at boiling temperature due to the conversion of liquid to vapour. The pressure increases the boiling point of water, so water at the bottom of the ocean remains liquid even at 400°C (Rothschild and Mancinelli, 2001). Marquis (1991) studied the effect of pressure on some microorganisms. Low pressure up to 13.8 MPa increases biological activity and even increases the optimal growth temperature by a few degrees. He also noted that at or near optimal temperature microorganisms show a great tolerance to high pressure (Marquis, 1991).

Rock matrix: The pores in rocks can be connected in different ways. In sandstone they are more like a system of channels; in carbonate rocks they can be poorly interconnected. Permeability of the fine sandstones is high – up to 1,000 mD; and it is very low for chalks – 1–20 mD. Permeability (ability of rock to transport fluid) is controlled by the geometrical structure of the rock and also by the distribution of the clay minerals. Pore size less than 0.5 nm will place severe restrictions on the ability of most of the bacteria to be transferred through a rock matrix especially for those types whose size is comparable with the size of pores or channels. Zvyagintsev (1970) working with some microbes has shown that placement in large capillaries (400 × 150 nm) increased the number of cells 7–10 times. But in small capillaries (5 × 3 nm) not only was an increase of cells observed but the size of the cells was reduced.

Nutrients: Any MEOR process will require the availability of essential nutrients in order for growth and metabolism to occur. The compatibility of the nutrients in substrates with brine and clays must also be studied to avoid plugging, swelling and transportation problems. Fermentative bacteria use glucose-, sucrose- or lactose-containing nutrients. The use of molasses as a substrate was proposed by Updegraff and Wren in 1953. Since then, molasses were used in several well tests as economic and effective nutrients for many types of bacteria. It is now generally believed that microorganisms cannot use petroleum components as food material under anaerobic conditions, at least within a time frame required for economical recovery. Moses et al. (1983) reports that anaerobic growth with petroleum as the sole carbon source can occur, but growth was hardly detected for several months.

Case Study: Applying *Clostridium tyrobutyricum* in MEOR

The focus of this investigation was the chalk rocks of the North Sea offshore oil-fields. The conditions of the Danish oilfields can be characterised as harsh for MEOR. Salinity increases up to 300 g/l from South to North due to the close location

to the salt domes (Kjetil et al., 1988). The porosity is up to 45% but the permeability is very low, less than 20 mD. The original temperature varies from field to field from 70 to $>100^{\circ}\text{C}$ but due to the injection of cool seawater the temperature of the oil layers decreased. The majority of MEOR well treatments in the world have been performed in sandstones. However, the carbonate rocks seem to be more promising for MEOR applications, as the injected microbes readily spread through fissures and fractures over a larger area; thus permeability can increase as the result of microbial rock dissolution.

Adaptation to Higher Salinities

Clostridia tyrobutiricum, which is a known gas and acid producer, was chosen for this study. Wagner (1991) successfully used these *C. tyrobutiricum* for the treatment of a field in Germany. He reported that the brines of the oil-saturated dolomites had a very high salt concentration; even the fissures and fractures were partially filled with salt. In the North Sea fields salinity depends on the distance from the salt domes. Salinity of the oilfields from the Danish North Sea sector (chosen to be probable candidates for MEOR treatment investigation) was 60 and 70 g/l.

To check the limit of salt for the chosen type of bacteria, two strains were obtained from the German Culture Collection (DMSZ). Using Hugh and Leifson technique, the test tubes were prepared containing substrate with different salinities from 5 to 70 g/l. After the bacteria were injected in the test tubes, they were sealed by paraffin to secure anaerobic conditions and incubated at 37°C . The gas production in the test tubes was detected by the movement of the paraffin plug. It was found that one strain was able to produce gas at a salinity of 30 g/l but a second strain was also able to produce gas at a salinity of 50 g/l and was selected for further analysis. No gas production was detected at higher salinities with either strain.

The experiment was repeated with the chosen strain in the flasks containing 400 ml of water and NaCl with final salinities of 40, 50, 60 and 70 g/l. Two additional flasks were prepared with 400 ml of seawater each. Seawater was used to represent injection water used. In all the bottles, 20 ml of molasses was also added as well as 5 ml of the culture with the exception of one flask which just had the seawater. The seawater had a salinity of 28 g/l and was highly populated with microbes. The idea not to inoculate the seawater with the strain was to check how much gas can be produced by adding molasses to the indigenous seawater communities. The experiment was conducted over 8 days. Figure 21.1 shows that more than 600 ml of gas was produced in the flasks with salinities 40 and 50 g/l which could be expected from the results of test tubes. Just 60 ml of gas was produced at the salinity 60 g/l during the first 3 days, and no gas was produced at the salinity 70 g/l. Seawater (+ molasses; – inoculum) produced 110 ml of gas, while seawater with *C. tyrobutiricum* and molasses added produced the highest amount of gas (860 ml). These results show that *C. tyrobutiricum* are able to produce large amounts of gas in seawater, as well as at 40 and 50 g/l but very little at 60 g/l.

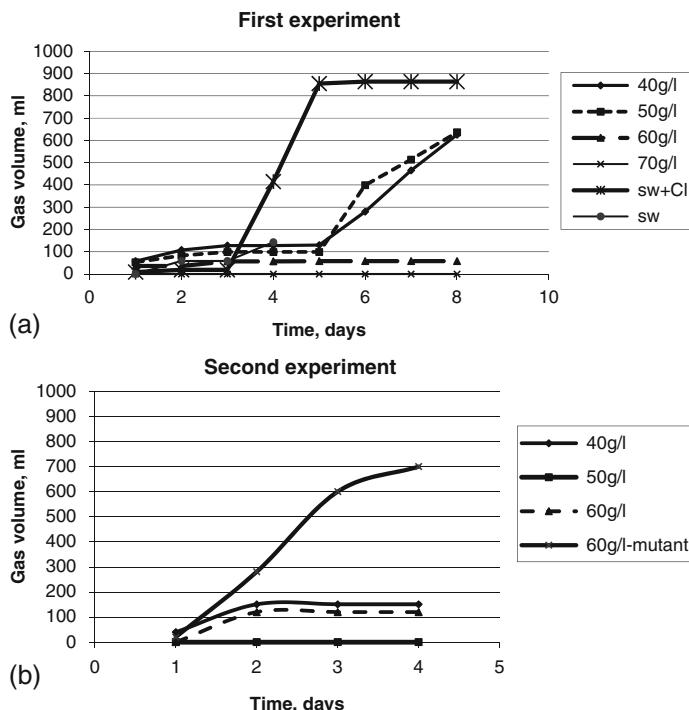


Fig. 21.1 Gas production at various salinities: 40, 50, 60 g/l – NaCl concentration; (a) first experiment: sw+Cl – *C. tyrobutyricum* in seawater; sw – seawater only; (b) second experiment: 60 g/l +mutant – bacteria taken from the flask with salinity of 60 g/l from the first experiment put again in the flask with salinity 60 g/l

An attempt was made to adapt the culture to higher salinities using four flasks containing 400 ml of water at final salinities of 40 g/l and 50 g/l and two flasks at 60 g/l. Molasses were also added; 5 ml of bacteria (from the flask with salinity 60 g/l from the first experiment) was inoculated into a new flask with a salinity of 60 g/l (Fig. 21.2). The bacteria, which earlier underwent experiments with salinity adaptation, are referred to as the ‘mutant’ to differentiate them from the original pure culture. The pure culture was inoculated into flasks with salinities of 40, 50 and 60 g/l as in the first experiment to check results of the first experiment and find out if any gas would be produced by mutant. The resulting graphs in Fig. 21.2 show that the pure culture at the salinity of 40 g/l produced just 160 ml of gas, while no gas production occurred at 50 g/l (more than was produced in previous experiment at 60 g/l). The adapted culture (mutant) produced the highest amount of gas (700 ml) at the salinity of 60 g/l. In the first experiment only 58 ml of gas was produced at this salinity. From this experiment, it is clear that the adaptation of *C. tyrobutyricum* strain to higher salinities is possible. The principle of the next adaptation experiments was to take cultures from flasks of high salinity (showing

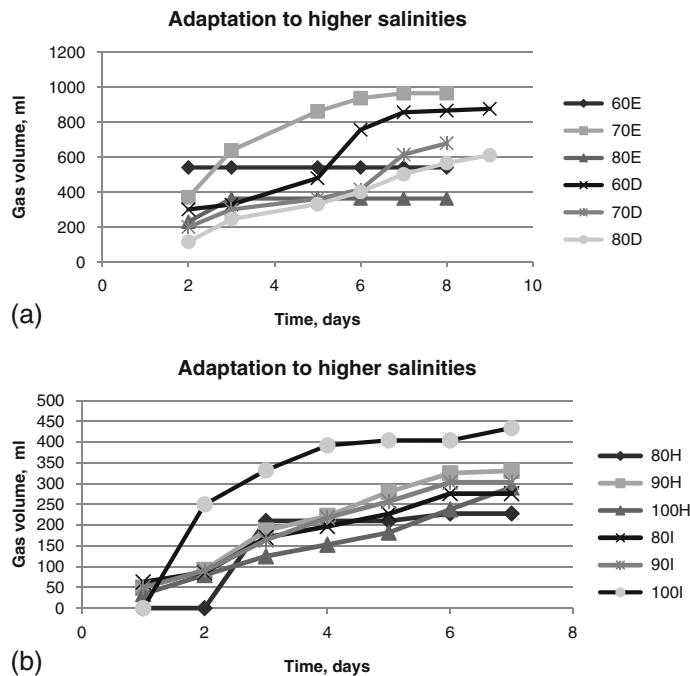


Fig. 21.2 The amount of gas produced by mutant during adaptation experiments: (a) at the salinities of 60, 70 and 80 g/l by mutants taken from the flask of salinity 60 g/l with mutant of the previous experiment (Fig. 21.1b) repeated in duplicate E and D; (b) at the salinities 80, 90 and 100 g/l (mutant H was taken from the flask 80D; mutant I from the flask 70E)

the highest amount of gas production) and sub-culture them into the same or higher salinities to check the reproducibility of this experiment.

Through a series of repeated adaptation experiments (Fig. 21.2), it can be seen that at 100 g/l salinity the mutants produced higher volumes of gas. The number of days of active gas production increased significantly. For example, 80D and 100H mutants produced some volume of gas every day. It must be noted that the adaptation process had several problems and several trials failed. For the adaptation to be more effective, the process should be continuous.

The alteration of ionic composition was also measured during 11 days of fermentation to determine which ions were important in adaptation to salinity. Our measurements showed that correlation exists between K⁺ and salinity (Fig. 21.3). The concentration of Na⁺ as a function of time is the highest at the salinity of 70 g/l and the lowest for 40 g/l because it is controlled by the concentration of NaCl in the solute. But the change of K⁺ concentration is controlled solely by microbes. On the last fermentation day the highest concentration of K⁺ was at salinity 70 g/l and the lowest at 40 g/l with intermediate salinities in between (Fig. 21.3b). That is

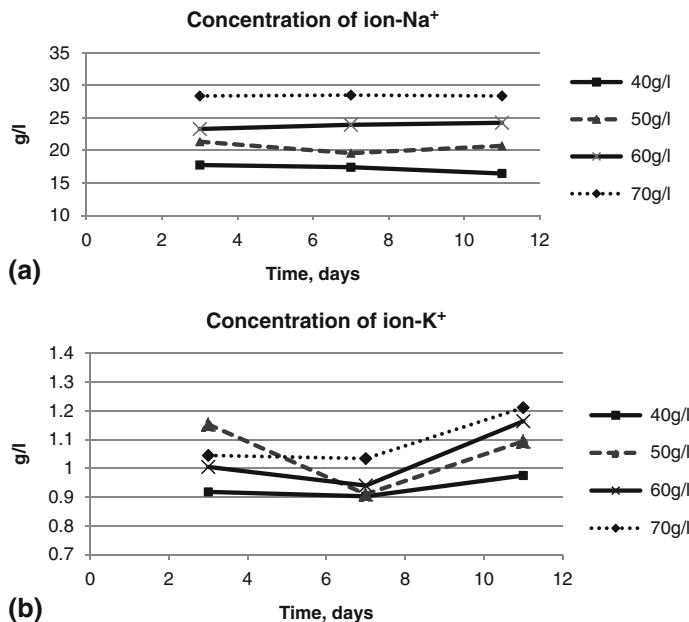


Fig. 21.3 Alteration of ion concentration during 11 days of fermentation at various salinities: (a) ions of Na^+ ; (b) ions of K^+

probably due to the work of NaK pumps running accumulation and release of Na^+ and K^+ ions inside and outside the cells.

Acid Production and Change of Permeability

Besides the gases (CO_2 and H_2), *C. tyrobutyricum* also produces butyric and acetic acids. The samples for measurements of the acid amounts were done at 40 g/l salinity from an 8 l fermentor with 400 ml of microbial solution added and 40 ml of molasses. Figure 21.4 shows that the volume of butyric acid production is much higher than that of acetic acid but the acetic acid contributes more to total acidity. The pH changed during the experiment from 5.4 to 8.2. When the pH of solution containing *C. tyrobutyricum* falls below 5, they start producing ethanol which inhibits further growth.

An experiment was also conducted to study if the acids produced during microbial growth could dissolve carbonates and hence change permeability. Four samples of tight chalk were added to a 5 l microbial culture and incubated for 2 weeks. Permeability was measured before and after the experiment. A large hole appeared as a result of the rock dissolution channelling inside one of the samples (Fig. 21.5). Permeabilities of the other three samples improved at 16% in average.

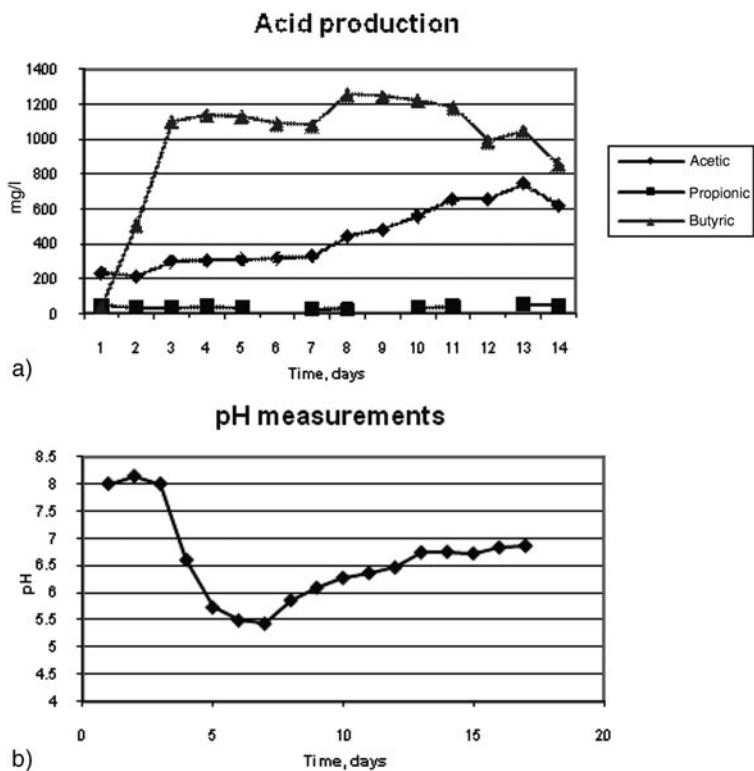


Fig. 21.4 Acid production by *C. tyrobutyricum*: (a) volumes of organic acids produced; (b) pH measurements

Fig. 21.5 Chalk sample after 14 days of exposure to microbial solution



Conclusions

The experiments on adaptation of cultures to higher salinity showed that growth limits at higher salinities can be significantly increased and produce sufficient volumes of gas. The mutant has better gas production abilities and salt resistance due to higher salt tolerance acquired during a series of experiments. Correlation was

observed between salinity and concentration of K⁺ ions during the process of adaptation to salinity. Metabolically produced acids can dissolve carbonates, improve permeability and enlarge porous space. If the problem of high temperature in reservoirs can be circumvented for *C. tyrobutiricum*, then this may be a good candidate for MEOR in chalk rocks.

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Chapter 22

How Specific Microbial Communities Benefit the Oil Industry

Anaerobic Microbial Processes and the Prospect for Methane Production from Oil

Lisa Gieg

Introduction

In strict anaerobic environments, oxygen is essentially non-existent. However, anaerobic microorganisms may thrive in such environments by metabolising organic or inorganic energy and/or carbon sources while respiring alternate electron acceptors such as nitrate, metals, or sulphate. Methanogenesis is the key electron accepting process in environments characterised by the absence of any electron acceptors other than CO₂. Geological evidence has shown that most of the Earth's petroleum resources have been biodegraded over millennia, the extents to which likely depended on nutrient and water availability, temperature, and the requisite microorganisms (Röling et al., 2003; Head et al., 2003; Hallmann et al., 2008). Gases of biological origin including methane are believed to be primary byproducts of microbial oil metabolism in petroliferous deposits where oil quality has diminished due to the preferential consumption of valuable 'light' hydrocarbons (Head et al., 2003; Milkov and Dzou, 2007; Jones et al., 2008). While this phenomenon has enormous economic implications for recovering high-value light oil, it also sets the precedent for a potential alternate energy recovery strategy – that is, recovering energy as methane gas that is biologically produced as the result of methanogenic oil biodegradation in petroleum reservoirs that are at their economic limits.

The idea of generating methane via crude oil decomposition by microorganisms in petroleum reservoirs was initially proposed in 1950 by Kuznetsov. Muller (1957) subsequently offered the first evidence that this metabolism exists in nature by showing that alkanes in crude oils could be microbially fermented to methane by a number of different inocula in controlled laboratory studies. Three decades later, Jack and colleagues (1985) examined the potential for the biomethanation of residual oil in marginal reservoirs as a potential energy recovery strategy and showed that numerous inocula could indeed bioconvert paraffinic oils to methane

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gas thus confirming Muller's results. The prospect of generating usable energy from marginal petroleum reservoirs by microbial conversion of oil to methane (natural gas) has received renewed attention in the past decade (Parkes, 1999; Suflita et al., 2004; Jones et al., 2008; Gieg et al., 2008) with the recognition that global petroleum supplies are diminishing while the demand for fossil fuel-based energy is increasing with world population growth. Future energy demands will no doubt be met via a suite of alternative and fossil fuel-based resources that can feasibly include an oil-to-methane bioconversion strategy in marginal petroleum reservoirs.

Methanogenic Hydrocarbon Metabolism

Methanogens are a specialised group of microorganisms that use only a few simple compounds such as acetate, formate, or H₂/CO₂ to form methane. Thus, hydrocarbon conversion to methane requires the involvement of other species that can enzymatically activate a hydrocarbon and metabolise it to methanogenic substrates. In such interspecies interaction, termed syntropy, the metabolism of the parent substrate by one or more species is thermodynamically unfavourable unless the produced products are kept at low concentrations by other species (McInerney, 1999). For example, the metabolism of a simple hydrocarbon such as toluene to the methanogenic substrates acetate and H₂ in the absence of electron acceptors is thermodynamically unfavourable based on a positive Gibbs free energy of the reaction (a), but when coupled with acetate- and H₂-consuming methanogenic reactions (b, c) the overall reaction becomes energetically feasible (d) ($\Delta G^\circ'$ calculated from values given in Thauer et al., 1977):

- (a) C₇H₈ + 7 H₂O → 3.5 CH₃COO⁻ + 3.5 H⁺ + 4 H₂ $\Delta G^\circ'$ (kJ/reaction) = +113.6
- (b) 3.5CH₃COO⁻ + 3.5 H₂O → 3.5 HCO₃⁻ + 3.5 CH₄ $\Delta G^\circ'$ (kJ/reaction) = -108.5
- (c) 4 H₂ + HCO₃⁻ + H⁺ → CH₄ + 3 H₂O $\Delta G^\circ'$ (kJ/reaction) = -135.6
- (d) C₇H₈ + 7.5 H₂O → 4.5 CH₄ + 2.5 HCO₃⁻ + 2.5 H⁺ $\Delta G^\circ'$ (kJ/reaction) = -130.5

Although it has been recognised since the 1920's that anaerobes are present in petroleum-related systems, the current understanding of how anaerobic microorganisms metabolise hydrocarbons for carbon and energy and thus may thrive in such systems has been garnered mainly through research efforts in the last two decades. This topic has been reviewed extensively (e.g. Widdel et al., 2006; Heider, 2007; Foght, 2008). Briefly, many compounds classified as monoaromatic hydrocarbons, alicyclic and aliphatic alkanes, alkenes, polycyclic aromatic hydrocarbons, and heterocyclic hydrocarbons can be metabolised under anaerobic conditions. Due to the lack of oxygen to serve as a co-reactant, anaerobes use a variety of unique enzymatic strategies to activate hydrocarbon bonds for metabolism that include fumarate addition, oxygen-independent hydroxylation, carboxylation, and methylation. Metabolites indicative of such reactions have been detected in a variety of petroleum-contaminated aquifers and thus may be useful indicators of in situ

anaerobic hydrocarbon metabolism (Beller, 2000; Gieg and Suflita, 2005). Signature anaerobic metabolites have also been detected in oil samples from biodegraded petroleum reservoirs (Aitken et al., 2004) and in production well fluids (Duncan et al., 2009) supporting the notion that anaerobic hydrocarbon metabolism occurs in such ecosystems.

The ongoing detection and identification of numerous and novel anaerobes, including methanogens, associated with petroleum reservoir fluids also supports this contention (e.g. Orphan et al., 2000; Magot, 2005; Nazina et al., 2006; Pham et al., 2009; Gray et al., 2009; Duncan et al., 2009). Most of the current knowledge on anaerobic hydrocarbon metabolism comes from studies with anaerobes incubated under nitrate- or sulphate-reducing conditions, while comparatively little is understood about how such metabolism proceeds in methanogenic environments. However, it is clear as indicated by the increasing number of reports on this topic that hydrocarbons can be metabolised under methanogenic conditions (Grbic'-Galic' and Vogel, 1987; Edwards and Grbic'-Galic', 1994; Zengler et al., 1999; Anderson and Lovley, 2000; Townsend et al., 2003; Siddique et al., 2006; Jones et al., 2008; Gieg et al., 2008).

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Chapter 23

How Specific Microbial Communities Benefit the Oil Industry

Case Study – Proof of Concept that Oil Entrained in Marginal Reservoirs Can Be Bioconverted to Methane Gas as a Green Energy Recovery Strategy

Lisa Gieg

Introduction

Conventional oil recovery techniques such as water flooding typically remove only up to 40% of the oil present in reservoirs. Enhanced oil recovery (EOR) techniques are considered tertiary strategies that may be applied to recover a greater volume of oil. In particular, the use of microorganisms to aid in oil production (microbial-enhanced oil recovery or MEOR) is considered a green energy recovery strategy since microbial processes do not require large amounts of energy input and can potentially produce large amounts of useful byproducts from inexpensive and renewable resources (Youssef et al., 2008). These byproducts can include the generation of biosurfactants, emulsifiers, acids, alcohols, and/or gases that can serve as agents for oil recovery. Recent reviews have summarised MEOR efforts undertaken since the 1950's with varying degrees of success (e.g. Jack, 1993; Belyaev et al., 2004; McInerney et al., 2005; Youssef et al., 2008). In MEOR schemes, petroleum reservoirs may be either stimulated with nutrients or inoculated with microorganisms with known activity to achieve desired effects (Youssef et al., 2008).

The idea of generating methane via crude oil decomposition by microorganisms in petroleum reservoirs was initially proposed in 1950 by Kuznetsov. In studies aimed at investigating the biomethanation of residual oil in marginal reservoirs as an MEOR strategy, Muller (1957) and Jack et al. (1985) offered initial evidence that this metabolism occurs in nature by showing that alkanes in crude oils could be microbially fermented to methane by a number of different inocula in controlled laboratory studies. More recent studies examining methanogenic hydrocarbon metabolism have confirmed this earlier work by providing more rigorous oil-to-methane mass balances and descriptions of the microbes involved

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(Zengler et al., 1999; Anderson and Lovley, 2000; Townsend et al., 2003; Siddique et al., 2006; Jones et al., 2008; Gieg et al., 2008). Geological evidence has shown that most of the Earth's petroleum resources have been biodegraded over millennia, mainly under methanogenic conditions (Röling et al., 2003; Head et al., 2003; Milkov and Dzou, 2007; Jones et al., 2008; Hallmann et al., 2008). The identification of diverse anaerobes, including methanogens, associated with petroleum reservoir fluids (e.g. Orphan et al., 2000; Nazina et al., 2006; Magot, 2005; Pham et al., 2009; Gray et al. 2009; Duncan et al., 2009) and metabolic evidence for anaerobic hydrocarbon metabolism (Aitken et al., 2004; Duncan et al., 2009) supports this contention. These observations coupled with the recognition that global petroleum supplies are diminishing while the demand for fossil fuel-based energy is increasing with world population growth have prompted renewed attention to the prospect of generating usable energy from marginal petroleum reservoirs by microbial conversion of oil to natural gas as an MEOR technology (Parkes, 1999; Suflita et al., 2004; Jones et al., 2008; Gieg et al., 2008).

In this case study (based on work reported by Suflita et al., 2004 and Gieg et al., 2008), the prospect that oil entrained in marginal reservoirs could be bioconverted into methane gas following inoculation with a hydrocarbon-degrading methanogenic consortium was investigated. The study consisted of a series of laboratory experiments utilising residual-oil bearing sandstone core material retrieved from a marginal oilfield that was inoculated with a methanogenic hydrocarbon-degrading culture. Techniques of anaerobic microbial cultivation were used to establish laboratory incubations. Methane concentrations were routinely monitored by gas chromatography (GC) in inoculated incubations relative to uninoculated controls to indicate the formation of methane as a result of residual oil biodegradation. Organic extractions of core-containing incubations followed by GC-MS (GC coupled with mass spectrometry) analysis were performed to quantify the consumption of oil components in methane-producing cultures. Tools of molecular biology (including DNA extraction, PCR amplification of the 16S rRNA gene, and sequencing) were used to identify members of the anaerobic inoculum capable of biodegrading oil to methane.

Experiments and Key Findings

The anaerobic consortium used to study methanogenic residual oil biodegradation originated from sediments collected from a gas condensate-contaminated aquifer undergoing anaerobic intrinsic hydrocarbon bioremediation (Gieg et al., 1999). The sediments were incubated with crude oil in the absence of added electron acceptors, and methane was monitored over time. Large amounts of methane were produced relative to sterilised controls over the course of the year. Hydrocarbon analysis showed significant depletion of alkanes and some PAHs relative to controls, convincingly demonstrating that crude oil components could be metabolised under methanogenic conditions by the aquifer population (Townsend et al., 2003).

To test the potential for this enrichment culture to metabolise oil entrained in marginal reservoirs, a sandstone core containing residual oil (designated Nowata

core) was obtained from a depth of approximately 200 m from a mature oilfield undergoing water flooding in Oklahoma, USA. The core sample had a residual oil saturation of approximately 30–40%, containing 0.013 g of oil per gram of core. Core material was crushed aseptically under anaerobic conditions, mixed with a mineral salts medium (containing nitrogen, phosphorous, trace elements, vitamins), and inoculated with the methanogenic culture. Relative to uninoculated controls, the inoculated core material produced approximately 2 mmol methane over the course of a year (Sufliita et al., 2004). The methanogenic consortium could be readily transferred to vessels containing freshly crushed residual oil-bearing sandstone core material (Gieg et al., 2008) with methanogenesis proceeding without lag. Methane concentrations were routinely monitored relative to uninoculated controls (incubations containing crushed core in medium only) in all transferred cultures. Methane production rates from the oil-bearing core material ranged from 0.15 to 0.40 $\mu\text{mol CH}_4/\text{day/g core}$ ($= 11\text{--}31 \mu\text{mol CH}_4/\text{day/g oil}$) with up to 3 $\mu\text{mol CH}_4$ produced per gram of residual oil. When pure crude oil from the Nowata formation was supplied to inoculum (e.g. in the absence of core material), methane production was significantly diminished, suggesting that core material surface was vital for the inoculum to proliferate. The grain size of the core material did not appear to matter since no significant differences in methane production were observed when the inoculum was incubated with the core material crushed to grain sizes ranging from $< 149 \mu\text{m}$ to $> 1.18 \text{ mm}$.

Throughout the experiments, no methane was produced when core material was not inoculated with the methanogenic oil-degrading culture. Further, only low levels of methane were formed when the inoculum was incubated in the absence of core material, suggesting that hydrocarbon decomposition within the core material was driving methane production. A time course experiment showed the progressive depletion of alkanes in the residual oil over 4 months (118 days) as quantified following organic extraction and GC–MS analysis (Gieg et al., 2008). The *n*-alkane-to-internal standard ratio of alkanes ranging from C₁₂ to C₂₉ decreased from 10.1 to 3.4 showing that approximately 2/3 of the *n*-alkane fraction of the residual oil in the sandstone was consumed, while the amount of methane concomitantly increased over time (Table 23.1). No significant changes in the uninoculated core samples were detected. With further incubation (up to 180 days), methane levels continued to increase even though the *n*-alkane-to-internal standard ratio did not decrease substantially, suggesting that oil fractions other than alkanes were also biotransformed to methane.

Experiments conducted with the Nowata core thus clearly demonstrated that microbial hydrocarbon consumption could lead to methane production if inoculated with the requisite consortium. However, in considering an MEOR strategy that would involve adding microbes to oil wells to recover energy as methane gas, it is necessary to determine how widespread this activity would be. Thus, other core samples obtained from a variety of residual oil bearing fields in Oklahoma were also used to challenge the inoculum for its ability to convert entrained hydrocarbons to methane gas (Gieg et al., 2008). Although the hydrocarbon composition was not monitored in these other core samples, significantly enhanced levels of methane

Table 23.1 The *n*-alkane-to-internal standard (IS, C₂₄D₅₀) peak area ratios and methane amounts measured during a time course experiment examining the loss of crude oil components in residual oil-bearing core material under methanogenic conditions in the presence and absence of an inoculum

Inoculated core		Uninoculated core	
Time (days)	<i>n</i> -alkanes/IS	CH ₄ (μmol)	<i>n</i> -alkane/IS
0	10.1	0.69	7.9
34	10.7	26.3	0
66	8.1	91.0	7.2
90	6.5	130.6	0
118	3.4	206.6	8.0
180	2.7	353.1	9.3

were produced when the core samples were inoculated relative to uninoculated controls in four of the five samples. Other experiments showed that the inoculum was able to produce significant levels of methane from residual oil-bearing core material containing up to 3% salt suggesting its potential usefulness in brackish reservoirs. Further, sulphate concentrations up to 10 mM did not impede methanogenesis (Gieg et al., 2008). This latter observation is important when considering an MEOR scheme via inoculation into fields containing sulphate as microbial production of sulphide would lead to oilfield souring.

Using 16S rRNA gene sequencing to identify the microbes in the inoculum, the predominant Eubacterial sequences affiliated most closely with genera of the sulphate-reducing bacteria *Desulfobulbus* (99% match to closest cultured species), *Desulfovorusinus* (96% match to closest cultivated species), and *Desulfovibrio* (94–97% sequence similarity with uncultured clones and 94% similarity with closest cultured species) and with the syntrophic bacteria *Desulfotomaculum* subcluster Ih (95% sequence similarity to *Cryptanaerobacter phenolicus*) and *Smithella* (92% sequence similarity with uncultured clones and with *S. propionica*). In addition, numerous clones affiliated closely with the fermentative bacteria *Chloroflexi* (sub-phylum I), *Clostridiales*, and *Bacteroidales* (Gieg et al., 2008). Archaeal sequences related to obligate acetate-using methanogens (98–100% sequence similarity to *Methanosaeta* sp.) were predominant in the consortium. However, some H₂-using methanogens (*Methanospirillum* and *Methanoculleus* sp., 92% sequence similarity to uncultured clones and cultivated species) were also detected following cultivation of the consortium on H₂/CO₂ and amplification with primers specific for the *mcrA* gene. This gene encodes methyl coenzyme M reductase that functions in the final step of the methane production pathway of methanogens. The species identified via sequencing efforts were not unexpected, as similar kinds of species have been noted in other alkane- or crude oil-utilising methanogenic cultures (Zengler et al., 1999; Jones et al., 2008). The sulphate-reducing bacteria, syntrophic bacteria, and fermentative bacteria identified are presumably involved in activating and metabolising the crude oil components to methanogenic precursors (such as acetate and H₂), whereas the methanogens consume these products to produce methane. The concerted action of such a mixture of species is required to make crude oil metabolism to methane

an energetically favourable process. Determining the metabolic roles played by the various community members will be necessary in order to use an inoculum such as this in methane recovery efforts from marginal domestic reservoirs.

Conclusions

This laboratory-based case study demonstrates the ‘proof of concept’ that crude oil entrained in mature reservoir samples can be converted to methane gas by the addition of a hydrocarbon-degrading methanogenic consortium. Although the various residual oil-bearing core materials tested were provided with nutrients such as nitrogen and phosphorus salts, CO₂, trace elements, and vitamins, methane production was not observed unless the core samples received the hydrocarbon-utilising inoculum. The results showed that when considering an oil-to-methane energy recovery process in mature reservoirs, inoculation may be required. However, much future work is required to determine how to scale up the laboratory-based observations for pilot- or field-scale applications in marginal wells.

Nevertheless, the rate data collected from this work (Gieg et al., 2008) may be used to determine how much methane can potentially be recovered from marginal conventional reservoirs by using the known volume of proven oil reserves in the United States (~ 375 billion barrels, US DOE, 2006) as an example. Assuming that 1% of residual oil supplies (e.g. 3.75 billions barrels) may be amenable to biomethane formation, and taking into account the measured methanogenesis rates of 11–31 µmol CH₄/d/g residual oil and the Nowata formation oil density (0.79 g/ml), the production of 3–13 billion cubic feet of CH₄ per day or 1–5 trillion cubic feet of CH₄ per year can feasibly be generated through this technology. Although this calculation is an oversimplification, it demonstrates how an oil-to-methane bioconversion process can potentially recover economically valuable energy.

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Chapter 24

How Specific Microbial Communities Benefit the Oil Industry

Dynamics of *Alcanivorax* spp. in Oil-Contaminated Intertidal Beach Sediments Undergoing Bioremediation

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Wilfred F.M. Röling, and Ian M. Head

Introduction

The industrial revolution has led to significant increases in the consumption of petroleum hydrocarbons. Concomitant with this increase, hydrocarbon pollution has become a global problem resulting from emissions related to operational use, releases during production, pipeline failures and tanker spills. Importantly, in addition to these anthropogenic sources of hydrocarbon pollution, natural seeps alone account for about 50% of total petroleum hydrocarbon releases in the aquatic environment (National Research Council, 2003). The annual input from natural seeps would form a layer of hydrocarbons 20 molecules thick on the sea surface globally if it remained un-degraded (Prince, 2005). By contrast with natural seeps, many oil spills, e.g. *Sea Empress* (Milford Haven, UK), *Prestige* (Galicia, Spain), *EXXON Valdez* (Prince William Sound, Alaska, USA), released huge amounts of oil (thousands to hundreds of thousand tonnes; Table 24.1) in a locally confined area over a short period of time with a huge acute impact on the marine environment. These incidents have attracted the attention of both the general public and the scientific community due to their great impact on coastal ecosystems. Although many petroleum hydrocarbons are toxic, they are degraded by microbial consortia naturally present in marine ecosystems.

Microbes with capacity to catabolise the hydrocarbons in petroleum have been described comprehensively (Floodgate, 1995) and a recent review lists 79 bacterial genera that can use hydrocarbons as sole source of carbon and energy, along with 9 cyanobacterial genera, 103 fungal genera and 14 algal genera that are known to degrade or transform hydrocarbons (Prince, 2005). Many hydrocarbon-degrading bacteria are metabolically versatile and degrade a wide variety of

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Table 24.1 Some major oil spill accidents between 1972 and 2002

Ship name	Year of accident	Place of accident	Oil spill size (tonnes)
Sea Star	1972	Gulf of Oman	115,000
Jakob Maersk	1975	Oporto, Portugal	88,000
Urquiola	1976	La Coruna, Spain	100,000
Hawaiian Patriot	1977	300 nautical miles off Honolulu	95,000
Nova	1985	Gulf of Iran	70,000
Khark 5	1989	Atlantic coast of Morocco	80,000
EXXON Valdez	1989	Prince William Sound, Alaska, USA	37,000
Sea Empress	1996	Milford Haven, UK	72,000
Prestige	2002	Galicia, Spain	63,000

organic compounds in addition to petroleum hydrocarbons (e.g. *Pseudomonas* spp.). However, specialist hydrocarbonoclastic bacteria exist, which almost exclusively use petroleum hydrocarbons as a carbon and energy source. Such specialist hydrocarbon-degrading genera include *Alcanivorax* spp., *Oleispira* spp., *Oleiphilus* spp., *Thalassolituus* spp. and *Cycloclasticus* spp. Since petroleum is a complex mixture of organic compounds, including both hydrocarbon and non-hydrocarbon components, its efficient degradation involves the action of consortia comprising many different microorganisms (Atlas, 1981; Floodgate, 1995; Röling et al., 2002).

Input of crude oil into aquatic environments triggers growth of petroleum hydrocarbon-degrading bacteria with immediate changes in the microbial communities (Coulon et al., 2007). However, availability of nutrients such as nitrogen (N) and phosphorus (P) play a rate-limiting role in microbial hydrocarbon degradation (Atlas and Bartha, 1981; Lee and Merlin, 1999; Young et al., 2001). Efficient hydrocarbon degradation and, therefore, bioremediation of spilled oil is more effective if optimum nutrient concentrations for microbial activity are provided and if the role of particular organisms in oil degradation is known. The identity and roles of several oil-degrading bacteria, and the efficacy of different intervention strategies for bioremediation of crude oil have been determined (McKew et al., 2007a, b). Marine *Gammaproteobacteria* from the genus *Alcanivorax* are globally prominent aerobic hydrocarbon degraders (Yakimov et al., 1998; Harayama et al., 1999; Röling et al., 2002; Röling et al., 2004; Cappello et al., 2007a; Nakamura et al., 2007). These studies have demonstrated that *Alcanivorax* spp. are specialist saturated hydrocarbon degraders which are strongly selected following addition of inorganic N and P to spilled oil in marine environments (Syutsubo et al., 2001). However, there have been few attempts to relate the effect of nutrient-induced bio-stimulation of hydrocarbon-degrading bacteria to quantitative changes in particular hydrocarbon-degrading populations and removal of hydrocarbons under field conditions. Such information will be useful to inform bioremediation models for developing improved bioremediation strategies. Therefore, the purpose of this study was to quantify and follow the dynamics of alkane-degrading *Alcanivorax* spp. in oil-contaminated beach sediments undergoing bioremediation.

Case Study Results

The present study deals with the effect of inorganic nutrient treatments on the dynamics of alkane-degrading *Alcanivorax* spp. in oil-treated beach sediment. An experimental field site located at Stert Flat in Somerset, UK, was divided into four plots (Fig. 24.1). The plots were designated as UC (no oil control without inorganic nutrient), OC (oil-treated control without inorganic nutrient), SR (treated with oil and slow-release fertiliser), and LF (treated with oil and liquid fertiliser). In situ CO₂ evolution as a measure of microbial activity and hydrocarbon degradation was determined. Quantification of petroleum hydrocarbons in sediment samples was determined by solvent extraction and gas chromatographic analysis (Röling et al., 2004; Swannell et al., 1995).

Changes in bacterial communities were assessed on the basis of Denaturing Gradient Gel Electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene fragments, and the abundance of *Alcanivorax* spp. was determined by qPCR of 16S rRNA genes using primers specific for *Alcanivorax* spp. previously identified at the field site (Röling et al., 2004). In addition, a selection of samples from a laboratory microcosm study of crude oil bioremediation, which used sediments from



Plot Number	Block Number	Treatment
1	Block 1	Oil and Fertiliser (SR)
2		Oil Only (OC)
3		No Oil Control (UC)
4		Fertilised Control (LF)
5	Block 2	No Oil Control (UC)
6		Oil Only (OC)
7		Fertilised Control (LF)
8		Oil and Fertiliser (SR)
9	Block 3	Oil and Fertiliser (SR)
10		Fertilised Control (LF)
11		Oil Only (OC)
12		No Oil Control (UC)



Fig. 24.1 Location, photograph and experimental details of the field site used in this study. The position of the Stert Flats site within the UK is indicated on the map. The layout of the treatment blocks at the site is superimposed on the photograph showing the intertidal zone of the field site. The experimental treatments within each block are provided in the *inset table*. Most of the data presented refer to block 2

Stert Flats, were analysed by DGGE of bacterial 16S rRNA gene fragments and the presence of alkane hydroxylase genes (*alkB*) was determined using PCR.

Effects of Inorganic Nutrient Treatment on Oil Degradation

CO₂ evolution and changes in oil composition were used to assess microbial activity and oil degradation in the experimental plots. CO₂ production was measured regularly for the first 14 days and the average amount of CO₂ produced per day in the different treatments was calculated. CO₂ evolution was dependent on the nature of the inorganic nutrients applied. The highest average daily rate of carbon dioxide production was obtained in the SR fertiliser-treated plot ($112.13 \pm 77.35 \mu\text{mol/day}$). This was approximately six times the value for CO₂ production in the LF-treated plot ($18.93 \pm 10.02 \mu\text{mol/day}$; Fig. 24.2; $p = 0.002$). By contrast the average CO₂ evolution rates from the no oil control plot without fertiliser (UC) and oil treated control without fertiliser (OC) plots were 12.12 ± 5.01 and $12.52 \pm 5.87 \mu\text{mol/day}$, respectively, and were not significantly different ($p = 0.881$). This demonstrated the positive effects of nutrient-stimulated bioremediation on CO₂ evolution, with the SR fertiliser having the greatest effect.

One week after oil treatment and just prior to inorganic nutrient treatment (termed day 0) total alkane content per gram of sediments was 608 ± 128.6 , 585.58 ± 23 and $585.67 \pm 92.15 \mu\text{g}$ in the OC, LF and SR plots, respectively, and these values were not significantly different (Fig. 24.3; $p = 0.941$, one-way ANOVA). However, 18 days after fertiliser treatment the concentration of *n*-alkanes had decreased in all treatment plots and showed a significant reduction in fertilised plots compared to day 0 values (Fig. 24.3). The greatest amount of alkane removal was seen in the SR fertiliser-treated plot which showed a $61.8 \pm 33.87\%$ reduction relative to day 0 ($p = 0.044$). The LF-treated plot exhibited a $39.68 \pm 27.02\%$ ($p = 0.056$)

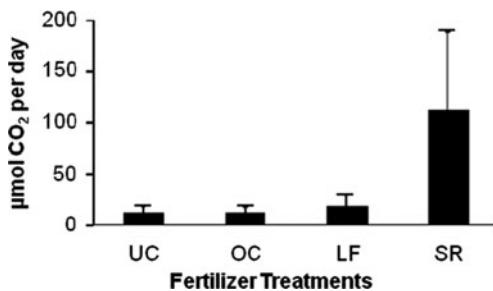


Fig. 24.2 Average daily carbon dioxide production in plots from a field trial of crude oil bioremediation. The data represent the daily CO₂ production rate averaged over the first 14 days of the experiment. UC – no oil control without inorganic nutrient, OC – oil-treated control without inorganic nutrient amendment, LF – oil-treated plots amended with liquid fertiliser, SR – oil-treated plots amended with slow-release fertiliser

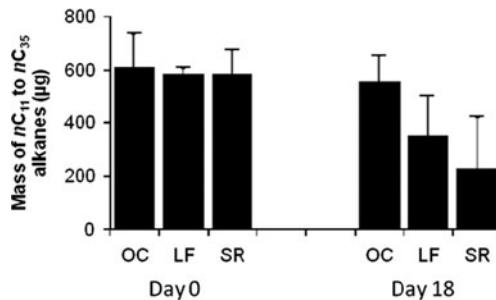


Fig. 24.3 Mass of alkanes (nC_{11} to nC_{35}) from crude oil in OC-, LF- and SR-treated plots on day 0 and day 18. The values are the average of measurements from three independent samples taken from replicate plots. OC – oil-treated control without inorganic nutrient amendment, LF – oil-treated plots amended with liquid fertiliser, SR – oil-treated plots amended with slow-release fertiliser

reduction in *n*-alkane content and the oil-treated control with no inorganic nutrients showed only $8.73 \pm 6.61\%$ ($p = 0.603$) decrease in *n*-alkane content. In addition, the nC_{17} to pristane ratio decreased from 2.044 ± 0.123 to 1.4 ± 0.38 ($p = 0.053$) in the SR plot, 2.04 ± 0.12 to 1.66 ± 0.24 ($p = 0.067$) in the LF plot and 1.95 ± 0.08 to 1.94 ± 0.095 ($p = 0.886$) in the OC plot relative to time zero for all treatments. The lowest nC_{17} to pristane ratio was observed in the SR fertiliser treatment followed by LF with only a slight difference in the case of the OC plots indicating the greatest degradation of *n*-alkanes in the SR fertiliser-treated plots. These results show a distinct effect of fertiliser treatment on the activity of the hydrocarbon-degrading microorganisms which is determined by the nature of the fertiliser applied.

Bacterial Community Dynamics and Quantitative Analysis of *Alcanivorax* spp.

The dominance of *Alcanivorax* 16S rRNA gene sequences (37.5% of the 40 clones screened) in clone libraries generated from SR fertiliser-treated plots (Röling et al., 2004) led us to quantify the abundance of *Alcanivorax* 16S rRNA genes in a range of samples from the oil spill bioremediation field trial (Röling et al., 2004). *Alcanivorax* was quantified by qPCR using *Alcanivorax*-specific 16S rRNA gene-targeted primers. Quantification of *Alcanivorax* spp. was performed on samples from oil-treated plots collected 5 days before SR fertiliser treatment (-5d) and in samples collected 1, 5, 11, 80, 107 and 315 days after SR fertiliser treatment and in samples taken on day 11 from the OC plot which had received oil but no inorganic nutrient treatment. The abundance of *Alcanivorax* spp. 16S rRNA genes increased from $4.92 \pm 0.28 \times 10^5$ per cm^3 on day -5, immediately after oil addition, to $1.90 \pm 0.22 \times 10^6$ 6 days after oil addition and 1 day after SR

fertiliser addition (Fig. 24.4a). Maximum *Alcanivorax* spp. 16S rRNA gene abundance ($2.49 \pm 1.28 \times 10^7$ per cm 3) was observed 11 days after SR fertiliser addition. The abundance of *Alcanivorax* spp. 16S rRNA genes on day 11 was significantly higher in the SR fertiliser-treated plots than in the OC control at day 11 which was treated with oil but no fertiliser ($4.38 \pm 0.94 \times 10^5$ per cm 3 ; $p = 0.009$). *Alcanivorax* spp. 16S rRNA gene abundance at day 11 in the OC control was not significantly different from their abundance immediately following oil treatment (Fig. 24.4a; $p = 0.516$). The *Alcanivorax* population in SR fertiliser-treated plot was thus around 50 times higher than in oil-treated plots that received no fertiliser. After

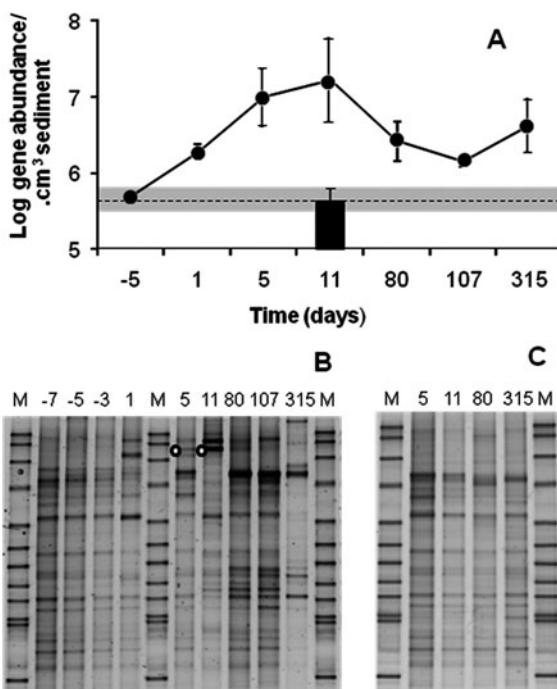


Fig. 24.4 (a) Abundance of *Alcanivorax* spp. 16S rRNA genes in SR plots 5 days before (-5d) and 1, 5, 11, 80, 107 and 315 days after slow-release fertiliser treatment. The abundance of *Alcanivorax* spp. 16S rRNA genes in oil-treated control plots which were not treated with fertiliser on day 11 are shown as a bar for comparison. The dotted line and horizontal grey bar represent the abundance and variance in *Alcanivorax* 16S rRNA gene abundance typically observed in beach sediments and beach sediments exposed to oil but not subject to inorganic nutrient treatment. (b) Changes in bacterial 16S rRNA gene-based DGGE profiles in a slow-release fertiliser-treated plot and (c) corresponding profiles from oil-treated plots which received no fertiliser treatment. Bands corresponding to *Alcanivorax* 16S rRNA gene fragments are indicated with a dot. The numbers above the lanes indicate the number of days after fertiliser treatment and M is a marker comprised of a mixture of bacterial 16S rRNA gene fragments. Parts b and c of this figure are modified from Röling et al. (2004) and are reproduced with permission

80 days *Alcanivorax* abundance had decreased to just over 10^6 per cm³ of sediment (Fig. 24.4a).

The change in abundance of *Alcanivorax* spp. was reflected in DGGE profiles of bacterial 16S rRNA genes from the oil-treated plots (Fig. 24.4b, c). The plots were treated with oil on day -7 and the SR plot was treated with fertiliser on day 0. Following oil treatment and prior to fertiliser addition (days -7 to -3) the bacterial communities remained relatively stable (Fig. 24.4b); however, upon fertiliser treatment the bacterial communities shifted dramatically (Fig. 24.4b; day 1 onwards) and bands corresponding to *Alcanivorax* 16S rRNA gene fragments (confirmed by sequence analysis after band excision and comigration with cloned *Alcanivorax* 16S rRNA gene fragments) became dominant in the profiles between days 5 and 11 (Fig. 24.4b, bands marked with circles). By contrast in oil-treated plots which were not treated with fertiliser, the bacterial communities remained relatively stable over several hundred days (Fig. 24.4c).

Qualitative Analysis of *Alcanivorax* and *alkB* Genes

16S rRNA gene-based DGGE analysis of oil-treated beach sediments amended with inorganic nutrients showed changes in the bacterial population selecting strongly for *Alcanivorax* spp. (Röling et al., 2004; Fig. 24.4b). Though nutrient treatment enhanced *Alcanivorax* spp. numbers (Fig. 24.4a), this did not necessarily reflect the metabolic capacity of *Alcanivorax* spp. to degrade petroleum hydrocarbon. In addition to demonstration of an increase in abundance of *Alcanivorax*, it was desirable to determine if this correlated with an increase in the abundance of genes for relevant hydrocarbon degradation pathways.

Alkane oxidation in many bacteria is initiated by an alkane monooxygenase/hydroxylase encoded by the *alkB* gene and its homologues (van Beilen et al., 2003; van Beilen and Funhoff, 2007). Therefore, oil degradation activity of specialist bacterial populations can better be understood by correlating changes in their 16S rRNA gene abundance with the functional *alkB* gene. The oil spill bioremediation field trial was complemented by a laboratory-based microcosm experiment. This was prepared using sediment from the intertidal zone of Stert Flats, UK, treated with oil and 1%N:0.1%P by weight of oil (Röling et al., 2002). Samples from the microcosms were analysed by DGGE of PCR-amplified bacterial 16S rRNA genes and PCR analysis of *alkB* genes. *Alcanivorax* spp. were not detectable 0 and 2 days after fertiliser treatment but by day 6, *Alcanivorax* had come to dominate the bacterial population (Fig. 24.5a); indeed, their dominance was even more pronounced than in the field experiment (Fig. 24.4b). Interestingly, *alkB* genes were detected only in samples showing predominance of *Alcanivorax* spp. (Fig. 24.5b) and the source of the *alkB* gene was confirmed by sequence analysis which demonstrated that the amplified *alkB* gene sequence had greater than 99.9% sequence identity with the *alkB1* gene of *Alcanivorax borkumensis* AP1 (data not shown).

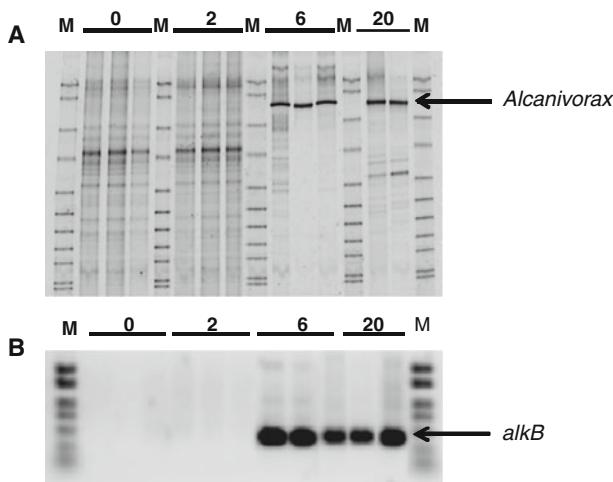


Fig. 24.5 (a) Changes in bacterial 16S rRNA gene-based DGGE profiles and (b) agarose gel electrophoresis analysis of *alkB* genes in crude oil treated beach sediment microcosms supplemented with 1% nitrogen and 0.1% phosphorus by weight of oil. Triplicate samples were analysed on day 0, 2 and 6 and duplicate samples were analysed on day 20. Bands corresponding to *Alcanivorax* spp. 16S rRNA gene fragments and *alkB* gene fragments are highlighted

Discussion and Conclusions

Marine ecosystems provide natural habitats for crude oil-degrading microorganisms. Although their numbers are typically low their growth is enhanced rapidly in response to inorganic nutrient amendment in hydrocarbon-polluted environments. Several studies have established the role of particular organisms as specialist hydrocarbon degraders. These include *Alcanivorax* spp. (Yakimov et al., 1998), *Oleiphilus* spp. (Golyshin et al., 2002), *Oleispira* spp. (Yakimov et al., 2003), which are alkane degraders, and *Neptunomonas naphthovorans* and *Cycloclasticus* spp. which are polycyclic aromatic hydrocarbon degraders (Hedlund et al., 1999; McKew et al., 2007b). Though there are many reports on the dynamics of *Alcanivorax* spp. in petroleum-contaminated environments (Kasai et al., 2002; Cappello et al., 2007a, 2007b), there have been relatively few attempts to analyse the abundance and dynamics of *Alcanivorax* spp. quantitatively in petroleum hydrocarbon-contaminated environments *in situ* (McKew et al., 2007a, b). In this study we have described the effect of nutrient amendment on the bioremediation activity of hydrocarbon-degrading organisms and quantitative changes in *Alcanivorax* populations in response to fertiliser (inorganic nutrient) treatment. Microbial activity and overall hydrocarbon degradation, determined by CO₂ evolution, was higher in fertiliser-treated plots showing the stimulatory effect of nutrient amendments on bioremediation activity. CO₂ production in SR fertiliser-treated plot was more than six times that in the LF-treated plot, whereas CO₂ production in LF fertiliser-treated plot was no different from the rate of CO₂ production in UC-treated plot and OC

plot. Previous work has shown that the lack of stimulation of oil degradation in response to liquid fertiliser treatment in the LF plots was probably due to leaching of the inorganic nutrients from the plots by tidal action while the SR fertiliser treatment resulted in better retention of inorganic nutrients which supported microbial activity for a longer period (Röling et al., 2004). Since the UC plot received no oil and no inorganic nutrient treatment, and the OC plot received oil treatment only, less CO₂ production was expected. Consistent with the data on CO₂ production, 18 days after inorganic nutrient addition 61.8 ± 33.87, 39.68 ± 27.02 and 8.73 ± 6.61% of the *n*-alkanes present were degraded in the SR, LF and OC plots, respectively.

Laboratory and field experiments performed using samples from the same site demonstrated a positive impact of nutrient amendment on oil degradation and concomitant selection for *Alcanivorax* spp. (Röling et al., 2002, 2004). Involvement of alkane hydroxylase, a product of the *alkB* gene, in alkane degradation is well known (van Beilen and Funhoff, 2007). Alkane hydroxylase initiates the first step in alkane degradation by oxidising the terminal carbon in the alkane chain to form a primary alcohol. Our results clearly demonstrated the stimulation of *alkB*-containing bacteria in fertiliser-treated oiled sediments in microcosms, and the detection of *alkB* genes closely mirrored the selection of *Alcanivorax* spp. revealed by DGGE analysis of 16S rRNA gene fragments from the same samples (Fig. 24.5). The current findings reinforce the importance of *Alcanivorax* spp. in biodegradation of petroleum hydrocarbons in marine environments, especially with respect to degradation of alkanes, a major constituent of crude oil. These results prompted us to quantify the abundance of *Alcanivorax* spp. in response to a bioremediation treatment in the field (SR fertiliser treatment of oiled sediments in the field). qPCR-based determination of *Alcanivorax* 16S rRNA gene abundance showed that *Alcanivorax* abundance was greatest in SR fertiliser-treated plots where they were more than an order of magnitude higher than in the OC plot and the SR fertiliser-treated plot 5 days before fertiliser treatment. In conclusion, the results presented here show that inorganic nutrient treatment of petroleum hydrocarbon-contaminated sites enhances the bioremediation process and that this is closely associated with a rapid increase in the abundance of *Alcanivorax* spp., which are known to be specialist petroleum hydrocarbon degrading marine bacteria.

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Chapter 25

How Specific Microbial Communities Benefit the Oil Industry

Significant Contribution of Methyl/Methanol-Utilising Methanogenic Pathway in a Subsurface Biogas Environment

Dariusz Strapoć, Matt Ashby, Ladonna Wood, Rick Levinson, and Bradley Huizinga

Introduction

Methanogenesis is considered the main terminal process of subsurface anaerobic organic-matter degradation. Previous geochemical studies have reported CO₂-reducing and acetoclastic methanogenesis as the predominant subsurface methanogenic pathways for primary and secondary biogenic gas generation (i.e. in oil biodegradation or coalbed methane settings). In lab-scale experiments and microbiology literature, however, methanogens have been shown to be able to utilise a wider variety of substrates, typically containing methyl groups, i.e. dimethyl sulphide (DMS), methyl amines (e.g. TMA), formate, and methanol. Additional methanogenic substrates include CO and other primary alcohols and secondary alcohols (Whitman et al., 2006; Fig. 25.1). Here, we describe a volumetrically important natural biogenic gas field in which these methylotrophic pathways have contributed significantly to biomethane formation.

Case Study: Results

Our recent observations from a single biogenic gas field in Alaska (Cook Inlet Basin; Fig. 25.2a) suggested a significant contribution of another methanogenic pathway in the subsurface environment. Low-maturity sub-bituminous organic debris in fluvial sediments (Fig. 25.2b) has been partially degraded to methane via three different methanogenic pathways: CO₂, acetate, and methyl/methanol utilising (Fig. 25.3a). The addition of a third subsurface-operative methanogenic

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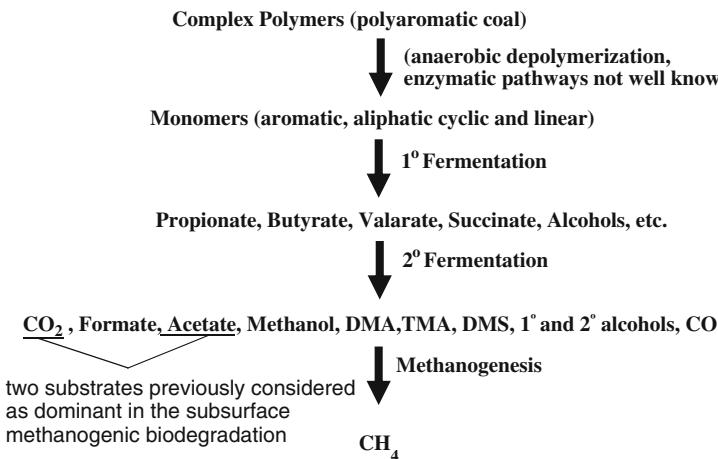
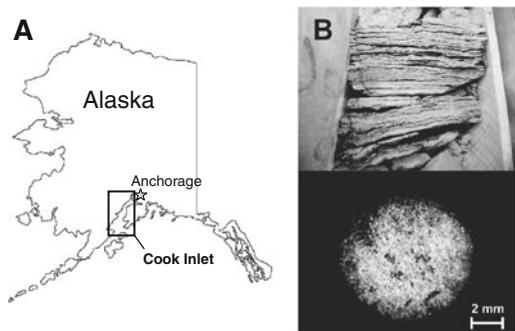


Fig. 25.1 Flow diagram showing decomposition of subsurface organic material, e.g. coal, down to methane. In grey – methanogenic substrates with significant contribution in the studied Cook Inlet microbial gas field and previously not considered as important in the subsurface environments

Fig. 25.2 Study area and lithology. (a) Geographic location of the Cook Inlet Basin. (b) Typical fragment of core and its photomicrograph from one of the producing wells in the Cook Inlet Basin; fluvial sandstone with organic debris and volcanic ash



pathway (methyl/methanol-utilising) is represented by obligate methylotrophic *Methanolobus* (König and Stetter, 1982; Whitman et al., 2006), recently also encountered in a coal (Doerfert et al., 2009) and a gas field (Mochimaru et al., 2009). Based on average results for the field, studied here, the clone library suggests roughly equal importance of each of the three methanogenic pathways (CO_2 , acetate, and methyl/methanol-utilising) as a source of methane for this gas field. However, between specific wells, the distribution of microbes involved in the dominant methanogenic pathway varies substantially and appears to be controlled by local geochemical conditions within the reservoir(s). The main environmental controls are reservoir temperature and water chemistry. Statistical analyses of integrated microbial and geochemical data suggest variation of methanogenic communities with environmental reservoir conditions.

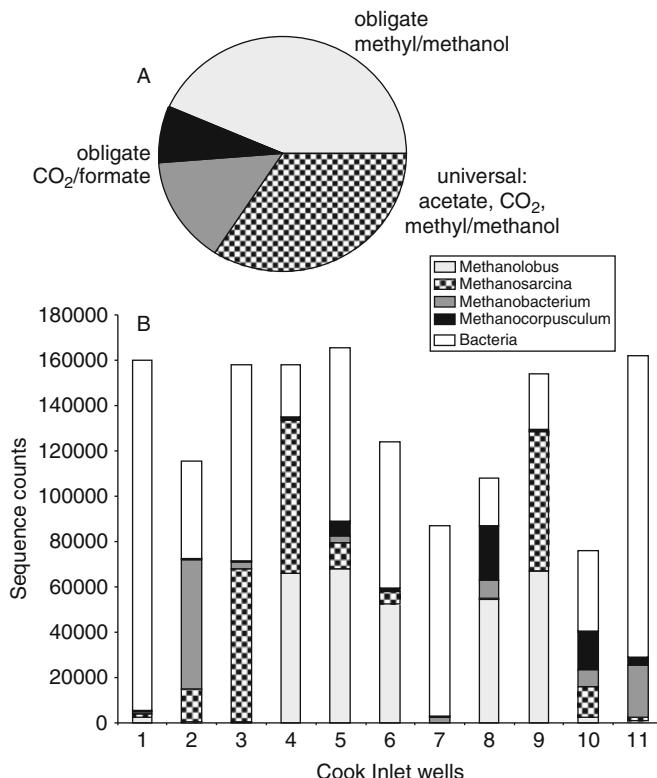


Fig. 25.3 16S rRNA pyrosequencing data for 11 Cook Inlet wells. (a) Average contribution of methanogenic Archaea in all 11 wells; significant fraction of *Methanolobus*. (b) Distribution of methanogens and total bacterial counts in all studied wells

In the Cook Inlet, biogenic gas has been formed from low-maturity terrigenous organic matter with vitrinite reflectance between 0.3 and 0.4% R_o . This gas is hosted in mostly fluvial sandstones and siltstones, containing variable amounts of organic debris and volcanic ash, with intervening interbeds of thin coals (10–30 cm). Sands with organic debris and volcanic ash form a poppy-seed cake texture (Fig. 25.2b). The association of nutritious volcanic ash with low-maturity coals and organic matter dispersed throughout fluvial high-permeability sands makes this formation an ideal bioreactor for microbial gas generation.

Microbial populations sampled from the formation water of gas-producing wells contained surprisingly significant abundances of archaeal methanogens utilising the methyl/methanol pathway. We observed that microbial enrichments from wells that contained highest fraction of methyl/methanol-utilising methanogens converted lignin mix (lignin and lignin monomers) to methane at highest rates, up to ~0.4 ml CH₄/l of culture medium per day (Fig. 25.4). Therefore, biodegradation of substrates, such as lignin mix, mimicking low-maturity coal suggests

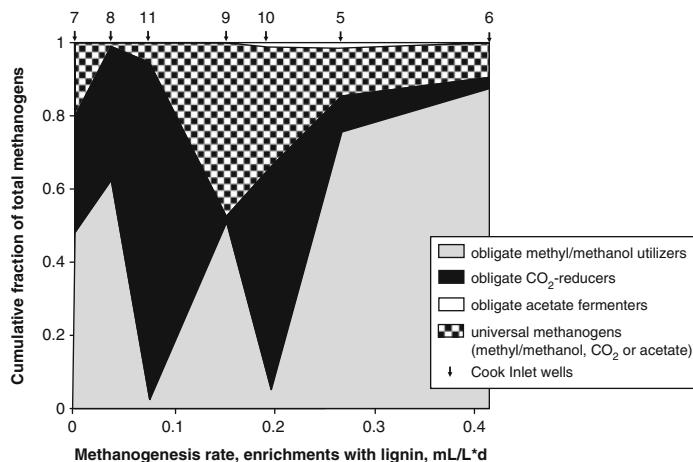


Fig. 25.4 Fractions of methanogens divided based on their methanogenic substrates and methanogenesis rates from lignin mix (lignin plus lignin monomers). Microbial populations from two wells with largest fraction of obligate methyl/methanol utilisers obtained highest rates of methanogenesis. Wells 1, 2, and 4 (not shown) had extremely low or no methane production

that methyl/methanol pathway may be the most efficient one in this particular biogenic gas field. In detail, the universal methanogens are represented by a couple of species from the *Methanosarcina* genus. *Methanosarcina*, in addition to acetate, may also use substrates obligatory to other groups of methanogens, i.e. CO₂ and methyl/methanol. Therefore, *Methanosarcina* can potentially switch between methanogenic pathways according to favourable chemical conditions of the formation water. Obligate acetate utilisers are represented by small populations of *Methanosaeta* (Fig. 25.4).

The methanogenic Archaea in 5 out of 11 studied Cook Inlet wells are dominated by *Methanolobus*, a methyl/methanol-utilising methanogen, with a fraction of *Methanolobus* reaching up to 88% (Fig. 25.3b). Additionally, *Methanosarcina*, a very common methanogen in studied wells, is also known to utilise the methyl/methanol pathway (Sowers et al., 1984). Dominance of sequence counts of methyl/methanol-utilising methanogens (*Methanolobus* and *Methanosarcina*) suggests the large contribution of biogenic methane might be produced by this pathway. Yet, potential contribution of acetate and CO₂ pathways cannot be ignored. Differentiation of the Cook Inlet wells based on their archaeal populations points out preferred methanogenic substrates (Fig. 25.5, insert). Wells separate into three clusters according to their dominant methanogenic pathway: obligate methyl/methanol-utilising, obligate CO₂ reduction, and acetate fermentation (Fig. 25.5, insert).

However, despite inferred pathway differentiation in the Cook Inlet wells, all stable isotopic fingerprints plot within surprisingly narrow range on the isotopic methane origin classification plot (Fig. 25.5). This may suggest that most of the gas

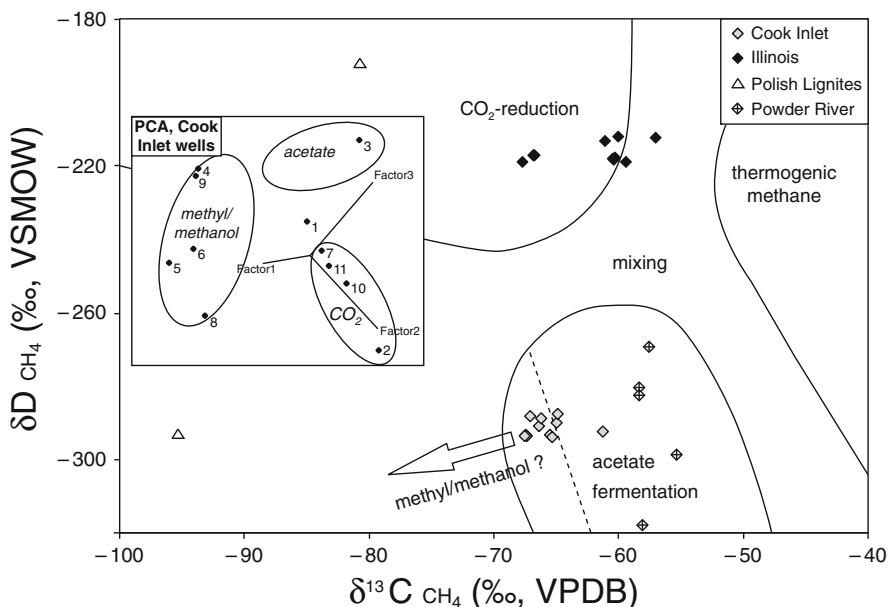


Fig. 25.5 Gas origin classification plot using stable isotopes of carbon and hydrogen. Solid lines after Whiticar et al. (1986). Dashed line – suggested approximate division of acetate and methyl/methanol fields. Arrow indicates inferred increase of methane generated via the methyl/methanol pathway. Insert diagram – principle component analysis (PCA) showing clustering of wells based on distribution of sequence counts of different methanogen species; as a result wells presumably clustered according to the dominant methanogenic pathway or substrate

in the field was and/or is being generated by one dominant pathway. Traditional classification of methane origin using carbon and hydrogen isotopes plots Cook Inlet biogenic gas within the acetate fermentation field (Fig. 25.5). Therefore, the methyl/methanol pathway was not previously noticed as isotopically distinct and potentially important in the subsurface environments. Our results suggest that methane generated via the methyl/methanol pathway may be slightly isotopically lighter than the typical acetate-derived one. Better differentiation of biomethane formation via the methylotrophic pathway versus the acetate-fermentation pathway requires further development.

Conclusions

Our study of volumetrically significant Cook Inlet biogenic gases shows that microbial conversion of subsurface organic matter (e.g. coal, shale, oil) to gas is far more complex than previously considered and confirms recent observations of *Methanolobus* in hydrocarbon-rich environments. In addition to the well-known CO₂-reduction and acetate-fermentation pathways, a third methanogenic

pathway utilising methyl/methanol substrate intermediates is important in certain low-maturity kerogen III-dominated settings. In fact, this under-recognised third methanogenic pathway may dominate in specific sub-surface environments depending on (i) the type and thermal maturity of the carbonaceous substrate and (ii) independently on in situ pressure, temperature, and geochemical conditions of the formation water.

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Part V

Fuel for the Future

Chapter 26

Fuel for the Future

Development of New Fuels, e.g. Biofuels

**Gitte Sørensen, Ketil Bernt Sørensen, Hans Ove Hansen,
and Sune D. Nygaard**

Introduction

Whether we like it or not, climate change is to some extent affiliated with the emission of green house gasses, and specifically CO₂ emissions, which are rising due to the global increased use of fossil fuels. As a result, political enthusiasm is high when it comes to implementing new initiatives aimed at better protection of the global environment. However, environmental concerns are just one aspect of the issues associated with the use of fossil fuels, since fossil fuels are a natural reserve and, therefore, a limited resource. Prognoses vary, but within the next decades the fossil fuel reserves will be exhausted leading to reduced oil production, rising oil prices, and the risk of international bellicose conflicts caused by adverse national interests. Additionally, fossil fuel as a natural reserve is unevenly distributed, meaning that a few countries possess the main energy reserve of the entire world. The incorporation of alternatives to fossil fuel into the existing fuel infrastructure is currently under intense development in the Western world, both to cut the oil dependency and to counter the depletion of oil reserves. This political enthusiasm to decrease the use of fossil fuel is emphasised by the fact that according to the International Energy Agency (IEA) more than 80% of the global primary energy consumption in 2007 accounts from fossil fuels and half of this is oil. More than 60% of the oil is used in the transport sector.

Biofuels are one group of alternatives to fossil fuels, and interest in these has risen dramatically during the last decades, showing that biofuels as an alternative to fossil fuels are highly potent. Biofuels are renewable fuels, i.e. they are made from biomass, and they are biodegradable. The most common biofuels are bioethanol and biodiesel. Bioethanol is a fuel based on alcohol produced by fermentation of materials rich in sugar and starch, such as sugar cane and wheat, or in the near future from ligno-cellulosic biomass, e.g. wood and wheat straw. The

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fermentation is an anaerobic conversion of sugar to alcohol, usually using yeast, *Saccharomyces cerevisiae*. Bioethanol can be used as a blend in gasoline or as 100% ethanol fuel; however, blends above 25% bioethanol require engine modifications. Biodiesel is produced by conversion of oil to fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEEs). The oil feedstock for this product normally originates from oleaginous plants such as rapeseed (*Brassica napus*), palm tree fruits (*Elaeis guineensis*), and soybean (*Glycine max*), from used vegetable oils, or from rendered animal fat. Biodiesel as an engine fuel can according to Escobar et al. (2009) and Jegannathan et al. (2009) be added to petrochemical diesel to form a blend or it can be used as the 100% pure biodiesel fuel in modified engine systems. The chemical structure of bioethanol and the most prevalent biodiesel components are given in Fig. 26.1. Besides bioethanol, or bioalcohols in general, and biodiesel, several other biofuels are being researched and a few of those are on the commercial market. Some to be mentioned are biogas, synthetic diesel fuels, 2,5-dimethylfuran, and biohydrogen. These will, however, not be discussed further here.

Biofuels for transportation purposes have according to Luque et al. (2008) been developed over a long time starting even with the invention of the internal combustion engine and diesel engines where ethanol and, initially, unmodified peanut oil were used as fuels, respectively. Biofuels were also common during World War II in machinery from both sides. First-generation biofuels are produced from feedstocks like corn, soy, or wheat through conventional conversion technologies such as fermentation and trans-esterification. Some challenges have, though, arisen with increasing production of biofuels since the basis of first-generation biofuel is feedstocks that otherwise would be readily available for human consumption or feed production. Cultivation of even more oil- and sugar-rich crops for production of

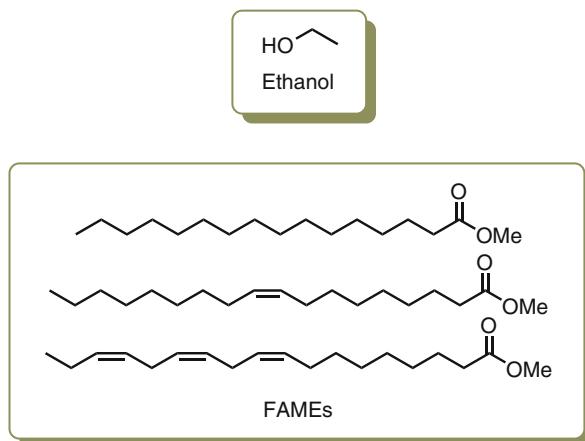


Fig. 26.1 Ethanol and examples of FAMEs, the components of bioethanol and biodiesel, respectively

biofuels and subsequent replacement of fossil fuel has been suspected for causing rising food prices and contributing to the shortage of food and feed witnessed in certain parts of the world. This has brought serious consideration to the necessity of choosing between using agricultural land for production of food and feed and for production of energy crops. Recognising these challenges has led to a massive development of biofuel feedstocks and production technologies in order to create what is commonly known as second-generation biofuels. These biofuels are often chemically identical to the first-generation biofuels but are contrary to those produced from sustainable, non-edible feedstocks or industrial waste products. Consequently, the production of second-generation bioethanol is therefore mainly based on cellulose-rich waste products, e.g. wheat straws, whereas biodiesel is produced from non-edible vegetable oil from plants like jatropha, or waste products, such as waste oils and fats. Using non-edible materials and marginal land for biofuel raw material is believed not to significantly affect food supply and cost, and, additionally, use of a cheaper waste feedstock is also believed by some researchers to reduce biofuel production costs significantly since oil cost usually constitutes a significant part of total biodiesel production costs. These issues are discussed by Ma and Hanna (1999), Luque et al. (2008), Pinzi et al. (2009), and Demirbas (2009).

Case Study: Production of Biodiesel at Daka Biodiesel

At Daka Biodiesel agricultural by-products are converted into environmentally friendly biodiesel and biofuel oils. Specifically, industrial waste in the form of rendered fat from production animals is transformed into biodiesel, i.e. FAME. Conversion of animal fatty acids to FAME poses some specific technical challenges compared to using plant oils. Some of these challenges are addressed in a joint industry project called Waste-2-Value. Specific is the content of free fatty acids (FFAs) that is much higher in animal fat compared to what is observed in traditional vegetable oils. If one used the traditional alkali-catalysed conversion of the fat into biodiesel the FFAs will form soap when reacting with the alkaline catalyst. This causes the reaction mixture to emulsify, thereby complicating further conversion to FAME and eventual purification.

To counteract soap formation the conversion of animal fat into biodiesel consists of a two-step process as given in the process flow diagram in Fig. 26.2. The first step in Daka Biodiesel's process is a pre-esterification of FFAs with sulphuric acid to convert the FFAs to FAME. This is followed by a second step where triglycerides from the rendered animal fat are trans-esterified, i.e. converted to FAME with methanol using sodium hydroxide or potassium hydroxide as the catalyst. The biodiesel is purified by sedimentation where the by-products glycerine and sodium or potassium sulphate are separated from the biodiesel. The crude biodiesel is further purified by distillation which results in pure biodiesel with a sulphur content of less than 10 ppm.

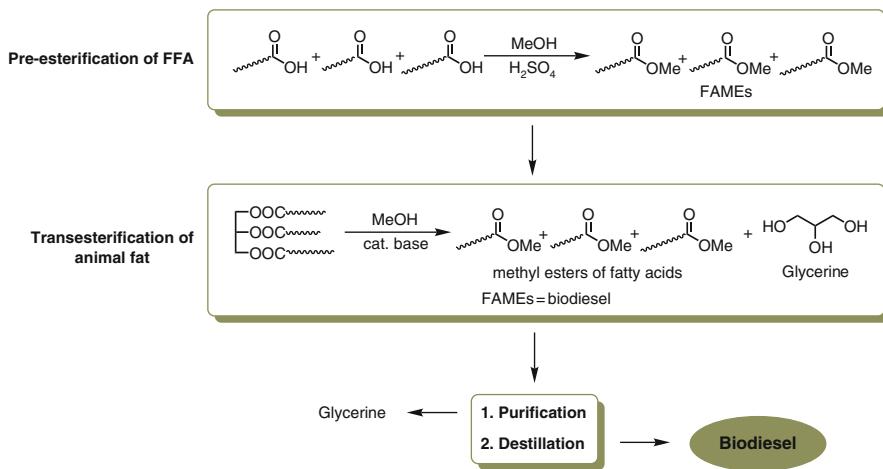


Fig. 26.2 Process flow diagram of the biodiesel production at Daka Biodiesel

Challenges Associated with the Use of Biodiesel

Low-Temperature Properties

Another challenge related to the use of animal fat as opposed to vegetable oil for biodiesel production is low-temperature usage since the freezing point of biodiesel is higher relative to that of most traditional diesel fuels. This is also the case for the cold flow properties of diesel fuels described by the cold filter plugging point (CFPP). CFPP defines the temperature at which a predefined filter is blocked to such a degree that diesel flow is significantly hindered according to standard measurements (ASTM D6371, IP309, EN-116).

The CFPP values of traditional fossil diesel fuels depend on the degree of refinement of the fuel which is a decision based on seasonal outdoor temperatures in the area where the diesel is to be put into use, i.e. spring, summer, fall, and winter diesel. Additionally, additives such as Arctic Express, Lubrizol, or Flozol are usually added to the fossil fuel to lower the CFPP value and improve other key parameters. Pure biodiesel, named B100, from Daka Biodiesel has a CFPP temperature of approximately 9°C, while biodiesel from vegetable sources according to Park et al. (2008) and Moser (2008) has CFPP values in the range between -20 and 12°C. Most of them, though, are considerably lower than biodiesel based on rendered animal fat because of the difference in FAMEs. Specific is the high content of fully saturated FAME molecules associated with a high CFPP value and vice versa. When biodiesel is added to fossil diesel the CFPP value of the blended fuel is generally observed to increase towards the CFPP value of the pure biodiesel. Studies on blends created from Daka Biodiesel's B100 based on animal fat and three different batches of fall diesel fuels have provided the results given in Fig. 26.3a.

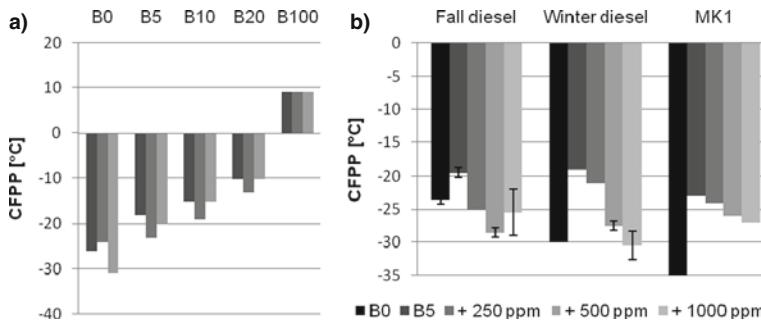


Fig. 26.3 (a) CFPP values obtained from mixing of three different standard batches of fall diesel with Daka Biodiesel based on animal fat. (b) CFPP values of three different diesel fuels. Each diesel fuel has been measured as B0 and B5 as well as B5 blends added 250, 500, and 1,000 ppm R408 from Infineum

Figure 26.3a shows that variations in diesel cause differences in cold flow properties when mixing with biodiesel. The general trend is, however, pronounced, showing that going from B0 to B100 increases CFPP values for all of the three fall diesel variations. This observation illustrates one of the major challenges associated with the use of biodiesel blends in colder climates. Biodiesel blends are on the other hand much easier to implement in the fuel infrastructure in warmer climates without risking problems with regard to filter blocking. To ensure that filter blocking does not become a problem even in colder climates like Denmark, a lot of effort has been put into testing the effect of a number of specific CFPP-improving additives. An example of an additive affecting the CFPP value of several biodiesel blends is the additive R408 from Infineum. Studies on the effect of this additive have been performed on five different B5 blends prepared from different diesel fuels. The promising results of the additive are shown in Fig. 26.3b.

The diesel fuels used for this study are all intended for use in Scandinavia, i.e. a cold climate. As seen from Fig. 26.3b the CFPP value of pure winter diesel (B0) is lower than for pure fall diesel, and the CFPP value of MK1 is even lower. The differences in the observed CFPP values of winter and fall fossil fuels correlate to the temperature changes from fall to winter. It is clearly seen that addition of the same amount of biodiesel to different types of diesel does not affect the CFPP value to the same degree. Likewise, addition of R408 does not counteract increases in CFPP to the same degree in all diesel fuels. In general, this shows that changing the fuel matrix to contain more biofuels is not necessarily easily done. What is also seen is the possibility to improve cold properties of B5 biodiesel blends significantly by addition of the right additives in reasonable amounts.

Stability of Biodiesel Blends

Biological growth has been a challenge to the storage and handling of fuels throughout the distribution chain for a long time. These challenges have in the past been

countered by developing good housekeeping practices for the storage and handling of fossil fuels. With the introduction of new and more biogenic fuels, new best practices must be developed as discussed by Schleicher et al. (2009) and Blin et al. (2007). To gain knowledge on the nature of new fuels and their properties, studies on the biomass in fossil diesel and biodiesel as well as blends of these have been performed at the Danish Technological Institute. Preliminary experiments have been carried out by varying storage conditions in order to monitor the effect on biological growth in blends of Daka biodiesel and fossil diesel. For reasons of comparison, similar experiments have been performed with neat fossil diesel fuels. Anaerobic incubations of B0, B5, and B20 mixtures have been studied with and without the presence of free water. The water used is collected from a petrochemical diesel storage tank in order to ensure presence of relevant diesel-degrading bacteria. Prior to the experiments, the water was diluted by a factor of 10 diluted by a factor of 10.

The microbiological growth in the incubations is followed using several ‘state-of-the-art’ techniques and molecular microbiological methods (MMM) such as DAPI-staining of microorganisms and quantitative polymerase chain reaction (qPCR) according to standard procedures as described by Porter and Feig (1980) and Gittel et al. (2009). Changes in microbial community were followed using DGGE as described by Muyzer et al. (1998).

Growth Studies

The growth study discussed here is primarily based on DAPI counts and visual evaluations of the anaerobic incubations at 30°C. Blends from fossil fall diesel and Daka biodiesel into B0, B2, B5, B10, and B20 blends have been followed with DAPI counts. The results of DAPI counts in anaerobic incubations with free inoculated water added are shown in Fig. 26.4.

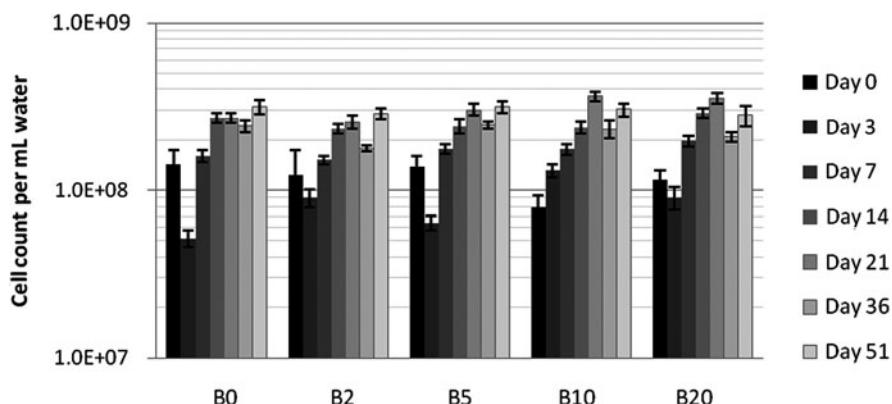


Fig. 26.4 Averaged DAPI counts for duplicates of B0, B2, B5, B10, and B20 anaerobic incubations with added free inoculated water over a period of 51 days

A general trend in the DAPI counts for all incubations is that an initial decrease in the cell count is observed from day 0 to day 3 which is believed to indicate the bacterial stress associated with mixing of the samples. This initial decrease in the bacterial number is followed by a quite fast growth in cell counts from day 3 to days 7 and 14. After this period, the microbial growth seems to level out at around 3×10^8 cells/ml water. The DAPI counts do not reveal any significant difference in the levels of microbial growth between the five tested blends from B0 to B20.

Visual observations indicated a significantly larger amount of biomass formed in incubations containing more biodiesel. This is exemplified in the photographs given in Fig. 26.5 where the B20 blend to the right has significantly more biomass in the water–oil interface seen as a blurry transition compared to the nearly clear transition between oil and water in the B0 blend.

This discrepancy between DAPI counts performed on the water phases of B0–B20 incubations and the visually observed growth of biomass may arise from the fact that the collected samples for DAPI countings are not representative of the actual growth. This might arise from the way the samples have been collected. The flasks containing the oil blend and water were shaken to disperse the formed biomass evenly in the aqueous phase. However, the flasks were allowed to rest for a short time after shaking in order to avoid contamination of the water sample with emulsified oil phase. Consequently, too long settling times before sampling might result in biomass accumulation in the interface between the oil and water again leading to uneven distribution of biomass and, hence, bad sampling. This may explain lower DAPI counts than expected according to visual observations.

The amount of microorganisms in the incubations was also followed by qPCR enumeration of 16S rRNA genes as described by Gittel et al. (2009). Results from anaerobic incubations are shown in Fig. 26.6. The water analysed at time 0 is the inoculation water from the diesel storage tank diluted by a factor of 10.

The bacterial qPCR results of the anaerobic incubations show compliance with the obtained DAPI results at the end of the experiment since DAPI and qPCR counts are in the same range. Methane formation was not observed during gas

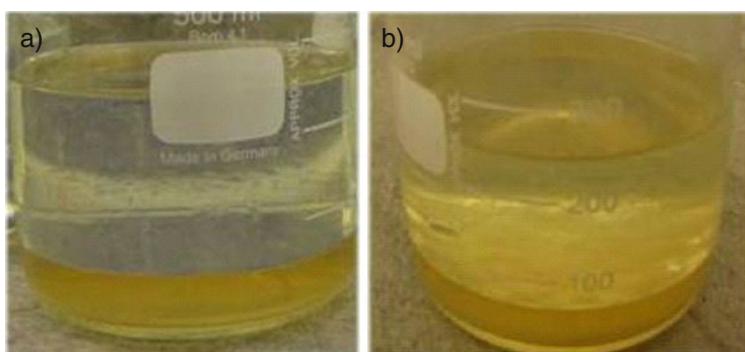


Fig. 26.5 Incubations of (a) B0 and (b) B20 after storage for 3.5 months. (Please consult with the online version of this chapter (which is in colour) for clarity of the water–oil interfaces)

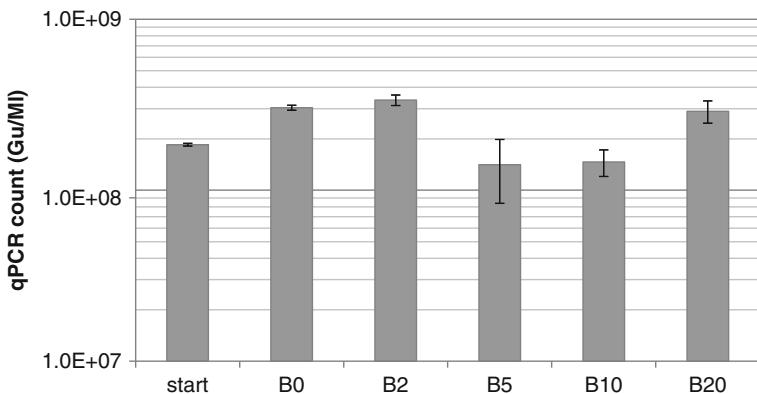


Fig. 26.6 Bacterial qPCR results from the diluted inoculation water at time 0 and blends after incubation for 51 days

chromatographic measurements, and *Archaea* remained below the detection limit of the assay, i.e. 10^3 genes/ml. This is in contrast to what is usually observed in anaerobic incubations without electron acceptors such as nitrate and sulphate where methane formation is abundant as a major end product of anaerobic metabolism. These findings will be subject to further studies.

Comparison of bacterial communities in the incubations was performed by DGGE analysis. A DGGE gel with PCR-amplified fragments of bacterial 16S rRNA genes from the starting material, i.e. the inoculation water, and duplicates of the five different blends after 51 days of incubation is shown in Fig. 26.7. By excision and

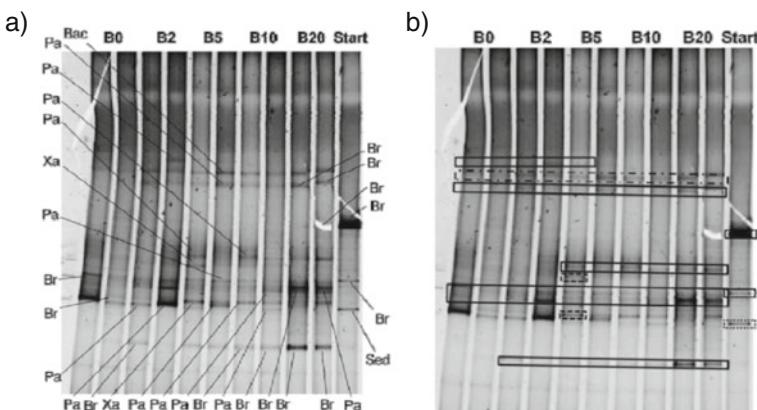


Fig. 26.7 DGGE gel. (a) The indicated bands were reamplified and sequenced for identification. Results are indicated as follows: Pa: *Paenibacillus*; Bac: *Bacteroidetes*; Xa: *Xanthomonas*; Br: *Brevundimonas*; Sed: *Sedimentibacter*. (b) The bands are affiliated with *Alphaproteobacteria* (— boxes), *Bacteroidetes* (— · — box), *Firmicutes* (..... box in Start), and *Gammaproteobacteria* (— · — · — boxes in B5)

reamplification a range of bands have been sequenced and identified, and the results are summarised in Table 26.1 and Fig. 26.7.

The DGGE analysis revealed a significant change in the bacterial community structure going from a community structure of a few bands in the inoculation water to a high overall bacterial diversity in the blends after incubation. Several of the detected bacteria are known to degrade complex organic molecules and polymers. The observed change in bacterial community structures indicates growth of microorganisms in the incubations which supports the growth results obtained by DAPI. Additionally, the DGGE analysis shows that some microorganisms are present only when biodiesel is present in the blend. This may be explained by the following arguments: (i) the microorganisms are able to grow on biodiesel but not on petrochemical diesel or (ii) the organisms growing in the incubations originated from the biodiesel.

The results of the identification of bacterial communities show that all groups of bacteria found in the incubations are ubiquitous in both marine and freshwater systems. They all possess multifaceted metabolisms and thereby do not add much functional information to the identification. Generally, the bands have been affiliated with *Alphaproteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Gammaproteobacteria*, as shown in Fig. 26.7b.

Table 26.1 Description of key bacteria detected in the incubations

	Start	Incubation	Affiliation	Physiology	Other information
<i>Brevundimonas diminuta</i>	1	8	<i>Alphaproteobacterium</i> <i>Caulobacterales</i> <i>Caulobacteraceae</i>	26°C, aerobic	
<i>Other</i>	1	1			
<i>Brevundimonas</i>					
<i>Bacteroidetes</i>	–	1		Aerobic/ anaerobic	
<i>Sedimentibacter</i> sp.	1		<i>Firmicutes</i>	Anaerobic	
<i>Paracoccus aminovorans</i>	–	6	<i>Alphaproteobacterium</i> <i>Rhodobacterales</i> <i>Rhodobacteraceae</i>	Aerobic, NO ₃ – red to NO ₂ –, not growing by nitrate red, 30–37°C	Dimethylformamide- utilising, closely related species are nitrate-respiring (Urakimi et al., 1990)
<i>Paracoccus FLN-7</i>	–	6	<i>Alphaproteobacterium</i> <i>Rhodobacterales</i> <i>Rhodobacteraceae</i>		Hexaflumuron- degrading
<i>Other</i>	–	1	<i>Alphaproteobacterium</i>		
<i>Paracoccus</i>			<i>Rhodobacterales</i>		
<i>Xanthomonas</i> sp.	–	2	<i>Gammaproteobacterium</i> <i>Xanthomonadales</i> <i>Xanthomonadaceae</i>		

Conclusions

This preliminary study (*vide supra*) has been aimed at exploring some of the properties of animal-derived biodiesel blends. These studies have clearly indicated that it is by no means trivial to produce biodiesel blends which can be used in colder climates, like Scandanavia. However, by carefully selecting the fossil fuel and biodiesel components, as well as additives present in the fuel blend, it is possible to tailor-make biodiesel blends that can be used under a wide variety of climatic conditions. The evaluations of the biological experiments indicate that addition of biodiesel to fossil diesel fuels results in growth of new types of bacteria compared to pure fossil diesel systems. The results presented are still rudimentary. However, detailed knowledge on microbiology related to the addition of biofuels is highly important in order to develop best-practice protocols for storage and handling of these novel fuels.

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Chapter 27

Fuel for the Future

Biodiesel – A Case study

Márcia T.S. Lutterbach and Mariana M. Galvão

Introduction

High crude oil prices, concern over depletion of world reserves, and growing apprehension about the environment, encouraged the search for alternative energy sources that use renewable natural resources to reduce or replace traditional fossil fuels such as diesel and gasoline (Hill et al., 2006). Among renewable fuels, biodiesel has been attracting great interest, especially in Europe and the United States. Biodiesel is defined by the World Customs Organization (WCO) as ‘a mixture of mono-alkyl esters of long-chain [C₁₆-C₁₈] fatty acids derived from vegetable oils or animal fats, which is a domestic renewable fuel for diesel engines and which meets the US specifications of ASTM D 6751’. Biodiesel is biodegradable and non toxic, produces 93% more energy than the fossil energy required for its production, reduces greenhouse gas emissions by 40% compared to fossil diesel (Peterson and Hustrulid, 1998; Hill et al., 2006) and stimulates agriculture.

Biodiesel can be used in its pure form (B100) or as a blend (BXX) with conventional petroleum diesel fuels. The blend is identified using B followed by the percentage of biodiesel in the finished product. B5 is the most common blend for public use in Europe and B30 is used extensively for special fleets. In the USA, B20 is widely used. In Brazil, the blend of biodiesel and diesel has been mandatory since 2008 according to Brazilian law no. 11097 of January 2005 (LAW no. 11097). Currently, diesel sold to a final consumer within Brazil must contain 4% of biodiesel (B4). The B5 requirement will probably be moved up to 2010.

Biodiesel can be manufactured from a wide range of renewable resources such as vegetable oils, animal fats, oil and grease wastes. In general, the oils used are those found in abundance in the producing country. There are many species of plants which can be used to produce biodiesel, such as castor oil, palm, sunflower, peanuts, soybeans and pinion tame, among others. Brazil is one of the countries that want to

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diversify the raw material used in the production of biodiesel. Besides the variation in the feedstock used, biodiesel can also vary according to the alcohol employed in the transesterification reaction. Biodiesel is often termed FAME (fatty acid methyl esters) if produced using methanol or FAEE (fatty acid ethyl esters) if produced using ethanol.

Biodiesel quality is regulated by national and international standards that determine product specifications. However, none of the standards specify limits or analytical methods for microbial contamination of biodiesel, even though there are some studies reporting biodiesel susceptibility to microorganisms and its high biodegradability (Gerpen et al., 1996; Zhang et al., 1998; Energy Institute, 2008). There is evidence that biodiesel is more prone to microbial spoilage but studies have been limited. The growth of microorganisms in the fuel distribution chain and in end use can cause severe problems such as fuel filter and fuel injection system clogging, microbiologically influenced corrosion (MIC) of the fuel system and fuel deterioration.

Since biodiesels are produced from natural feedstock, which present great variability, the risk of microbiologically influenced corrosion (MIC) and fuel biodegradation occurring in the presence of biodiesel (and biodiesel–diesel blends) are perhaps higher than that in conventional fuels. In order to prevent and control the occurrence of these problems in the presence of biodiesel and its blends, it is essential to understand the types of microorganisms found in biodiesel and the compounds of biodiesel that may be critical for their growth.

This work presents an example of how the application of molecular microbiological methods (MMM) can contribute to a better understanding of microbial diversity in biodiesel samples from different feedstock and different blends. Different aerobic bacterial strains were isolated and identified by MMM by the Laboratory of Biocorrosion and Biodegradation (LABIO) from biodiesel samples (Tables 27.1 and 27.2).

Three aerobic bacterial strains were isolated from biodiesel samples: *Cupriavidus pauculus*, *Staphylococcus pasteuri* and *Bacillus cereus*. Two of them are different from the bacteria usually recovered from diesel samples (*C. pauculus* and *S. pasteuri*).

More than 60% of aerobic bacterial strains isolated from biodiesel samples at LABIO were identified as *Cupriavidus* sp. These bacteria have been found in biodiesel produced from different raw materials (cottonseed oil and soybean oil). According to Mazur et al. (2009), bacteria from this genus are able to use biodiesel as a carbon source and are also known to produce a high content of extracellular polymeric material (EPS). For these reasons, this bacterium was selected for further studies on biodiesel degradation and materials corrosion at LABIO.

A *Staphylococcus* sp. genetically related to our *S. pasteuri* strain has been detected in oil-contaminated samples (Gutiérrez et al., 2008). This fact may indicate the ability of this strain in biodegrade biodiesel. The other bacterium found, *B. cereus*, is known to degrade the aliphatic and aromatic portions in diesel. This species has been previously isolated from corrosion residues of a transmission

Table 27.1 Morphological observation of bacterial colonies and optical microscopy of Gram stain from strains isolated from biodiesel samples

Strain	Sample	Bacterial colonies (non-selective agar media)	Gram	Identification
0055	Biodiesel from cottonseed oil			<i>Cupriavidus pauculus</i> (99%)
0056	Biodiesel from cottonseed oil			<i>Staphylococcus pasteuri</i> (100%)
0100	Biodiesel from soybean oil			<i>Bacillus cereus</i> (99%)

Table 27.2 Morphological observation and optical microscopy of the fungus strain isolated from biodiesel sample

Strain	Sample	Sabouraud Agar	Microscopy (10X)	Microscopy (40X)	Identification
0083	Biodiesel				<i>Aureobasidium pullulans</i> (99%)

pipeline carrying diesel fuel (Muthukumar et al., 2007). *B. cereus* has also been employed in other studies of biodiesel susceptibility to microbial degradation. The authors reported that *B. cereus* prevailed throughout the test period in B100 probably due to their sporulation capacity (Klofutar and Golob, 2007).

Besides the bacterial strains, a black fungus was also detected in two biodiesel samples. The sequence analysis of the regions containing ITS 1, ITS 2 and the 5.8S ribosomal RNA genes showed that the fungus strain isolated has a 99% similarity with *A. pullulans* (Table 27.2). *A. pullulans* are able to produce and secrete the polysaccharide known as pullulan. Pullulan produces high-viscosity solutions at relatively low concentrations and can be utilised to form oxygen-impermeable films, thickening or extending agents or adhesives (Lee et al., 1999).

Microorganisms that produce polymers are of great concern for fuel quality since these microbes have been identified as the cause of a number of failures such as clogged pipelines, vents, filters and irregular functioning of the automatic

measurement equipment (Klofutar and Golob, 2007). Lee and collaborators (2009) have also found this fungus in B100 samples.

The isolation and identification of culturable microorganisms from biodiesel samples is a good approach to better comprehend microbial contamination of biofuels. Since these microorganisms can be cultured, different assays can be performed to study microbial participation/activity in the biodegradation of these fuels. However, it is now well recognised that only a small fraction of all microorganisms have been isolated and characterised (Wayne et al., 1987; Ward et al., 1992). Certainly, the lack of knowledge of the real conditions under which most of the microorganisms grow contributes to the limited percentage of culturable microbes. Therefore, to obtain a better understanding of the role of microbial diversity in a given environment other approaches which complement the traditional microbiological procedures are needed.

In this study, Denaturing Gradient Gel Electrophoresis (DGGE) was employed to determine the microbial diversity in biodiesel from different feedstock and different

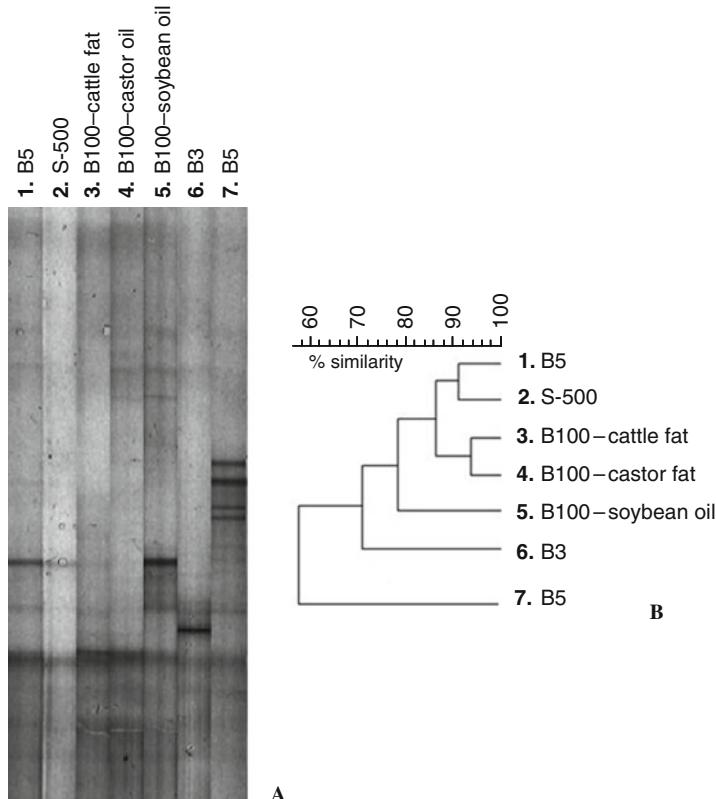


Fig. 27.1 DGGE profiles of PCR-amplified 16S rRNA gene fragments of bacterial communities from seven different fuels (a) and dendrogram of DGGE profiles of the bacterial communities (b)

blends. DGGE is a genetic fingerprinting technique in which DNA fragments of the same length but with different sequences can be separated based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (Muyzer and Smalla, 1998). (See Chapter 5, this volume.)

The DGGE analysis of PCR-amplified 16S rRNA gene fragments for seven samples of fuels is shown in Fig. 27.1. The cluster analysis was based on Pearson's correlation index. Lane 1 shows the microbial diversity of a B5 sample prepared from the mixture of S-500 diesel (lane 2) and B100 (data not shown). Since 95% of this B5 sample was composed of the same S-500 diesel, these fuels shared approximately 91% similarity. Biodiesels (B100) produced from three different feedstock were analysed (lanes 3, 4 and 5). It seems that biodiesels from cattle fat and castor oil share more similarities in microbial diversity than biodiesel from soybean oil. However, more samples should be tested to confirm this observation.

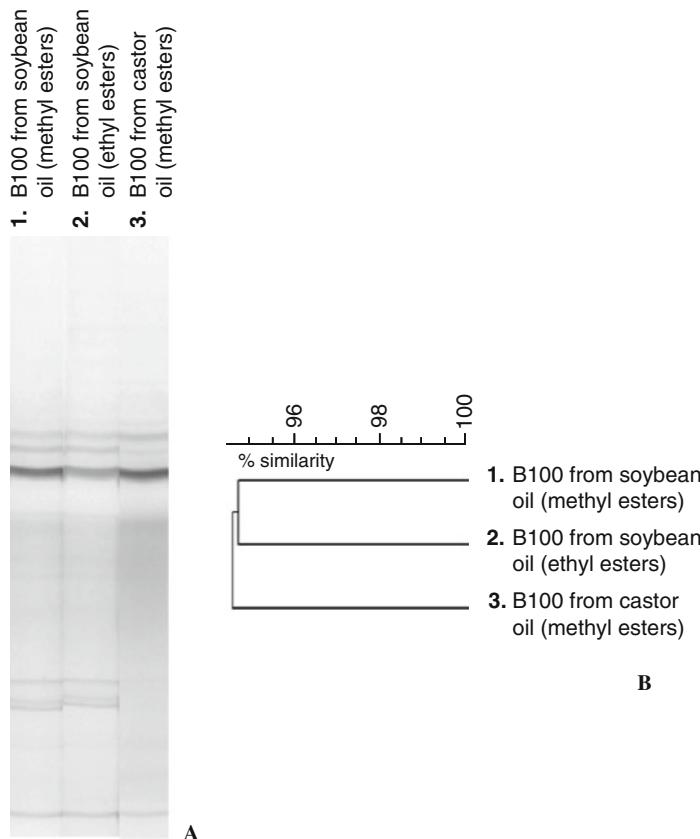


Fig. 27.2 DGGE profiles of PCR-amplified 16S rRNA gene fragments of bacterial communities from three biodiesel samples (a) and dendrogram of DGGE profiles of the bacterial communities (b)

Introduction of biodiesel to conventional fuel has been found to increase the number and types of microorganisms growing in the fuel mixture (Muthukumar et al., 2007). When comparing the B3 sample (lane 6) and the B5 (lane 7), more bands can be seen in B5. This fact would indicate the presence of a more diversified bacterial community in biodiesel–diesel blends with higher biodiesel content.

Three other biodiesel samples were analysed through DGGE (Fig. 27.2). The samples included two B100 from soybean oil – differing only in the alcohol employed in the transesterification reaction – and one B100 from castor oil produced using methanol. Cluster analysis of the bacterial DGGE profiles indicated that the two soybean oils showed more than 95% similarity with each other (Fig. 27.2b). These results suggest that the diversity of microbial communities in biodiesels produced from the same feedstock (regardless the alcohol employed) is closely related.

Conclusions

Much of the experience obtained with conventional fuels is based on years of research and testing. At the present time, many new alternative fuels from different feedstock and manufacturing processes are being developed and brought to market with little or no work concerning the potential impact of microbial activity in distribution, storage and use of these fuels. The use of biodiesel is now mandatory in several countries and the constant increase in the percentage of biodiesel in biodiesel–diesel blends is being encouraged. Although there are many international standards for biodiesel specifications, none of the standards specify limits or analytical methods for microbial contamination of biodiesel, even though there are several studies reporting biodiesel susceptibility to microorganisms and its high biodegradability.

Most of the microorganisms isolated from biodiesel samples and identified through molecular biology at LABIO are potentially harmful for the quality of biofuels and should be regarded with caution. Therefore, the study of the microorganisms present in biodiesel and their role in the fuel degradation and tank/equipment corrosion is fundamental. Further studies should also consider the different raw materials employed for biodiesel production as well as the alcohol employed in its transesterification. These variations should be better investigated in order to determine their influence on microbial contamination.

The microbial diversity present in biodiesel and its blends is yet not well understood. The PCR-DGGE technique can be a powerful biomolecular tool since it may overcome the limitations of selective growth that are encountered in culture-based studies and may provide valuable information on microbial diversity in biodiesel samples. Although the use of biodiesels is a global reality, there is still a lot of work that needs to be carried out since little is known about the interaction of microorganisms with this new substrate. In conclusion, new fuels present new challenges to be overcome to avoid new problems.

Acknowledgements We thank Petrobras for funding this work.

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Chapter 28

Fuel for the Future

Unlocking New Fuel Resources

Richard Johnson and Corinne Whitby

Introduction

With worldwide production of light crude oil reserves expected to last ~50 years, a global energy crisis may ensue. In fact, a recent report by the UK Energy Research Centre stated that ‘conventional oil production may peak before 2030 with a significant risk of a peak before 2020’ (October, 2009). Therefore, there is an increasing need to look towards the use of alternative fuel resources like the heavy/super heavy crude oils and oil sands. In Canada and Venezuela there are vast oil sands (containing 2,100 billion barrels) that are already being exploited (Clemente and Fedorak, 2005). During oil sand refining, bitumen is extracted, which results in large amounts of contaminated waters (known as tailings pond waters, TPW) being generated. TPW contain complex mixtures of toxic, alicyclic, aliphatic and aromatic acids known as naphthenic acids (NAs) (Fig. 28.1). TPW have to be stored in large ponds for several years until their toxicity is reduced to acceptable levels and consequently they pose a significant environmental risk. TPW contain NA concentrations between 40 and 120 mg/l (Herman et al., 1994) and with the volume of process water expected to reach 1 billion m³ by 2025 (Hadwin et al., 2006), removal of NAs is of great environmental importance.

NAs are highly recalcitrant compounds that persist in the environment for many years. They have been shown to be toxic to many organisms, including rats (Rogers et al., 2002); fish (Dokholyan and Magomedov, 1984); and microorganisms (Clemente et al., 2004). NAs may also cause severe engineering problems. For example, the presence of NAs in oil increases the total acid number (TAN), devaluing the oil and making it less saleable. NAs can also form calcium naphthenate deposits which can block pipelines and processing equipment causing billions in lost revenue as well as the cost of replacing equipment. NAs are highly corrosive (due to the carboxylic acid group and the polar nature of NAs) which can also cause equipment failure.

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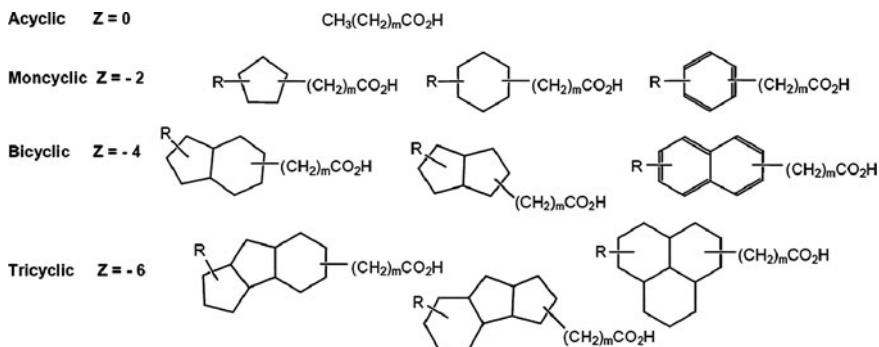


Fig. 28.1 Examples of NAs (Whitby, 2010)

NAs and their salts have a range of uses in industry such as wood preservatives. Consequently, NAs may enter the environment from a number of different anthropogenic sources. Recent evidence suggests that NAs originate either from the natural products of archaeal cell wall lysis and breakdown (Lutnaes et al., 2006) or from the metabolites and breakdown products of aerobic and anaerobic metabolism of crude oils (Head and Jones, 2003).

Chemical Composition and Methods of Characterising NAs

It was previously believed that NAs followed the general formula of $\text{C}_n\text{H}_{2n-Z}\text{O}_2$ where n equals the carbon number and Z equals the number of hydrogen atoms displaced by the ring structure (in the case of alicyclic NAs). NAs in the $Z = 2$ group contain one ring, $Z = 4$ contain two rings, $Z = 6$ three rings and so on (Fig. 28.1). This accounts for all the monoprotic alicyclic and aliphatic components of NAs. However, NAs also consist of polyprotic acids (i.e. acids that donate more than one proton per acid molecule) as well as aromatic acids. This definition therefore does not fit with current knowledge, and the term NAS should be applied to encompass the whole acid hydrocarbon fraction of crude oils. Due to the complexity of NA mixtures, separating and quantifying them has been complex. Methods used include gas chromatography coupled with flame ionisation detection (GC-FID) (Headley et al., 2002; Herman et al., 1994); high-performance liquid chromatography (HPLC) (Clemente et al., 2003); Fourier-transform infrared spectroscopy (FT-IR) (Clemente and Fedorak, 2005; Holowenko et al., 2001); and gas chromatography coupled to mass spectroscopy (GC-MS) (reviewed by Headley et al., 2009).

Case Study: Biodegradation of Aliphatic and Aromatic NAs

Since NAs cause many environmental and commercial problems, a number of strategies have been developed to remove them, including the use of ozone (Scott et al., 2007). However, on a large scale, ozonation is expensive to implement. The

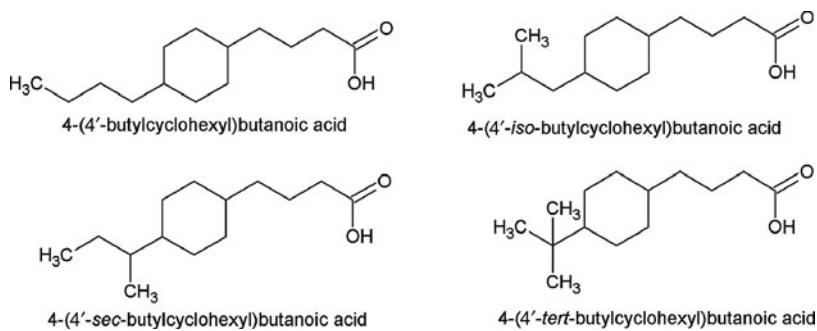


Fig. 28.2 Structure of aliphatic NAs (Smith et al., 2008)

application of microorganisms to remove NAs from contaminated systems is a more cost-effective approach compared to other physical/chemical methods. Many studies have analysed the aerobic biodegradation of commercial and environmental mixes of NAs (Biryukova et al., 2007; Clemente et al., 2004; Del Rio et al., 2006), while other studies have used laboratory-synthesised NA compounds such as cyclohexane carboxylic acid as substrates (Rho and Evans, 1975; Taylor and Trudgill, 1978), which may not be representative of those NAs found in the environment. Smith et al. (2008) synthesised a range of C₁₄ carboxylic acids which were thought to be more representative of environmental NA mixtures (Fig. 28.2) and were acutely toxic to oyster embryos.

The study used a marine sediment that had previous exposure to long-term hydrocarbon contamination as an inoculum in aerobic NA-degradation experiments (Fig. 28.3). The study showed a clear relationship between biodegradation rates

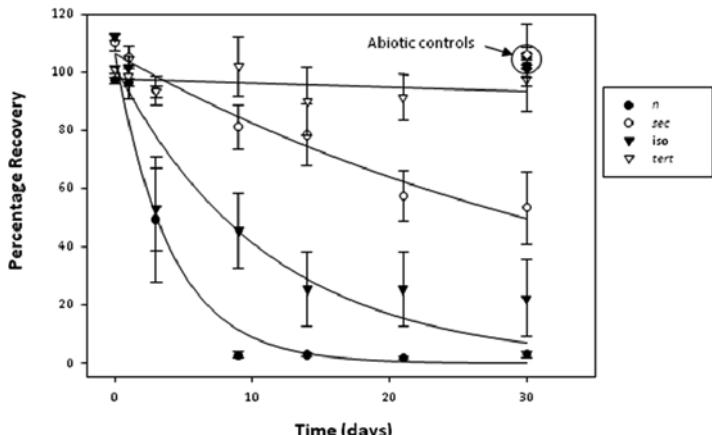


Fig. 28.3 Biotransformation of aliphatic NAs expressed as percentage NA recovered over time. NAs: *n*-BCHBA (closed circles), *iso*-BCHBA (closed triangles), *sec*-BCHBA (open circles) and *tert*-BCHBA (open triangles) (Smith et al., 2008)

and side chain branching of four butylcyclohexylbutanoic acid (BCHBA) isomers whereby, little degradation of the highly branched 4(4'-*tert*-butylcyclohexyl)butanoic acid (*tert*-BCHBA) occurred (even after 30 days), compared to the least branched 4(4'-*n*-butylcyclohexyl)butanoic acid (*n*-BCHBA) where >97% was degraded after 9 days (Smith et al., 2008). 4(4'-*iso*-Butylcyclohexyl)butanoic acid (*iso*-BCHBA) and 4(4'-*sec*-butylcyclohexyl)butanoic acid (*sec*-BCHBA) with intermediate degradation rates of 77 and 47%, respectively, degraded after 30 days (Smith et al., 2008).

Production of metabolites corresponded directly with degradation rates (*n*-BCHBA produced the highest percentage of metabolites, followed by *iso*-, *sec*- and *tert*-BCHBA) (Fig. 28.4). During NA degradation a major metabolite with a retention time between 16.70 (metabolite of *tert*-BCHBA) and 17.31 min (metabolite of *n*-BCHBA) was produced and tentatively identified as butylcyclohexylethanoic acid (Smith et al., 2008).

The shifts in the microbial communities during NA degradation were characterised using DGGE and clone libraries. DGGE profiles from the enrichments grown on *n*-BCHBA demonstrated the presence of three bands that were absent in the inoculum (or in low abundance) but became predominant after 30 days and may be associated with NA degradation. These bands had 96% 16S rRNA gene sequence similarity to *Pseudomonas aeruginosa*. DGGE profiles from the enrichments grown on *iso*-BCHBA demonstrated the presence of five additional bands (present after 30 days enrichment) and had 96% 16S rRNA gene sequence similarity to an uncultured *Alphaproteobacterium*.

Three bands which were predominant at 30 days compared to the inoculum in the enrichment culture grown on *sec*-BCHBA had 97–100% 16S rRNA gene sequence

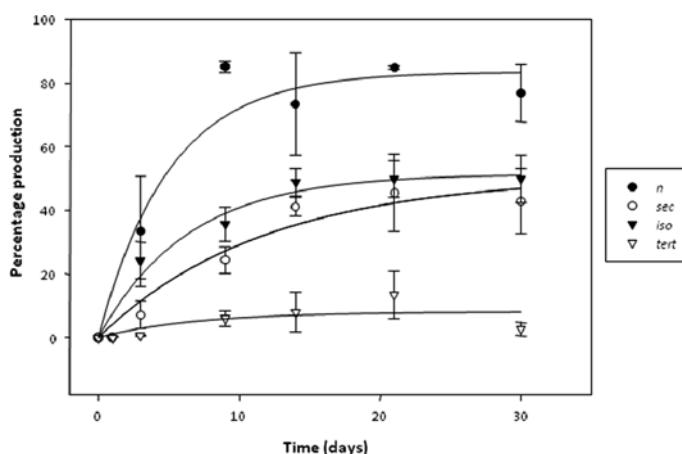


Fig. 28.4 Percentage production of major metabolites (butylcyclohexylethanoic acids) during biotransformation of aliphatic NAs expressed as percentage of the initial amount of substrate. NAs: *n*-BCHBA (closed circles), *iso*-BCHBA (closed triangles), *sec*-BCHBA (open circles) and *tert*-BCHBA (open triangles) (Smith et al., 2008)

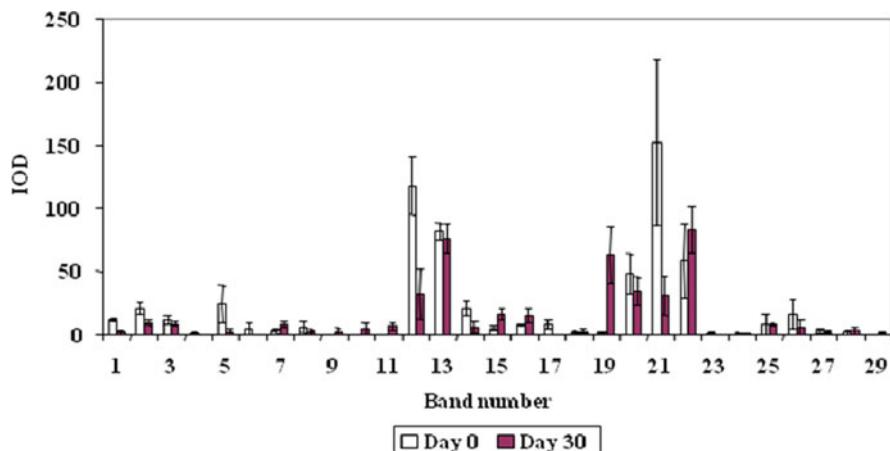


Fig. 28.5 Scanning densitometry of DGGE band profile of the 16S rRNA genes PCR-amplified from an enrichment culture grown in the presence of *tert*-BCHBA. *IOD* integrated optical density

identity to uncultured bacterium clones. The DGGE profiles obtained from the cultures grown on the *tert*-BCHBA were also diverse and scanning densitometry analysis of the DGGE banding profile obtained is shown (Fig. 28.5).

The initial culture was predominated by bands 12 and 21. Growth on the *tert*-BCHBA selected for a dominant band at day 30 compared to day 0 (band 19) which had 97% 16S rRNA gene sequence identity to a *Pseudomonas* sp. and may represent an important *tert*-BCHBA-degrading microorganism.

Analysis of clone libraries identified a diverse number of microorganisms. A total of 90 clones were screened using DGGE analysis and 28 clones were selected for sequence analysis. Two clones (denoted N1 and N3) recovered from the *n*-BCHBA enrichment after 30 days had 97–98% sequence similarity to *Pseudomonas* sp., thus supporting the DGGE data that *Pseudomonas* sp. may be involved in NA degradation. A further six clones recovered from the *tert*-BCHBA enrichments had high 16S rRNA gene sequence identity (98–99%) to uncultured bacterium clones. In addition, three clones (denoted T3, T5 and T27) also from the *tert*-BCHBA enrichment had 97–98% 16S rRNA gene sequence identity to an *Alphaproteobacterium*. All the partial 16S rRNA gene sequences (850 bp) that were recovered were aligned against database 16S rRNA gene sequences using Seaview (Galtier et al., 1996). Phylogenetic analysis was performed using PHYLIP 3.4 using Jukes–Cantor DNA distance and neighbour-joining methods (Jukes and Cantor, 1969; Saitou and Nei, 1987). Bootstrap analysis was based on 100 replicates using SEQBOOT (PHYLIP 3.4). A neighbour-joining tree was then constructed using Treeview (WIN32) version 1.5.2 (Page, 1996) (Fig. 28.6). The Eubacterial 16S rRNA gene sequences from the clones were spread throughout the *Alpha*-, *Beta*- and *Gammaproteobacteria* demonstrating the diversity of microorganisms which may be involved in NA degradation.

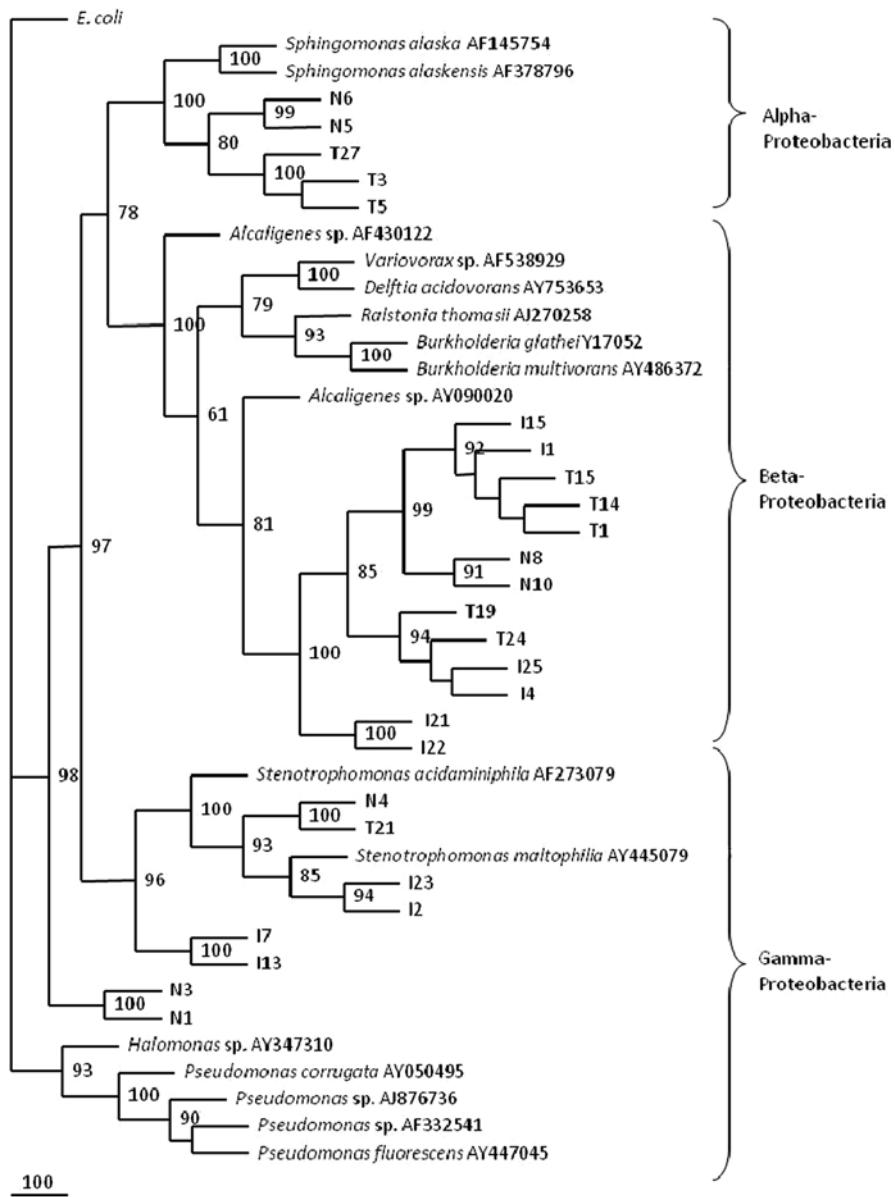


Fig. 28.6 Phylogenetic analysis of the 16S rRNA genes from selected clones. Prefix T denotes clones derived from *tert*-BCHBA enrichment; prefix N denotes clones derived from *n*-BCHBA enrichment; prefix I denotes clones derived from the inoculum. Named species indicate reference database sequences

Conclusions

Many NAs used in previous studies may not be representative of those NAs found in the environment. In this case study, >97% of the *n*-BCHBA was degraded compared to *tert*-BCHBA (only 2.5% was degraded) after 30 days incubation. Such differences in the degradation rates may be due to the increased branched structure in the alkanoic acid side chain causing steric hindrance. DGGE and clone library analysis identified members of the NA-degrading microbial consortia with high 16S rRNA gene sequence identity to either previously uncultured bacteria or *Pseudomonas* spp. Phylogenetic analysis of the 16S rRNA gene sequences demonstrated the NA-degrading community structure was diverse and spanned the *Alpha*-, *Beta*- and *Gammaproteobacteria*. However, such high microbial community diversity found in this study may be due to microorganisms ‘cross-feeding’ on the metabolites produced during NA degradation and further analysis is required. In addition, DGGE bands which decreased in band intensity during enrichment on NAs may represent genotypes susceptible to NA toxicity, and further research is required.

It is generally accepted that beta-oxidation is one of the main pathways involved in NA degradation (Quagrainne et al., 2005). The metabolites produced and identified in this study were consistent with beta-oxidation of the alkanoic side chain with the removal of two CH₂ groups. This further confirms that beta-oxidation is a major metabolic route for the biotransformation of NAs.

Future Perspectives

The use of molecular techniques in our studies to characterise the microbial communities capable of degrading aliphatic and aromatic NAs is a major advance and we are now starting to gain an insight into the community structure, diversity and activity of the microorganisms, involved in the degradation of these recalcitrant NA fractions. Such information is crucial if we are to better understand the pathways and functional genes involved in NA degradation. This information would enable us to optimise NA-degrading microbial consortia and pure cultures more efficiently. This would facilitate the future development of more cost-effective strategies for exploiting these novel fuel resources more effectively and in an environmentally responsible manner.

Acknowledgements We thank Max Frenzel for his technical assistance.

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Appendix: Description of the Methods Used by Some of the Contributing Authors in This Book

Chapter 2: Sampling and Nucleic Extraction Procedures from Oil Reservoir Samples

Materials

Jerry cans (10 l), silicon hose (ca. 2 m length, 1 cm diameter), silicon hose (ca. 2 m length, 0.4 cm diameter), connectors and steel tubes (1 cm diameter, connectors: 1–0.4 cm), fabric pieces (15 × 15 cm), displacement pump (Cole parmer, USA), hollow fibre filter (Spectrumlabs, mediakap-5), ice box.

Methods

Sterilise the silicon hoses, connectors, steel tubes and pieces of fabric at 121°C. Take a piece of sterile tubing (1 cm diameter) and place an 8 cm long sterile piece of steel piping in one end. Wrap a piece of sterile fabric around the piece of pipe. This is the nozzle used to suck the water fraction from the bottom of jerry cans. The piece of fabric is used to prevent large pieces of material and the larger part of the oil fraction from entering the hose. Sterilise the complete nozzle with hose again at 121°C and store it. Use sterile 10 l jerry cans. Take samples using the jerry cans at the well or processing site (do not forget to discard the first produced water fraction). Fill the jerry cans up to the top and seal them directly to avoid O₂ intrusion. Take samples to the laboratory as soon as possible. Let the oil and water fractions separate by natural flotation. Connect the 1 cm diameter tube to a displacement pump (holding a pump head that allows a 1 cm thick tube). Use a connector to connect the thick tube to the narrow tube. Flush the system with a fraction of your production water. Now cut the narrow tube into two and put a clean filter in between. Set the pump rate at 4 ml/s. Keep the nozzle at the bottom of the jerry can. Allow 4 l of liquid to pass through the filter. Stop the flow and disconnect the filter, wrap it in parafilm and place on ice. Connect a second clean filter, and filter again 4 l of water. Store one filter in the freezer. Repeat the steps above and connect a new piece of tubing. Filter your next sample.

DNA Extraction of Brine Water Samples Using the MoBio Soil DNA Extraction Kit (Qiagen)

Materials

Sterile plastic Petri dishes, sterile 1.5 ml eppendorf tubes, a surgical knife (allowing blades to be replaced), a gas burner suited for laboratory use, centrifuge, ice box, MoBio soil DNA extraction kit (Qiagen).

Methods

Filter cartridges were opened using a heated surgical knife under a flame. The released suspension was collected in a sterile Petri dish (around 2 ml); 1 ml amounts of this suspension were collected in sterile 1.5 ml eppendorf tubes that were subsequently placed on ice. The filters were then backwashed with buffer according to the manufacturer's instructions. The suspension was again collected in eppendorf tubes and placed on ice. The filter lamellas were squeezed, releasing an additional volume of suspension. Finally the filter lamellas were cut using sterilised scissors; also these pieces were collected and placed on ice. Two selected 1 ml collected amounts from the released suspension and the backwashed suspension were centrifuged at 13,200 rpm for 2 min. From these centrifuged amounts, 900 µl of supernatant was removed. The pellet was resuspended into the remaining supernatant. The collected materials now were subjected to DNA extraction using the MoBio soil DNA extraction kit (Qiagen). These materials were as follows: released suspension, centrifuged suspension, backwashed suspension, centrifuged backwashed suspension, water from the filter and cut lamella pieces. A change in the protocol was as follows: the bead beating was extended by 5 min. Since the samples contained a lot of liquid, after bead beating, the volume of the sample was divided into two, and subsequent steps up, until the actual DNA retrieval step were carried out, and then combined.

DNA Extraction of an African Oilfield Core Sample Using the MoBio Soil DNA Extraction Kit

Materials

Bottle of liquid N₂, dry ice, mortars and pestles (sterilised at 160°C for 5 h), sterile chisels and spatulas (121°C), a surgical knife (with blades), a gas burner suited for laboratory uses, centrifuge, ice box, safety glasses, MoBio soil DNA extraction kit (Qiagen).

Methods

Sterilise four pestles and mortars at 160°C for 5 h (wrapped in aluminium foil). Keep a bottle of liquid N₂ on the table. Unpack the core under a flame. Scrape off the top layer of material using a sterile surgical knife. Unpack one of the mortars and pestles. Cut the core using a sterile chisel and a hammer. Pour liquid N₂ in one of the mortars. Take pieces of released core material and put one in the N₂-filled mortar. Crush the sample with the pestle keeping the sample submerged in liquid N₂. Place the core back on dry ice. Take 1 g samples from the crushed material and place it directly in a bead beater tube from the MoBio soil DNA extraction kit. Repeat steps with new core pieces using sterile chisels, mortars and pestles. Reduce the amount of time; the core is exposed to temperatures above 0°C.

Pre-treatment Steps for the DNA Extraction of Two Dutch Oilfield Core Samples

Materials

Sterile spatulas (121°C), sterile surgical knife (121°C), skimmed milk (VWR), poly deoxinocinic-deoxycytidylc (pdIdC, Sigma Aldrich), powersoil DNA extraction kit (Qiagen), dry ice, a gas burner suited for laboratory use, various wash solutions:

Wash solution 1:	50 mM Tris–HCl (pH 8.3)	500 µl
	200 mM NaCl	400 µl
	5 mM Na ₂ EDTA	100 µl
	0.05% Triton X-100	5 µl
Fill with demi-water or MilliQ water to 10 ml		
Wash solution 2:	50 mM Tris–HCl (pH 8.3)	500 µl
	200 mM NaCl	400 µl
	5 mM Na ₂ EDTA	100 µl
Fill with demi-water or MilliQ water to 10 ml		
Wash solution 3:	50 mM Tris–HCl (pH 8.3)	500 µl
	5 mM Na ₂ EDTA	100 µl
Fill with demi-water or MilliQ water to 10 ml		

Methods

Sterilise a surgical knife at 121°C. Unpack the core under a flame; keep the core on dry ice. Scrape off the top layer of material using a sterile surgical knife. Take 0.25 g of sample and place it directly in a bead beater tube. For the washing pre-treatment,

collect 5 g of sample and place it in a 15 ml tube. Skimmed milk pre-treatment: per g of soil sample, 80 ng of skimmed milk was added. Poly deoxinocinic-deoxycytidylc (pdIdC): per g of soil sample, 10 µg pdIdC was added.

Methods: Wash Protocol

Make wash solutions with the compositions as given below: 5 g of sediment was washed with 10 ml of wash solution 1, by vortexing for 2 min. The mixture was centrifuged for 3 min at 13,200 rpm. Both steps were repeated with wash solutions 2 and 3. Transfer 0.25 g of washed soil sample into a bead beater tube. After the various pre-treatments (described above), the powersoil DNA extraction kit (Qiagen) was applied. No modifications were done regarding the standard protocol as supplied by the manufacturer.

Chapter 5: Application of PCR-DGGE: Case Study on an Oilfield Core Sample

A Touchdown PCR Amplification of Bacterial 16S rRNA Gene Fragments

Materials

Sterile PCR tubes (eight tube strips), sterile 1.5 ml eppendorf tubes, T1 thermocycler (Biometra, Germany), *Taq* PCR master mix (containing *Taq* polymerase and free nucleotides, Qiagen), nuclease-free water (Qiagen), primers (BAC-341F+GC, BAC-907Rm) (50 mM stock), template DNA from a sampled source.

Methods

Execute protocol per sampled environment. Make serial dilutions of the template DNA. Add 25 µl of 2 × master mix to PCR tubes. Add 1 µl of each primer to the PCR mix. Add 1 µl of each dilution as template DNA in the different PCR reactions. Fill the tube with nuclease-free water until a volume of 50 µl is reached. Place all PCR tubes in a T1 thermocycler; start the specific PCR program.

Eubacterial Touchdown PCR Program

A denaturation step of 5 min at 95°C was followed by 20 cycles of denaturation at 95°C for 1 min, annealing for 1 min at a start temperature of 65°C and chain elongation for 20 s at 72°C. During these first 20 cycles, the temperature of each annealing step in each new cycle was decreased by 0.5°C in order to prevent PCR bias. The final annealing temperature after 20 cycles was 55°C. This touchdown

program was followed by 12 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and chain elongation at 72°C for 20 s. The program was finished with a 15 min final elongation step at 72°C. After this final elongation, the temperature was decreased to 10°C. PCR products were analysed using a 1.5% (w/v) agarose gel run at 100 V for 45 min.

PCR Amplification of Archaeal 16S rRNA Gene Fragments

Materials and Methods

As above, except the primers used were specific for archaeal 16S rRNA fragments, i.e. Primers (Parch-519 fm and ARC-915R+GC).

Archaeal PCR Program

A denaturation step of 5 min at 95°C was followed by 32 cycles of denaturation at 95°C for 30 s, annealing for 40 s at 57°C and chain elongation for 40 s at 72°C. The program was ended with a final extension step of 10 min at 72°C. After this final elongation step, the temperature was decreased to 10°C. PCR products were analysed using a 1.5% (w/v) agarose gel run at 100 V for 45 min.

DGGE Analysis of Bacterial and Archaeal 16S rRNA Gene Fragments

Materials

Denaturing Gradient Gel Electrophoresis system, e.g. DCode system (Bio-Rad) or PhorU system (Ingeny), blue light safe imager (Syngene), C-box docking system and associated genesnap software (Synoptics), peristaltic pump, a gradient former. We use a combination of peristaltic pump (Model EP-1, Bio-Rad) and gradient former (Model 385, Bio-Rad) to cast gels. Fluorescent DNA stain, Gelgreen (Biotum).

Preparation of the Acrylamide/Bisacrylamide Stock Solutions and Required Reagents

Formamide (de-ionised)

- Acrylamide/bisacrylamide stock solution (37.5:1, 40%) (e.g. Bio-Rad 161-0149). (*Acrylamide is a powerful neurotoxin and should be handled with care.*)

- 10% ammonium persulphate solution (ammonium persulphate 1.0 g, water to 10 ml). Aliquot into single-use portions and store at -20°C.
- TEMED is bought as a ready-to-use solution (e.g. from Fluka or Bio-Rad)
- 50 × TAE buffer (2 M Tris, 2 M acetic acid, 50 mM EDTA; pH 8.3)
- 1 × TAE running buffer. (Dilute 1 volume of 50 × TAE buffer with 49 volumes of MilliQ water.)

Prepare 6% (w/v) acrylamide/bisacrylamide gradient solutions according to the amounts of reagents shown below:

	0% Urea/formamide	80% Urea/formamide
Acrylamide/bisacrylamide	15 ml	15 ml
50 × TAE (pH 8.3, stock)	2 ml	2 ml
Urea	—	33.6 g
Formamide	—	32 ml
MilliQ	Fill to 100 ml	Fill to 100 ml

Filter the solution (e.g. through a Schleicher & Schuell folded filter 595 1/2) and store at 4°C in a dark bottle.

Notes Before Running a DGGE Gel

To achieve the maximum resolution in DGGE patterns of unknown samples it is recommended to determine the best gradient conditions. This requires running perpendicular denaturing gels with the unknown sample to define the range of denaturant concentrations that allow the best separation possible. Gradients ranging from ca. 10–20 to 70–80% denaturant concentration (urea and formamide; UF) result in a good separation of fragments obtained by PCR with the mentioned bacterial and archaeal primers and provide a security margin for fragments melting at unexpectedly high denaturant concentrations at the same time. It is strongly recommended to run time-travel experiments when starting DGGE analysis in combination with a perpendicular gradient DGGE analysis, to check for optimal separation. (See DGGE manual by Bio-Rad for further details.)

Assembly and Casting of Parallel Denaturing Gradient Gels

- Clean the glass plates and spacers with water and soap. Rinse them with demineralised water.
- Wipe the glass plates first with 70% ethanol and then with acetone. Use a dust-free cloth (e.g. Kimwipes). Do not wipe any plastics (e.g. spacers, combs) with acetone.

- Wipe the spacers (1 mm thickness) with ethanol and let them dry, then sparingly smear grease (high vacuum grease; Dow Corning, Auburn, MI, USA) along one of the long edges, such that around 2 mm is covered with a thin grease film on each face of the spacer.
- Place the large glass plate on a clean surface, and put the spacers onto the left and right margins, such that the greased edges face the outside edge of the glass plate.
- Put the small glass plate on top of the spacers, to form a ‘sandwich’.
- Align the spacers and the glass plates in such a way that they are flush at the bottom of the sandwich (this can be done on an even surface, or in the aligning slot of the casting stand).
- Attach the clamps to the sandwich, tighten the clamp screws (finger-tight) and put the sandwich in the casting stand, fix in casting slot by turning the levers. Before proceeding to the next step, make sure that the device used for casting the gradient gel is installed and you are familiar with the procedure described below. The work has to proceed quickly, otherwise you run the risk of gel solutions polymerising before casting is finished.
- Connect the tubing of the pump with the outflow chamber of the gradient chamber. Attach an injection needle to the LuerChapter lock of the outlet tubing of the pump and insert the needle between the glass plates in the middle of the gel sandwich.
- Prepare the high and low denaturant solutions for the gradient as required in disposable plastic tubes. Using 1 mm thick spacers, 12 ml each is recommended.
- The gradient gel will finally be overlaid with a 0% denaturant acrylamide solution (prepare 5 ml), otherwise the presence of the denaturants hinders the formation of appropriate wells for loading the sample.
- Add ammonium persulphate and TEMED to the gradient solutions. Add 60 μl of ammonium persulphate and 8 μl of TEMED to each solution; directly pipette these into the solutions. Close tubes and mix thoroughly by inverting several times.
- To inspect the gradient, add 120 μl of gradient-dye solution to the high denaturant solution. Close the tube and mix by inverting several times.
- Close the connection pipe between the two chambers of the gradient chamber; make sure the pump is not running. Pour the high denaturant gel solution into the outflow chamber of the gradient chamber.
- To remove air bubbles in the connection pipe, slowly open the pipe by turning the lever aside until the air has been expelled from the pipe and a drop of high denaturant gel solution is visible on the bottom of the second chamber. Then close connection pipe, and pipette back all high denaturant gel solution back to the outflow chamber using a clean pipette tip.
- Carefully add the low denaturant gel solution into the second chamber.
- Turn on the magnetic stirrer at 250 rpm, then turn on the pump and slowly open the connection pipe, such that no extra high denaturant gel solution enters the second chamber. Cast the gel with ca. 4 ml/min. The last 1 ml of the gradient gel will not be mixed properly (due to remains of high denaturant gel solution in the

connecting pipe of the gradient chamber), hence avoid delivery of that last bit of gradient solution, as it will disturb the top of the gradient gel.

- Remove needle from gel sandwich. Rinse gradient chamber and pump tubing with water to remove residual gel solution.
- Clean a comb (1 mm thick) with ethanol and let it dry.
- Add 25 µl of ammonium persulphate and 5 µl of TEMED to 5 ml of 0% denaturant gel solution.
- Close the tube and mix.
- Carefully overlay the gradient gel with about half of the 0% denaturant gel solution using a 1,000 µl pipette.
- Insert the comb at an angle to avoid the formation of air-bubbles.
- Completely fill the gel sandwich with the remainder of the 0% denaturant gel solution. Let the solution polymerise for at least 2 h.

DGGE Set-Up Preparation

- Fill the electrophoresis tank with approximately 7 l of 1 × TAE buffer.
- Insert the core (the device that can hold a DGGE gel).
- Two gels can be attached to the core and run at the same time. If only one gel is run, attach a buffer dam at the other site. The buffer dam can be made of a large and small glass plate without spacers and held together by the sandwich clamps.
- Carefully place the lid (i.e. the electrophoresis/temperature control module) on the electrophoresis tank. Take care that the end of the stirring bar comes in its proper position.
- Switch on the DCode system with the on/off button on the electrophoresis/temperature control module.
- Switch on the buffer recirculation pump and the heating element. Set the temperature to 60°C and set the ramp rate to 0. The buffer will reach the temperature in about 1 h.
- Prepare the samples by adding between 5 and 10 µl of gel-loading solution. Mix the samples and spin briefly.
- Remove the comb slowly, when the acrylamide gel is polymerised.
- When the buffer has reached 60°C, switch off the electrophoresis unit, wait at least 15 s before removing the lid and place the lid on the lid stand.
- Take out the core, pre-wet the sandwich clamps of the gel sandwich and attach to core.
- Replace the core in the electrophoresis tank.
- Take a 25 ml syringe, pull up the buffer from the electrophoresis tank, attach a needle and rinse the wells of the denaturing gel to remove traces of non-polymerised acrylamide.
- Load the samples into the wells with a 50 µl Hamilton syringe.
- Thoroughly rinse the syringe with electrophoresis buffer between the different samples.

- Put the lid on the buffer tank and turn on electrophoresis unit and connect the cords to the power supply.
- Run the gel at constant voltage of 10 V for 10 min while the temperature is brought back to 60°C.
- Run the gel at a constant voltage of 100 V for 18 h. The amperage should be around 35 mA. In case of archaeal gene fragments use 200 V for 5 h.
- After 18 h (5 in case of the archaea), turn off the power supply and the electrophoresis unit. Wait at least 15 s before removing the lid. Take out the core and detach the gel sandwich.
- Remove carefully one of the glass plates as well as the spacers. Stain the gel on the glass plate with Gel Green (Biotum, USA) DNA staining solution.
- Transfer the gel to a blue light safe imager.
- Make photos of the gel in a C-box doc system (Syngene) using the associated genesnap software (Synoptics).
- Take several photos of the gel with varying exposure times (optimal, underexposed and overexposed). Underexposed photographs may help to define very intense bands, while overexposed photographs may help to identify additional faint bands.

Excision of DGGE Bands (and Re-amplification)

Materials

Surgical knife, gas burner for laboratory use, a blue light safe imager, sterile eppendorf tubes, dark room, nuclease-free water (Sigma Aldrich), Exosap-IT enzyme solution (USB Europe), T1 thermocycler (Biometra, Goettingen, Germany), *Taq* PCR master mix (containing *Taq* polymerase and free nucleotides, Qiagen), primers (as earlier described during the original PCR, optional similar primers can be used without the GC-clamp).

Methods

After documentation of the denaturing gel, make a printout of the gel and mark all bands that are to be excised and sequenced. Assign each band a number and label a corresponding number of 0.5 ml reaction tube, accordingly. Transfer the gel onto a blue light safe imager. Sterilise the scalpel using the gas burner flame blade with ethanol and allow it to cool. Shortly after, cut out band of interest and pick it up with the blade or with forceps. Transfer the gel piece to the labelled tube. Continue excising bands as described, until all bands of interest have been excised. (Sterilise the blade in between each excised band.) Add 25–50 µl of nucleic acid free water, spin down and incubate at 4°C overnight. Use water from the supernatant as template for reamplification with the same primers as for the PCR for DGGE (optional without GC-clamp), store the remainder at –20°C. After the reamplification, remove

free nucleotides and primers from the PCR product pool using the Exosap-IT enzyme cocktail. Dilute the cleaned PCR product accordingly and perform gene sequencing.

Exosap-IT Enzyme Program

To each reamplification reaction 1.6 µl Exosap-IT enzyme solution was added. PCR tubes were placed in a T1 thermocycler. They were kept at a temperature of 37°C for 30 min to activate the enzymes subsequently followed by an enzyme denaturation step at 80°C for 15 min; 1 µl of each purified product was checked for purity and concentration on a 1.5% agarose gel. The PCR products were diluted accordingly to obtain a 50 ng/µl PCR product solution and subsequently sequenced.

Chapter 6: Application of Clone Libraries: Syntrophic Acetate Degradation to Methane in a High-Temperature Petroleum Reservoir

Methods

Sample Collection

Production fluids were collected directly from the production wells into sterilised bottles and immediately prepared for chemical analyses, radioisotope and culture studies. The bottles were filled to capacity with the oil–water–gas mixture and stored at 4°C prior to analyses. For DNA isolation, water was separated from crude oil by decantation at room temperature. Triton X100 (0.1% v/v) and *n*-hexane were added to the samples. After hexane extraction, the water phase was fixed with ethanol (1:1 v/v) and stored at 4°C prior to analyses.

Culture Media and Growth Conditions

Anaerobic bacteria were cultivated in Hungate tubes with pure argon as the gas phase; an exception was the medium with H₂/CO₂ for methanogens. The numbers of cultivable anaerobic microorganisms were estimated using the method described previously (Nazina et al., 2007). Enrichment media were either degassed by boiling under a stream of O₂-free argon (for fermentative bacteria) or reduced with Na₂S·9H₂O (for sulphate-reducing bacteria and methanogens); pH was adjusted to 7.0–7.2. Syringes were used to inoculate the media with the samples of formation water and were incubated at 60°C. Microbial growth was assayed by phase-contrast microscopy and by measuring the content of H₂, sulphide and CH₄ in the media for anaerobic organotrophs, sulphate reducers and methanogens, respectively (Bonch-Osmolovskaya et al., 2003). The chemical composition of the formation water was analysed as described previously (Bonch-Osmolovskaya et al., 2003).

Isolation and Identification of Pure Cultures

Pure cultures of methanogens were grown in a liquid mineral medium with H₂/CO₂ (Nazina et al., 2007) supplemented with ampicillin (2 mg/ml). The same media supplemented with sodium pyruvate (40 mM) was used for isolation of anaerobic bacteria from methanogenic enrichments growing on acetate. For reconstruction of syntrophic methane production by the binary culture, the medium with sodium acetate (80 mM) was used. The isolates were identified by 16S rRNA gene sequencing as described below.

Radioisotope Method

The rates of sulphate reduction and methanogenesis in formation waters were determined by radioisotope method using labelled Na₂³⁵SO₄, ¹⁴CH₃-COONa and NaH¹⁴CO₃ as described previously (Bonch-Osmolovskaya et al., 2003).

Construction and Analysis of 16S rRNA Gene Clone Libraries

DNA Extraction

Total DNA was extracted from formation water and cultures using the DiatomTM DNAPrep kit ('BioKom', Russia). Microbial biomass from approximately 2 l of the water phase of production fluids and from 100 ml of enrichment cultures was collected by centrifugation (Beckman JA10, 8,000g × 1 h). The cell pellet was resuspended in MilliQ water and was twice frozen with liquid molecular nitrogen and thawed (at 65°C). The cell lysate was then supplemented with guanidine hydrochloride and incubated at 65°C for 1 h. The sorbent (Diatomid/silica) was added to the cell lysate. After DNA sorption, the supernatant was removed and the adsorbent was washed using a buffer (pH 7.0) and 70% (v/v) ethanol. The total DNA preparation was dissolved in MilliQ water and used for 16S rRNA gene amplification.

16S rRNA Gene Amplification and Cloning

16S rRNA genes were amplified by PCR using the primers specific for *Bacteria* (8-27f [5'-AGAGTTGATCCTGGCTCAG-3'], 519r [5'-G(T/A)ATTACCGCGGC (T/G)GCTG-3'] and 1492r [5'-TACGGYTACCTTGTACGACTT-3']) (Weisburg et al., 1991) and *Archaea* (A109F [5'-ACG/TGCTCAGTAACACGT-3'], A1041r [5'-GGCCATGCACCW CCTCTC-3']) (Kolganova et al., 2002). The final 50 µl reaction mixture contained 5 µl of template DNA, 0.5µM of each primer, 1 × DNA polymerase buffer, 2.5 mM MgCl₂, 0.1 mM of each deoxynucleoside triphosphate and 1 U of *Taq* polymerase (Perkin-Elmer). Polymerase chain reaction cycles were performed on a Mastercycler (Eppendorf, Germany) as follows: after 5 min of initial

denaturation at 94°C, nucleic acids were amplified for 30 cycles (30 s of denaturation at 94°C, 30 s of annealing at 50°C and 30 s–1.5 min of elongation at 72°C) followed by a final extension step at 72°C for 8 min. Archaeal 16S rDNA was amplified at 35 cycles of PCR. PCR products were separated on 0.8% (wt/vol) agarose gel and stained with ethidium bromide. The amplicons were cloned into a pGEM-T vector (pGEM-T Easy Vector Systems, Promega) according to the manufacturer's instructions.

Sequencing and Phylogenetic Analysis

Inserts of selected clones were amplified by PCR with T7 and SP6 plasmid primers. The same primers were subsequently used to sequence bacterial PCR products. Archaeal PCR products were sequenced with the A109f primer. The sequencing was performed on an ABI 3100 Avant Genetic Analyser with the BigDye Terminator V3.1 (Applied Biosystems, USA).

Sequence data were aligned using the CLUSTALW v.1.75 package, with clones having similarities of 97% or above grouped into operational taxonomic units (OTUs). The clones were homology-searched using BLAST and the GenBank database of NCBI (<http://www.ncbi.nlm.nih.gov>). Chimeras were detected using the CHIMERA-CHECK program from the Ribosomal Database Project (<http://rdp.cme.msu.edu>). The sequences were edited using the BioEdit software package (<http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html>).

Nucleotide Sequence Accession Numbers

The sequences identified in this study were submitted to the GenBank database under the following accession numbers: DQ657903–DQ657906 (pure cultures) and FJ898323–FJ898364 (clones).

Design of Specific Primers

To analyse the predominant phylotypes in the clone libraries, we designed two specific primers. Primers M400r (GAAAAGCCACCCCGTTAAGA) and Sph196r (CTCGGCGATAATCTTGGAC) were developed to detect *Methanothermobacter* sp. and *Sphingomonas* sp., respectively. The primers were designed using the BioEdit and Lasergene v. 5.06 software packages. Melting temperature was analysed with the Oligo v. 6 program and used in the PCR procedure. The specificity of the primers was then tested by using DNA extracts from strains and clones with 16S rDNA inserts representing different species. Each specific primer gave positive PCR results for the corresponding target 16S rDNA and negative PCR results for non-target 16S rDNA.

Chapter 9: Quantitative Reverse Transcription PCR (qRT-PCR)

Construction of Quantitative Real-Time PCR Standards

Target sequences of 123 and 1,412 bp corresponding to the *dsrA* and *dsrAB* genes, respectively, were amplified from a *Desulfovibrio desulfuricans* 342 culture (DNA extraction performed as described in Section 2, but using 2 ml of culture). PCR reactions were performed in a final volume of 50 µl containing 1–2 µl of template, 0.4 µM of both primers (DSR1F-DSR2QR for *dsrA* or DSR2F-DSR4F for *dsrAB*), as in Chin et al. (2004, 2008), 0.1 mM of deoxynucleoside triphosphates, 2.5 units of *Taq* polymerase and 1.5 mM of MgCl₂ (buffer supplied with the enzyme: Qiagen®). Thermocycling consisted of an initial denaturation at 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s, with a final extension of 72°C for 10 min. Amplification was confirmed by electrophoresis on a 2% (w/v) 1 × TAE agarose gels.

The ssDNA (single-stranded DNA) template used as a standard in the qPCR was obtained by cloning of the target sequences into a plasmid, followed by PCR amplification with the vector primers, in vitro transcription and reverse transcription in order to get the corresponding cDNA.

The target sequences of 123 and 1,412 bp corresponding to the *dsrA* and *dsrAB* genes, respectively, were inserted into a pCR® 2.1 linearised plasmid (Invitrogen) and the plasmid was then used to transform competent TOP10 *Escherichia coli* cells (Invitrogen). Insert-containing transformants were selected on Luria-Bertani agar plates with 100 µg/ml of ampicillin and 40 mg/ml of X-Gal. Insert-containing transformants were determined by performing a colony PCR with the vector primers M13 forward and reverse, which resulted in a PCR product of the target sequence flanked on both sides. PCR reactions were performed in a final volume of 50 µl containing 0.4 µM of both M13 primers, 0.1 mM of deoxynucleoside triphosphates, 2.5 U of *Taq* polymerase and 1.5 mM of MgCl₂ (buffer supplied with the enzyme: Qiagen®). Thermocycling consisted of an initial denaturation at 95°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension of 72°C for 10 min. Amplification was confirmed by electrophoresis on 2% (w/v) 1 × TAE agarose gels. The amplicon was purified using the QIAquick PCR purification kit (Qiagen) and quantified with a Nanodrop ND-1000 spectrophotometer.

cDNA templates were created by in vitro transcription of the corresponding flanked amplicon obtained as described above followed by reverse transcription of the mRNA using the vector M13 reverse primer. As the pCR® 2.1 plasmid used for cloning the targets contained a T7 promoter downstream of the M13 forward primer, the in vitro transcription of the purified flanked amplicon with the T7 RNA polymerase included in the MEGAshortscript TM T7 kit (Ambion) could be performed. The DNA template was removed by treatment with the TURBO DNase included in the kit and the resulting mRNA was quantified with the Nanodrop spectrophotometer. cDNA was obtained by reverse transcription of 1 µl of mRNA

with 1 µl of Bioscript reverse transcriptase (Bioline) and 20 pmol of M13 reverse primer to produce a flanked cDNA template.

The copy numbers for mRNA transcripts were calculated assuming a molecular mass of 330 Da for RNA.

cDNA standard curves were created by producing a dilution series of template ranging from 10^4 to 10^{11} amplicons/µl.

Total Nucleic Acid Extraction

To ensure that the extractions were free of RNases, all the solutions were prepared with diethyl-pyrocarbonate-treated water, all the glassware employed was baked for 8 h at 180°C, bottle plastic lids were treated with 2 M NaOH and only RNase-free plastic tubes were used.

Total nucleic acids were obtained by centrifuging at 5,000 rpm for 10 min at 4°C. To the pellet obtained, 0.5 ml of 0.1 M sodium phosphate buffer (pH 8.0) and 0.5 ml phenol–chloroform–isoamyl alcohol (25:24:1) were added and the suspension was transferred to Lyzing Matrix B tubes (Bio 101 System, Q-Biogene®). Cells were bead beaten in a Mikro-dismembrator-U (B. Braun, Biotech International) at 2,000 rpm for 30 s and centrifuged again (13,100 rpm for 1 min at 4°C). Up to 450 µl of the aqueous upper layer was carefully pipetted and mixed by inversion with 1 volume of chloroform–isoamyl alcohol (24:1). After centrifugation (13,100 rpm for 1 min at 4°C), 400 µl of the upper phase was transferred to a clean tube. For DNA precipitation, 0.1 volume of 3 M sodium acetate and 2.5 volume of absolute ice-cold ethanol were added to the previous aqueous phase and the mixture incubated on ice for 30 min. Nucleic acids were pelleted by centrifugation (13,200 rpm for 25 min at 4°C) and subsequently washed twice with 1 ml of ice-cold 70% ethanol. The pellet obtained was air-dried and resuspended in 40 µl of diethyl-pyrocarbonate-treated water. From this suspension, 20 µl was kept at -20°C for gene copy number quantifications (this would be the total nucleic acids), and to obtain the RNA, a sub-sample of 20 µl was digested with TURBO DNase (Ambion) according to the manufacturer's instructions. PCR was performed to ensure there was no contaminating DNA. The control PCR was carried out using 0.4 µM of primers 27F and 1492R targeting the 16S rRNA sub-unit, 0.1 mM of dNTPs, 0.5 mM of Qiagen PCR buffer, 2.5 units of *Taq* polymerase and 1 µl of RNA sample. The mixture was denatured at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s, with a final step of 10 min at 72°C. The RNA extraction method followed is well established and has previously been shown to produce good reproducibility of RNA extractions (McKew et al., 2007).

For oilfield samples, 2 l of produced water (treated with RNAlater® (Ambion) was filtered through 0.22 µm filters for each well, and filters subjected to nucleic acid extraction. Filters were introduced directly into Lyzing Matrix B tubes and the appropriate buffers added.

Reverse Transcription Reaction

cDNA was produced by reverse transcription of 1 µl RNA with 1 µl Bioscript reverse transcriptase (Bioline) and two different kinds of primers: 0.2 µg of random primers (Invitrogen) or 20 pmoles of the specific primer (DSR2QR or DSR4R) following the manufacturer's instructions.

Quantitative Real-Time PCR Analysis

It should be noted that the efficiency of the reaction was much lower for gene *dsrAB* due to its large size. This product consists of 1,412 bp, while the recommended product size for qPCR is 75–250 bp. Products longer than 250 bp involve lower sensitivity, therefore the method has been optimised for short amplicons, never longer than 500 bp in any case. Efficiency and reproducibility were much better with the *dsrA* gene (123 bp), therefore this gene sub-unit was used as the template for producing the standards, and primers specific for *dsrA* were used to detect *dsrA* in the produced water samples. *dsrA* gene (1F-2QR primers; 123 bp) and 16S rRNA gene (945F-1087R primers; 140 bp) were tested for quantification of DNA copies by qPCR. The *dsrA* gene and 16S rRNA gene were cloned into vectors and PCR amplified in order to produce a product, which was used as a standard in the qPCR (absolute quantification), as for cDNA standards. Following RNA extraction and processing, qRT-PCR was performed on produced water samples, and the copy number for the *dsrA* gene calculated by comparison with the standard curve.

Chapter 12: Monitoring and Preventing Reservoir Souring Using Molecular Microbiological Method (MMM)

DNA Extraction and PCR Amplification

DNA extracts were obtained from produced water samples (triplicate extracts) and from sulphate-reducing prokaryotic enrichment cultures (single extracts). Samples of 100–250 ml PW and 5–12.5 ml enrichment culture were filtered onto white polycarbonate filters (0.2 µM pore size, Nuclepore, Whatman, UK). DNA was extracted by combining bead beating, enzymatic and chemical lysis with the FastDNA® Spin Kit for Soil (Q-Biogene, Carlsbad, CA) (Foesel et al., 2008).

Archaeal and *bacterial* 16S rRNA genes were amplified using the universal primer 1492R (Loy et al., 2002) together with 8F for *Bacteria* (Juretschko et al., 1998) and 21F for *Archaea* (DeLong, 1992). PCR reactions contained 20 µl of dH₂O (Sigma-Aldrich), 25 µl of *Taq* DNA Polymerase Master Mix Red (Ampliqon, Copenhagen, Denmark), 1 µl of bovine serum albumin (10 mg/ml, Amersham Biosciences), 1 µl each of forward and reverse primers (10 pmol/µl each, MWG

Biotech) and 1 µl of template DNA. Thermal cycling conditions were as follows: initial denaturation at 96°C for 4 min, 25–28 cycles of 30 s denaturation at 94°C, 45 s annealing at 57°C (bacterial 16S rRNA genes) or 56°C (for archaeal 16S rRNA genes) and 2 min elongation at 72°C. Cycling was completed by a final elongation step at 72°C for 10 min. PCR amplification of the *dsrAB* genes was performed as described previously using primer mixtures of DSR1F and DSR4R (Loy et al., 2002; Kjeldsen et al., 2007). Thermal cycling conditions were as follows: 5 min at 96°C, 35 cycles of 30 s at 48°C, 45 s at 48°C and 2 min at 72°C, completed by a final elongation step of 10 min at 72°C.

Sequencing and Phylogenetic Analysis

Amplicons of 16S rRNA genes and *dsrAB* genes were purified with the Gen Elute™ PCR Clean up Kit (Sigma) and cloned using the pGEM®-T Vector system (Promega) for 16S rRNA genes and the TOPO® XL cloning kit (Invitrogen) for *dsrAB* genes. Plasmids were isolated and purified with the QIAprep Spin Miniprep Kit (Qiagen). Inserts were sequenced using the vector primers M13F (16S rRNA) and M13R (*dsrAB*). Sequences (750–850 nt) were checked for possible chimeric origin using Bellerophon (Huber et al., 2004). Initial alignment of amplified sequences and close relatives identified with BLAST (Altschul et al., 1990) was performed using the SILVA alignment tool (Pruesse et al., 2007), for the 16S rRNA gene sequences. *dsrAB* gene sequences were translated into amino acid sequences and manually aligned in ARB (Ludwig et al., 2004). Operational taxonomic units (OTU) were determined with DOTUR (Schloss and Handelsman, 2005) and defined as clone sequences sharing >97% nucleotide (for 16S rRNA) and >97% amino acid (for DsrAB) sequence identity. Coverage, species richness and diversity indexes were calculated using SPADE (<http://chao.stat.nthu.edu.tw>). For each OTU of the 16S rRNA gene clone libraries generated from produced water samples, at least one representative full-length sequence (>1,450 nt) was determined. The similarity of 16S rRNA clone libraries was statistically evaluated using LIBSHUFF (<http://libshuff.mib.uga.edu/>). Phylogenetic trees based on 16S rRNA gene sequences (> 1,300 nt) and deduced DsrAB amino acid sequences (540 unambiguously aligned sequence positions) were created based on distance analysis using the neighbour-joining algorithm of the ARB program package (Ludwig et al., 2004). Different filters were applied to select sequence positions for the phylogenetic analyses. The robustness of inferred topologies was tested by bootstrap resampling using the same distance model (1,000 replicates). Short sequences were added to the generated 16S rRNA gene- and the DsrAB amino acid based trees applying maximum parsimony criteria without changing the overall tree topologies. A broad range of taxa was included in the phylogenetic analyses; some of the reference sequences were removed from the presented phylogenetic trees to enhance clarity.

Quantitative PCR Assays

Bacterial and *archaeal* 16S rRNA genes were amplified with the primer set 8F/338R for *Bacteria* and 806F/958R for *Archaea* (Lipp et al., 2008, and references therein). For the quantification of bacterial and archaeal *dsrAB* gene copies, the primers Del1075R and Arch1830F (Gittel et al., 2009) were applied. These primers were used in combination with DSR1F and DSR4R, respectively (Wagner et al., 1998). Quantification of copy numbers of the different genes was performed with a Roche Lightcycler® 480 instrument. Each reaction (20 µl) contained 1 µl DNA template (~3 ng DNA), 1 µl bovine serum albumin (10 mg/ml, Amersham Biosciences), 1 µl of each primer (100 pmol/µl each), 6 µl dH₂O and 10 µl of Lightcycler® 480 SybrGreen I Mastermix (Roche Molecular Biochemicals). Cycling conditions varied depending on the primer set (see Table 1 for details). In general, initial denaturation (10 min, 95°C) was followed by 40 cycles of 30 s at 95°C, 30 s at 55–58°C, 15–40 s at 72°C and completed by fluorescence data acquisition at an assay-specific temperature used for target quantification. Product specificity was confirmed by melting point analysis (55–95°C with a plate read every 0.5°C).

Plasmids containing either the 16S rRNA gene or *dsrAB* gene target sequences were used as standards. DNA concentrations for the initial standard as well as unknown samples were calculated from triplicate measurements with a Nanodrop™ spectrophotometer (Thermo Fisher Scientific). The gene copy number in the initial standard was calculated from the DNA content, the length of the cloned gene fragment and the mean weight of a base pair (1.1×10^{-21} g) and adjusted to 10^8 target molecules per 1 µl. Gene copy numbers in unknown samples were determined based on standard curves constructed from 10-fold serial dilutions of the standard. Amplification efficiencies were calculated from the slope of standard curves. Detection limits for the various assays (i.e. lowest standard concentration that is significantly different from the non-template controls) were less than 50 gene copies for each assay. Samples, standards and non-template controls were run in triplicate.

Chapter 15: Identification of H₂S-Producing Bacteria in Corrosion Product of a Gas Pipeline

Methods

In order to obtain a pure culture, the dilution to extinction technique was employed. Successive decimal dilutions were prepared from the positive culture; aliquots of each dilution were then inoculated in Postgate E medium. The dilution technique was performed twice from the last positive dilution tube. This methodology was followed by streaking onto plates and the isolate was purified by repeating the streaking three times. Plates were incubated in an anaerobic jar. The purity of the isolate was confirmed by microscopic observations.

The DNA from the pure strain was extracted using a microbial DNA isolation kit. After extraction, the 16S rDNA was selectively amplified by polymerase chain reaction (PCR) from purified genomic DNA using the bacterial forward primer SAdir (5'-AGAGTTGATCATGGCTCAGA-3') corresponding to positions 8–28 of *E. coli* 16S rRNA and the bacterial reverse primer, S17rev (5'-GTTACCTTGTACGACTT-3').

PCR reaction mixtures included 29 µl of water, 5 µl of 10 × PCR buffer (supplied with the enzyme), 4 µl of deoxynucleoside triphosphate mix (at a final concentration of 0.2 mM), 1.5 µl of MgCl₂ (supplied with the enzyme), 2.5 µl of the primers SAdir and S17rev (each primer at a final concentration of 0.5 µM), 1 U of *Taq* polymerase and 5 µl of template DNA. The thermal cycling consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 0.5 min, primer annealing at 55°C for 0.5 min and elongation at 72°C for 0.5 min. Cycling was completed after a final elongation step at 72°C for 10 min. The 16S rRNA gene products were analysed by electrophoresis in 1% (w/v) agarose gels.

The PCR products were further purified using a PCR cleaning kit and sequenced.

The sequence of the 16S rDNA was compared to those available in the GenBank database by using the Advanced BLAST search program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to identify the strain.

Chapter 23: Case Study – Proof of Concept that Oil Entrained in Marginal Reservoirs Can Be Bioconverted to Methane Gas as a Green Energy Recovery Strategy

Methods

Establishment of Laboratory Incubations Under Anaerobic Conditions

Incubations to study methanogenesis from residual-oil bearing core material were prepared using techniques for the cultivation of anaerobic microorganisms. This included the use of an anaerobic glove bag (containing 5% H₂ in N₂ and palladium catalyst), an anaerobic gas mixture (20% CO₂ in N₂) and aseptic technique (autoclaving for sterilisation, flaming surfaces prior to sampling).

(a) Preparation of anaerobic medium

The medium used for the anaerobic incubations contained, per 100 ml: 5 ml of solution 1 (K₂HPO₄, 10.0 g/l), 5 ml of solution 2 (MgCl₂, 6.6 g/l; NaCl 8.0 g/l; NH₄Cl, 8.0 g/l; CaCl₂·2H₂O, 1.0 g/l), 0.1 ml resazurin (0.1%), 1 ml of a trace element solution and 1 ml of a vitamin solution.

The trace element solution contains, per 1 water: 0.5 g EDTA; 3.0 g MgSO₄·6H₂O; 0.5 g MnSO₄·H₂O; 1.0 g NaCl; 0.1 g CaCl₂·2H₂O; 0.1 g

FeSO₄·7H₂O; 0.01 g CuSO₄·7H₂O; 0.01 g Na₂MoO₄·2H₂O; 0.01 g H₃BO₃; 0.005 g Na₂SeO₄; 0.003 g NiCl₂·6H₂O.

The vitamin solution contains per 1 2.0 mg biotin; 2.0 mg folic acid; 10 mg pyridoxine-HCl; 5.0 mg thiamine-HCl; 5.0 mg riboflavin; 5.0 mg nicotinic acid; 5.0 mg DL calcium pantothenate; 0.01 mg vitamin B12; 5.0 mg PABA; 5.0 mg lipoic acid; 5.0 mg mercaptoethane-sulphonic acid.

These components were mixed in a glass vessel, the pH was adjusted to approximately 7.2 with 0.1 N HCl and the solution was boiled to removed dissolved oxygen. The solution was cooled on ice under a stream of 20% CO₂ in N₂ until room temperature, then 0.35 g NaHCO₃ was added (per 100 ml). The vessel was capped under the gas mixture. Medium was reduced by the addition of a 2.5% solution of cysteine sulphide (2 per 100 ml) and sterilised by autoclaving for 20 min. Once cooled, the medium was brought into the anaerobic glovebag.

(b) Preparation of incubation vessels

Glass tubes (Balch tubes) or serum bottle openings were sealed with foil and autoclaved. Blue butyl rubber stoppers were also autoclaved in foil. Once cooled, these were brought into the anaerobic glovebag at least a day prior to using for experiments.

(c) Preparation of core material and construction of incubations

Intact core samples were stored in the laboratory at room temperature but were placed in an anaerobic glove bag for at least 1 week prior to use in experiments. To prepare the core material for incubations in the anaerobic glove bag, interior core portions were dissected with sterilised chisels and the resulting rock fragments were crushed to varying extents with sterilised mortars and pestle before dispensing into sterile serum bottles or tubes. To obtain core material of different grain sizes, crushed core was shaken through appropriately sized sieves that had been autoclaved. Most incubations contained core material that had been crushed to a grain size of less than 1.18 mm. The amount of core material added to serum bottle incubations ranged from 5 to 20 g. Sterile medium was dispensed to bottles containing the crushed core material using sterile pipettes (10–25 ml). The serum bottles or tubes were then closed with sterile butyl rubber stoppers, crimp-sealed, removed from the glove bag and the headspace of each was aseptically exchanged three times with 20% CO₂ in N₂ to remove traces of H₂ and provide buffering capacity for the medium. Inoculum (1–2 ml) was then aseptically added to the appropriate incubations.

Methane Measurements

Methane concentrations were monitored by gas chromatography (GC) on a Varian 3300 gas chromatograph (GC) equipped with a flame ionisation detector (FID) and a packed stainless steel column (6' × 1/8" diameter, Poropak Q, 80/100, Supelco, Bellefonte, PA). The injector and column temperatures were held at 100°C and the detector was at 125°C. One-millilitre syringes were used to sample 0.2 ml of headspace from incubations for injection into the GC. A calibration curve was

prepared prior to each sampling event by analysing methane standards ranging from 0.2 to 15% CH₄ = 84–6,590 µM CH₄.

Organic Extractions and GC-MS Analysis

To determine the loss of residual oil components in a time course experiment, duplicate inoculated incubations were sacrificed at each time point by opening the bottles and immediately adding an aliquot of methylene chloride. At time 0 and at every other time point, duplicate uninoculated core-containing incubations were also sacrificed. A standard compound (C₂₄D₅₀, Isotec, Miamisburg, OH) was added to all bottles prior to extracting the residual oil from the core material with a total of four aliquots of methylene chloride. The organic layers were pooled, dried over sodium sulphate and concentrated under N₂. Analysis of the resulting oil samples was by GC-MS on an Agilent 6890 model GC coupled with a 5973 model mass spectrometer. One microlitre of each extracted oil sample was injected at 270°C, and components were separated on an HP-5MS capillary column (30 m × 0.25 mm, 0.25 mm film thickness, Agilent Technologies, Inc.) by initially holding the oven at 45°C for 5 min, increasing the temperature at a rate of 8°C/min to 270°C and holding at this final temperature for 15 min. To quantify the alkane fraction of the residual oil extracts, the GC peak areas of alkanes ranging from C₁₂ to C₂₉ were summed and then divided by the GC peak area of the added C₂₄D₅₀ to obtain an *n*-alkane-to-standard ratio. Using this method of quantification, a decrease in the resulting ratio relative to controls indicates biodegradation.

Molecular Microbiological Methods

A brief overview of the DNA extraction method, PCR amplification, cloning and sequencing is provided. More details on these method are described in Gieg et al. (2008).

Culture fluids from the residual oil-grown inoculum (2–4 ml) were added to sterile microfuge tubes containing sterile silica beads, centrifuged to pellet the cells and the supernatant was removed. Commercially available DNA extraction kits (e.g. MOBIO Laboratories, Inc., FastDNA spin kit for soil, Quantum Biotechnologies, Inc., both Carlsbad, CA) were used to extract DNA from the cell pellets on different occasions. The protocols used bead beating and chemical means to lyse cells. Controls for sterility included processing 1–2 reagent controls to which no cells were added. When aliquots from reagent control tubes were used as template DNA in PCR reactions used to create the clone libraries as described below, no visible PCR products were formed.

Three 16S rRNA gene libraries (two *Eubacterial* and one *Archaeal*) and three *mcrA* (methyl coenzyme M reductase) gene libraries were constructed following DNA extraction from the inoculum. Universal *Eubacterial* primers sets (Herrick et al., 1993; Johnson, 1994) were used to create the PCR products for cloning *Eubacterial* libraries. Primers ARC333 and 958r were used to obtain *Archaeal* 16S

rRNA gene sequences (Struchtemeyer et al., 2005). The *mcrA* libraries were created using primers ME1 and ME2 (Hales et al., 1996). These primers were also used to amplify *mcrA* sequences after enrichment for H₂-utilising methanogens. The PCR products were cloned into the TOPO-TA vector (Invitrogen, Carlsbad, CA). Cloned DNA was directly amplified from transformed cells using flanking M13 vector sequences and purified by spin filter centrifugation (Montage PCR Purification Units, ThermoFisher Scientific). Sequencing was performed at the University of Oklahoma DNA Sequencing Facility on an ABI Model 377 automated sequencer. For all libraries, initial phylogenetic assignments were made following BLASTN searches (Basic Local Alignment Search Tool, Altschul et al., 1990). Sequencher® (Windows Version 4.2, Gene Codes Corp., Ann Arbor, MI) was used to trim vector regions from the cloned sequences and to examine each cloned sequence for the presence of universally conserved regions (primer regions). Chimera-Check, Bellerophon and Pintail (Maidak et al., 2001; Huber et al., 2004; Ashelford et al., 2005) were used to check for putative chimeric sequences that were then removed from the data set. Phylogenetic placement of 16S sequences was further examined using the Classifier program. Sequences from BLASTN search that most closely matched the sequences from the clones and sequences of selected outgroup strains were trimmed to a common region of approximately 800 bp, aligned using CLUSTAL X (ver. 1.81) and corrected manually as needed.

Chapter 24: Dynamics of *Alcanivorax* spp. in Oil-Contaminated Intertidal Beach Sediments Undergoing Bioremediation

Methods

Experimental Design

A field experiment was carried out in the upper intertidal zone at Stert Flats, a mudflat in Somerset, UK (Chapter 24, Fig. 1) with fine sand. Full details of the experimental design are presented in Röling et al. (2004), and a summary is presented here. An area of mudflat was divided into three replicate blocks (Chapter 24, Fig. 1), each containing four plots which were treated as follows: UC (no oil control without inorganic nutrient treatment), OC (oil-treated control without inorganic nutrient treatment), LF (treated with oil and liquid fertiliser) and SR (treated with oil and slow-release fertiliser). Data from block 2 are reported in the present study. For oil-treated plots (OC, LF, SR), 63 cm × 96 cm × 7 cm Nitex nylon mesh enclosures were anchored to a steel pole and filled with oil-treated beach sediment. These enclosures were closed with a Nitex nylon mesh lid using safety clips. Identical Nitex bags filled with untreated beach material were added to the UC plots. A smaller Nitex bag (30 cm × 30 cm × 2.5 cm) was inserted within each enclosure for measuring CO₂ production. The oil was mixed with the sediment as described by Röling et al. (2004) and the liquid fertiliser and slow-release fertiliser plots were treated with fertiliser 1 week after oil amendments.

The microcosm experiment was as described in Röling et al. (2002).

Carbon Dioxide Measurements

Carbon dioxide evolution as a measure of microbial activity was monitored on a daily basis for 14 days by using an *in situ* respirometry method (Swannell et al., 1995).

Oil Chemistry

Triplicate samples were collected from each plot on day 0 and day 18 after fertiliser treatment and were frozen at -20°C until analysis. Hydrocarbon extraction from 10 g of frozen sediments, their analyses and quantification by gas chromatography-fitted with flame ionisation detector were carried out as described (Röling et al., 2004; Swannell et al., 1995). Before extraction the sediments were spiked with surrogate standards (squalane and 1,1-binaphthyl) to determine extraction efficiency.

Nucleic Acid Extraction

Triplicate 20 g samples were collected randomly from each plot and stored at -20°C until analysis. Sub-samples (0.5–0.6 g) of frozen sediment were used for DNA extraction as described previously (Röling et al., 2004).

Primer Design

The PCR primers for amplifying 16S rRNA gene (A16S.1237f and A16S.1357r) and *alkB* gene (*alkBf* and *alkBr*) were designed using the probe and PCR primer design software tool Primrose (Ashelford et al., 2002).

PCR and Denaturing Gradient Gel Electrophoresis (DGGE)

16S rRNA gene fragments from the bacterial population were amplified by PCR in a final volume of 50 μl containing 0.2 μM of primer 2 and primer 3 (Muyzer et al., 1993), and separation of the amplified gene fragments by DGGE was performed essentially as described in Röling et al. (2002). Alkane hydroxylase genes were detected by PCR using primers *alkBf* (5'-GSNCAYGARYTSRGBCAYAAR-3') corresponding to positions 939–959 of the *Alcanivorax borkumensis alkB1* gene (AB110225) and *alkBr* (5'-CCRTARTGYTCVABRTARTT-3') corresponding to positions 1,354–1,336 of the *A. borkumensis alkB1* gene (AB110225). The predicted PCR product size is 416 bp; 50 μl PCR reactions contained 1 \times PCR buffer (Bioline), 1.5 mM MgCl₂ (Bioline), 1 U *Taq* DNA polymerase (Bioline), 0.2 μM of each forward and reverse primer, 0.2 mM dNTPs and 10–15 ng of template DNA. The temperature cycles were 1 cycle at 95° for 4 min followed by 30 cycles of 94° for 1 min, 55° for 1 min, 72° for 1 min and final extension at 72° for 10 min.

Quantitative PCR Analyses

Quantitative PCR (qPCR) of 16S rRNA genes from *Alcanivorax* spp. was performed by using a newly designed primer pair. The primers were designed to detect sequences related to a 16S rRNA gene clone (SR11-d6; GenBank Accession no. AF548762) representative of the *Alcanivorax* sp. that was known to be predominant in oil and inorganic nutrient-treated Stert Flats sediments from previous studies carried out at this location (Röling et al., 2004). The primer sequences were forward primer (A16S.1237f 5'-GCAGTACAGAGGGCAGCA-3'), reverse primer (A16S.1357r 5'-TTCTGACCCCGCGATTACTA-3'). The forward primer was designed to be specific for *Alcanivorax* spp. 16S rRNA give sequences that are highly similar to clone SR11-d6 and was an exact match to 39 bacterial sequences in the RDP database including 29 of 411 *Alcanivorax* spp., 2 sequences from the *Alteromonadales* and 5 from the *Bacteroidetes*. The reverse primer was designed to be highly specific for clone SR11-d6 and was an exact match to this sequence. The probe also targets 43 other bacterial sequences in the RDP database comprising 4 other sequences from the *Proteobacteria*, 12 from the, *Firmicutes*, 26 from the *Planctomycetes* and 1 from the *Acidobacteria*. The two primers in combination, however, target only the SR11-d6 sequence. qPCR was done using an iCycler (iQTM5 multicolor, Bio-Rad, Hemel Hempstead, UK). Each 20 µl reaction mixture contained 10 µl iQTM supermix (Bio-Rad), 10 pmoles each of forward and reverse primers, 0.2 µl of 100 × SYBR-green and 3 µl of template DNA. PCR reactions were run as follows: one cycle of initial denaturation at 95°C for 7 min followed by 40 cycles of 95°C for 30 s, 61°C for 60 s and 72°C for 30 s. Triplicate no template control reactions were also run to determine detection limits, and melt curve analysis was conducted at the end of the qPCR assays to detect any non-specific amplification products. A standard curve for qPCR was generated from a PCR-amplified *Alcanivorax* 16S rRNA gene from a 16S rRNA gene clone library obtained from Stert Flat sediments. The 16S rRNA gene fragment was gel purified (QIAquick PCR purification kit, Qiagen) and quantified spectrophotometrically, and a dilution series ranging from 10⁰ to 10⁸ genes was prepared.

Statistical Analysis of Data

Statistical analyses were performed using Minitab 15 statistical software. Unless mentioned otherwise all the analyses represent the results of a 2-Sample t-test.

Chapter 25: Significant Contribution of Methyl/Methanol-Utilising Methanogenic Pathway in a Subsurface Biogas Environment

Methods

Water samples were collected at the well heads of gas-producing wells. Prior to sampling, 1 l plastic bottles were sparged with argon. Subsequently small amounts

of resazurin (redox indicator) and cysteine (reducing agent) were added in the anaerobic chamber and the bottles were autoclaved. Upon collection of co-produced water samples bottles were filled up leaving very little or no headspace. After water collection, excess oxygen was treated with injection of sterile Na₂S, if needed. Samples were stored and transported cold but not frozen. In the laboratory, water samples were centrifuged and cell pellets were DNA extracted for the 454 pyrosequencing.

Chapter 26: Development of New Fuels, e.g. Biofuels

Experiment Set-Up

The experiments are performed in 1,000 ml Duran® bottles containing a volume of 100 ml inoculated water and 300 ml biodiesel blend. The air volume above is filled with N₂, and the incubations are stored at varying temperatures and evaluated over a period of 3.5 months.

The inoculated water consists of water collected from a petrochemical diesel storage tank at Samtank A/S in Aarhus, Denmark, and water sterilised by filtration in the relation 1:100. The diesel used is obtained from OK a.m.b.a. while the biodiesel is animal fatty acid methyl esters produced at Daka Biodiesel a.m.b.a. The experiments are performed as triplicates.

Method for Measurement of Cold Filter Plugging Point According to EN 116:1997

The cold filter plugging point (CFPP) describes the highest temperature at which a given volume of fuel fails to pass through a standardised filtration device.

Materials

- Heptane
- Acetone
- Lintless filter paper, 5 ± 1 µm retention
- Standardised CFPP measurement equipment/apparatus, including
 - Pipette connected to filter unit; pipette of clear glass with calibrating mark corresponding to a volume of 20 ± 0.2 ml at a point 149 ± 0.5 mm from the bottom of the pipette and a brass filter unit with internal Ø 4 ± 0.1 mm and a Ø 15 mm plain weave stainless steel wire mesh gauze according to ISO 3310-1
 - Thermometers
- Cooling bath
- Vacuum source and vacuum regulator

Methods

Before the measurement 50 ml of the sample is filtered at ambient temperature through dry lintless filter paper. The filter unit, test jar, pipette and thermometer are washed with heptane, rinsed with acetone and dried in a stream of filtered air before each test.

- Set the cooling bath to $-34 \pm 0.5^\circ\text{C}$.
- Pour the filtered sample into the test jar to the 45 ml mark.
- Place the test jar with lid carrying pipette with filter unit into the specified jacket and place this in the cooling bath.
- Connect the pipette to the vacuum source and apply an air flow of 15 l/h using the vacuum regulator.
- Immediately after placing the jacket with sample in the cooling bath, start the measurement. For each 1°C below the first temperature the sample is drawn into the pipette through the filter unit using the vacuum source. The corresponding times are logged in order to measure the temperature at which the filter is blocked by wax crystals to such a degree that the time for filling the pipette exceeds 60 s or the sample fails to return completely to the test jar before the sample is cooled by a further 1°C . The temperature at which the last filtration was commenced is recorded as CFPP.
- If CFPP has not been determined before reaching the temperature of the cooling bath, lower the cooling bath temperature to $-51 \pm 1.0^\circ\text{C}$. If CFPP is still not determined, then lower the temperature to $-67 \pm 2^\circ\text{C}$. If CFPP is not reached when sample temperature has passed -51°C , discontinue the test and report CFPP as ‘not plugged at -51°C ’.
- If the first measurement blocks the filter, repeat the measurement at higher temperature on a fresh sample.

Chapter 27 Biodiesel – A Case Study

Methods

Microbial Isolation and Identification

Aerobic bacterial strains and fungi were isolated from positive culture by the streak-plate technique in Plate Count agar (OXOID CM0325) and Sabouraud dextrose agar (OXOID), respectively.

DNA from the pure strains were extracted using a microbial DNA isolation kit. After extraction, bacterial 16S rRNA gene was amplified by PCR using the primer set Sadir (5'-AGAGTTGATCATGGCTCAGA-3', forward) and S17 Rev (5'-GTTACCTTGTACGACTT-3', reverse). For fungi, the internal transcribed spacer (ITS) 1 and 2 and 5.8S rRNA genes were amplified using the

primer set ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3', forward) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3', reverse) (White et al., 1990).

PCR reaction mixtures included 29 µl of water, 5 µl of 10 × PCR buffer (supplied with the enzyme), 4 µl of deoxynucleoside triphosphate mix (at a final concentration of 0.2 mM), 1.5 µl of MgCl₂ (supplied with the enzyme), 2.5 µl of the primers (each primer at a final concentration of 0.5 µM), 1 U of *Taq* polymerase and 5 µl of template DNA. The thermal cycling consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 0.5 min, primer annealing at 55°C (for bacteria) and 50°C (for fungi) for 0.5 min and elongation at 72°C for 0.5 min. Cycling was completed after a final elongation step at 72°C for 10 min. The PCR products were analysed by electrophoresis in 1% agarose gels.

The PCR products were further purified using a PCR cleaning kit and sent to the University of São Paulo (USP) for sequence analysis.

The sequences were compared to those available in the GenBank database by using the Advanced BLAST search program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to identify the strain.

DGGE Analyses

Two hundred millilitres of biodiesel was filtered over a 0.2 µm pore-size regenerated cellulose membrane to collect microbial cells. The membrane filter was washed free of fuel with 50 ml of a 0.1% sterilised detergent solution (Tween 80[®]) and rinsed with 100 ml of ¼ Strength Ringer's Solution.

Community genomic DNA was extracted directly from the membranes. The membrane was cut and immersed in 900 µl of lysis buffer (40 mM EDTA, 50 mM Tris-HCl pH 8, 0.75 M sucrose). Lysozyme was added (1 mg/ml final concentration) and the suspension was incubated at 37°C for 45 min. After the addition of Proteinase K (0.2 mg/ml final concentration) and sodium dodecyl sulphate (1% final concentration), the samples were incubated at 55°C for 1 h (Servais et al., 2003). DNA was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v:v:v) and was precipitated by the addition of 1/10 of the volume of 3 M sodium acetate and 2 volumes of cold isopropanol followed by incubation at -20°C for 12 h. The DNA was recovered by centrifugation at 10,000 × g for 30 min and the pellet was washed with cold 70% ethanol, dried and resuspended in 50 µl of sterile distilled water. The purity and concentration of the extracted DNA was assessed by the absorbance method at 260 and 280 nm.

A nested PCR protocol was employed. On the first PCR, fragments of approximately 1,500 bp were amplified using the same PCR conditions as described above for bacteria identification employing the primer set SAdir and S17 Rev. On the second PCR, 16S rRNA gene fragments (approximately 550 bp) were amplified by PCR, using the bacterium-specific primers 341F and 907R with a GC clamp attached to 341F's 5' end (Muyzer et al., 1993). The volumes of PCR mixture were 50 µl:1 × PCR buffer (supplied with the enzyme), 0.2 mM of each dNTP, 1.5 mM of MgCl₂, 0.5 µM of each primer, 2.5 U Taq DNA polymerase and 10–100 ng genomic DNA template, DNase and RNase free water. Touchdown PCR was performed:

initially 5 min at 94°C, followed by 30 cycles of 1 min at 94°; 1 min at 65–55° (reducing temperature by 1°C per cycle for 10 cycles plus 20 cycles at 55°); 1 min at 72°, followed by 10 min at 72° (Kousuke and Manabu, 2004). After PCR, the size of the products was verified on 1% agarose gel. PCR products were then purified using a PCR clean-up Kit.

DGGE was performed with DCode system (Bio-Rad Co., USA). PCR products were loaded onto 6% (w/v) polyacrylamide gel in 0.5 × TAE buffer. The 6% (w/v) polyacrylamide gel (acrylamide/bisacrylamide 37.5:1) was made with denaturing gradients ranging from 45 to 65% (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run at 60°C for 18 h at 70 V. After electrophoresis, the gels were stained for 30 min with Sybr Green® and photographed on a UV transillumination table with a Gel Documentation System.

Chapter 28: Unlocking New Fuel Resources

Methods

Synthesis of Naphthenic Acids

The naphthenic acids used in this study were 4-(4'-*n*-butylcyclohexyl) butanoic acid (*n*-BCHBA), 4-(4'-*iso*-butylcyclohexyl) butanoic acid (*iso*-BCHBA), 4-(4'-*sec*-butylcyclohexyl)butanoic acid (*sec*-BCHBA) and 4-(4'-*tert*-butylcyclohexyl) butanoic acid *tert*-BCHBA). These were synthesised to >90% purity using a modification of the Hawthorn synthesis (Vogels, 1989; Smith, 2006).

Environmental Sampling

Sediment samples (50 g) were obtained in January 2003, from Devonport, Avonmouth and Portishead (south-west UK), ONS Island (north-west Spain) (courtesy of GreenPeace) and Drill cuttings (supplied by MI Drilling fluids™). Sediments were transported to the laboratory at 4°C within 2 h of sampling and stored at -20°C.

Enrichment for Naphthenic Acid Degrading Communities

Starter enrichment cultures were prepared for the preliminary experiments using 1 g wet weight of each sediment inoculated into 50 ml minimal medium containing (g/l): MgSO₄, 0.2; (NH₄)₂SO₄, 0.5; KH₂PO₄, 0.5; K₂HPO₄, 1.5; NaOH, 0.02; Na₂EDTA, 0.12; ZnSO₄, 0.004; CuSO₄, 0.001; Na₂SO₄, 0.0001; Na₂MoO₄, 0.001; MnSO₄ 0.0004; CoCl₂ 0.0001 (pH 7.0) and supplemented with each individual naphthenic acid: *n*-BCHBA, *sec*-BCHBA and decahydronaphthoic acid (naphthoic acid hydrogenated in-house; purity >90%). Bottles were crimp sealed using PTFE seals to prevent volatilisation of the hydrocarbons. Enrichment cultures were incubated statically in the dark at 25°C. The degradation experiment was prepared by

inoculating 1 ml of the Devonport starter enrichment culture in triplicate flasks for each sampling point containing 25 ml of the same media described previously and supplemented with 50 µl (0.2 mg) of either *n*-BCHBA, *sec*-BCHBA, *iso*-BCHBA or *tert*-BCHBA dissolved in methanol (HPLC grade, Rathburn Chemicals Ltd., Scotland) (final concentration 4 mg/ml). Flasks were crimp sealed and incubated in the dark at 25°C. Triplicate samples were analysed at 0, 1, 3, 9, 14, 21 and 30 days. Abiotic controls using the same media containing the individual naphthenic acids but no inoculum and procedural blanks inoculated with the microbial consortium but containing no naphthenic acids were also analysed under the same conditions.

Naphthenic Acid Extraction and Gas Chromatography Analysis

Triplicate flasks containing 1-adamantecarboxylic acid (99%; Aldrich, UK) and eicosane (*n*-C₂₀; 99%; Aldrich, UK) dissolved in methanol (final concentration 1 mg/ml) were used as internal standards. Samples (25 ml) were removed from the enrichment cultures, centrifuged at 4,000g for 10 min. The supernatant was transferred to a glass quickfit boiling tube (50 ml), acidified to pH 2 (conc. HCl) and extracted with ethyl acetate (3 × 15 ml). Extracts were combined, dried (Na₂SO₄; overnight) and evaporated to near-dryness (Buchi, 40°C), transferred to GC vials and gently blown down to dryness (N₂). BSTFA (20 µl) was added to each sample and samples heated (70°C; for about 20 min). Samples were resuspended in dichloromethane (1 ml) and examined using gas chromatography-flame ionisation detector (GC-FID) and GC-mass spectrometry (GC-MS) comprising a HP5890 Series II GC (Hewlett-Packard, USA) fitted with a HP5970 Series Mass Selective Detector and HP7673 autosampler. GC-MS conditions were 1 µl splitless injection; injector temperature 250°C; column Agilent Ultra (12.5 m × 0.20 mm × 0.33 µm); GC programme was as follows: 40–300°C at 5°C/min, hold 10 min; head pressure 70 kPa; electron multiplier 1,600 V. Quantification was performed by comparing analyte peak areas to those of standards.

Direct DNA Extraction and PCR Amplification of the 16S rRNA Genes

DNA was extracted from both sediments and enrichment cultures using a bead beating method (Stephen et al., 1996). DNA pellets were washed in 2 volumes of 70% (v/v) ethanol, air dried and resuspended in 30 µl of ultrapure water (Sigma). PCR amplifications were performed in a PX2 thermal cycler (ThermoHybaid). PCR amplification of the 16S rRNA genes was performed using the primers and cycling conditions described by Muyzer et al. (1993). PCR reaction mixtures (50 µl) contained 50 ng of DNA template, 2 µl of each primer (10 pmol/µl) and 25 µl of Master Mix (Promega) and 19 µl ultrapure water (Sigma). PCR products were analysed by electrophoresis on 1% 1 × TAE agarose gels at 100 V for 30 min (Sambrook et al., 1989). Gels were viewed using AutoChemiSystems, LabWorks 46 Gel Pro Application Software (UVP).

Denaturing Gradient Gel Electrophoresis (DGGE) and Sequencing Excised Bands

DGGE analysis was performed using a DCode system (Bio-Rad) and 8% (w/v) polyacrylamide gels in 1 × TAE (Sambrook et al., 1989), using a gradient between 40 and 60% (100% denaturant contains 7 M urea and 40% formamide). Gels were run at 75 V overnight at 60°C. Gels were washed for 30 min in a fixing solution (10% ethanol and 0.5% acetic acid), stained for 20 min in a staining solution (0.2% AgNO₃), washed in developing solution for 10 min (1.5% sodium hydroxide and 0.8% formaldehyde) and in fixing solution for 5 min. Scanning densitometry of DGGE band profiles was performed using the AutoChemiSystems, LabWorks 46 Gel Pro Application Software (UVP). DGGE bands were excised, placed in 25 µl of sterile ultrapure water and incubated overnight at 4°C. The eluted DNA was used PCR amplified and the PCR products purified using a QIAquick PCR purification kit (Qiagen) and sequenced (MWG-Biotech, Germany).

Generation of 16S rRNA Gene Clone Libraries

The 16S rRNA gene libraries were constructed using DNA extracted from the inoculum and from day 30 of the enrichment cultures incubated with *tert*-BCHBA and *n*-BCHBA. PCR amplification was performed using the primers (PA/PH') and PCR cycling conditions described by Edwards et al. (1989). The PCR products were ligated into pGEM-T Easy Vector (Promega) and transformed into high efficiency competent *E. coli* JM109 cells according to the manufacturer's instructions (Promega). The transformed cells were plated on LB plates containing 100 µg/ml of ampicillin, 80 µg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 0.5 mM IPTG (isopropyl-β-D-thio-galactopyranoside) as recommended by the manufacturer (Promega). Twenty-eight clones were selected for 16S rRNA gene sequence analysis.

Phylogenetic Analysis of the 16S rRNA Genes

Partial 16S rRNA gene sequences (850 bp) were aligned against related sequences retrieved from GenBank using SEAVIEW (Galtier et al., 1996). Phylogenetic analysis was performed using PHYLIP 3.4 using Jukes–Cantor DNA distance and neighbour-joining method (Jukes and Cantor, 1969; Saitou and Nei, 1987). Bootstrap analysis was based on 100 replicates using SEQBOOT (PHYLIP 3.4). Tree construction was performed using Treeview (WIN32) version 1.5.2 (Page, 1996).

Nucleotide Sequence Accession Numbers

All partial 16S rRNA gene sequences obtained in this study have been deposited in GenBank under the following accession numbers: DQ926663–DQ926690.

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