Subsurface Ecosystems – Oil triggered life

Opportunities For The Petroleum Industry

Geert Marinus van der Kraan

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PROEFSCHRIFT

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Voor mijn ouders, Daniëlla en onze kleine jongen

'Geert was een leergierige jongen met eigen ideeën en oplossingen'
Leen van Loon, leraar Prinses Margriet basisschool, te Kats (2010)

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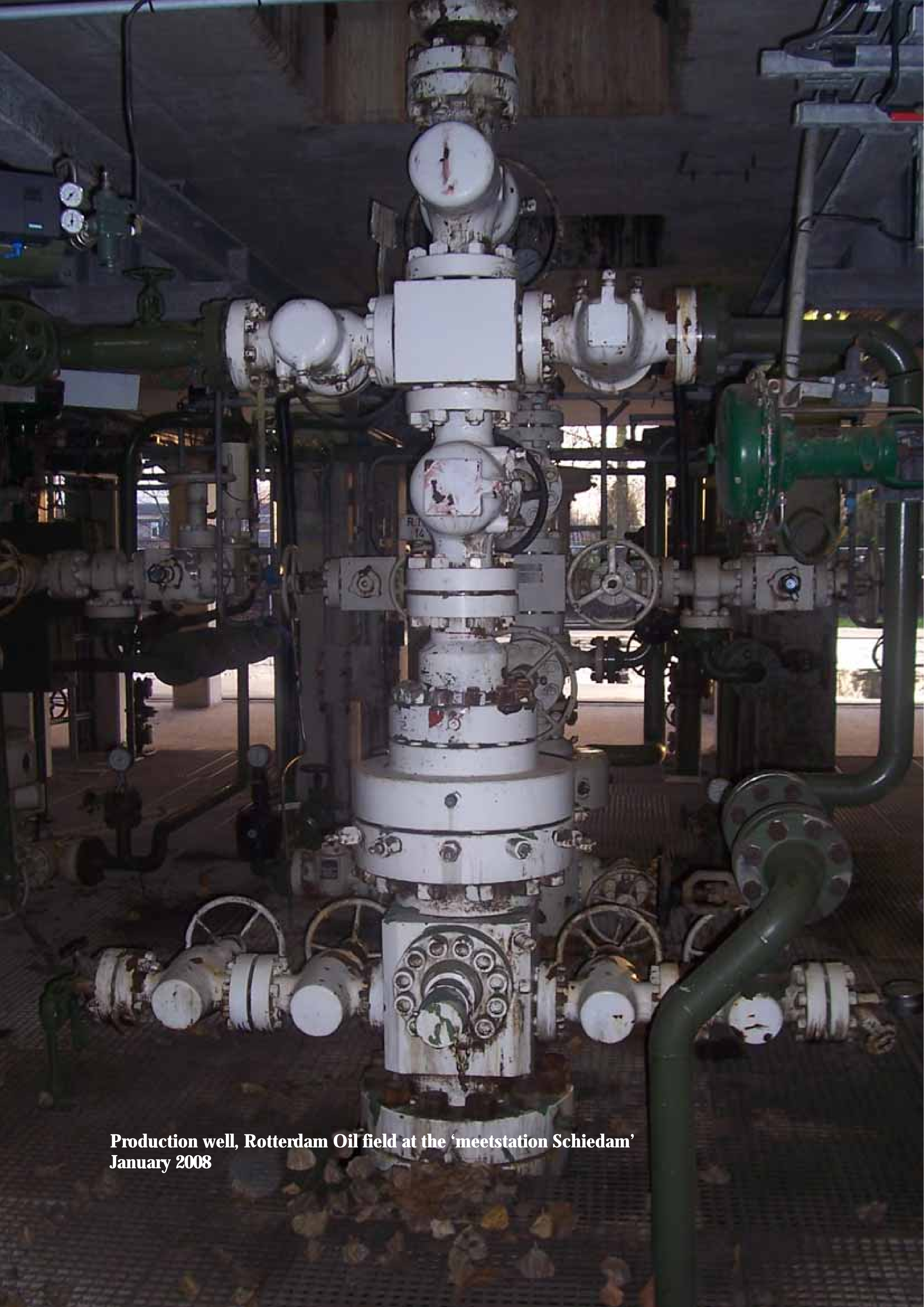

Production well, Rotterdam Oil field at the 'meetstation Schiedam'
January 2008

1

General introduction

Geert M. van der Kraan

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Bacteria from hydrocarbon seep areas growing on short-chain alkanes.

Gerard Muyzer & Geert M. van der Kraan.

Trends in Microbiology, Vol 16, Pages 138-141, 2008.

Life in oil-associated ecosystems

This thesis deals with various aspects regarding microbial life in oil-associated ecosystems and the applications regarding an improved oil recovery that might be developed using the presence and activities of this life. This introduction aims at putting the described research into a broad framework that comprises the field of petroleum microbiology. Therefore various notions in both disciplines are discussed. Also it is described how the various papers, that form the chapters of this thesis, are intermingled.

Crude oil

The simplest definition of crude oil is: a mixture of different molecules comprising mainly the elements carbon (C) and hydrogen (H). Oil is often indicated as petroleum of which the main constituent is $(-CH_2-)_n$. It is commonly found as a liquid contained in various geological formations (reservoirs) worldwide. Since molecules in petroleum are comprised of hydrogen and carbon, it is a highly flammable liquid (Fig. 1). Hydrocarbons are its most important constituent. The number of molecule types that can be created using carbon and hydrogen is endless. This is due to the fact that both elements mainly form covalent bonds, meaning that electron pairs are shared with other atoms. In addition to hydrogen and carbon, petroleum molecules also can contain the elements sulphur (S), nitrogen (N) and oxygen (O) in low quantities. Also the petroleum mix contains other organic compounds (e.g. oxygenated hydrocarbons), albeit that hydrocarbons are the most abundant. Therefore the liquid we call oil or petroleum is incredibly complex, holding molecules of different molecular weights and structures (Table 1). Nowadays petroleum and the products of petroleum have become part of our everyday life. Petroleum stands at the basis of our current society. We are heavily dependent on petroleum as 40 percent of our energy requirement is derived from it. Also petroleum is a resource for numerous products and applications that can be found all around the World. 90 percent of all chemicals used today are made from petroleum. This is due to the fact that oil has a high energy density, is easy to use and overall has a flexible chemistry; it is the driver of today's transportation and chemical industry. Today a society without petroleum is unthinkable as it has penetrated in all its aspects. Although the origin of our current oil-based economy lies in the year 1859, with the discovery of the first oil well by Edwin Drake (Bakas, 2007), the origin of oil itself lies millions of years in the past. It is now widely accepted that the black liquid we call oil is a remainder of small photosynthetic algae like for example diatoms (Fig. 2) that, after their death, were buried by deposition of sediments and subsequently converted into a material called kerogen through a process of thermogenesis at elevated pressures and temperatures that prevail in the deep

subsurface (Lorant, 2002). This process is slow and commonly this transformation takes millions of years. Kerogen is subsequently transformed into the oil and gas deposits we find today. Although oil deposits have been formed throughout the entire history of the Earth, most oil originates from the Carboniferous time frame (Fig. 3). This geological period began 359 million years ago thereby ending the Devonian period and ended with the beginning of the Permian period, about 299 million years ago. During the Carboniferous, the Earth was covered with dense swamps and warm shallow seas. The creation of these swamps was triggered by an increase in photosynthetic activity of plants and algae, which had already started in the Devonian era. As a consequence of this increased activity a large separation between carbon and oxygen was established. The O_2 level reached a stunning 35% and the basis for major coal beds and oil deposits were created by the presence of these swamps and shallow seas (as a comparison the air we today breathe holds 21% oxygen). At the end of the Devonian time frame CO_2 levels were high (1000-1500 p.p.m.), but dropped significantly during the Carboniferous time frame (300 p.p.m.), triggering at the end of the Carboniferous, a mass extinction (Beerling & Berner, 2000), (Beerling, 2002). Since the current oil deposits were formed millions of years ago out of biomass they also have been given the name fossil fuels.

Figure 1: *Oil bottle (Crude oil from the Rotterdam oil field)*

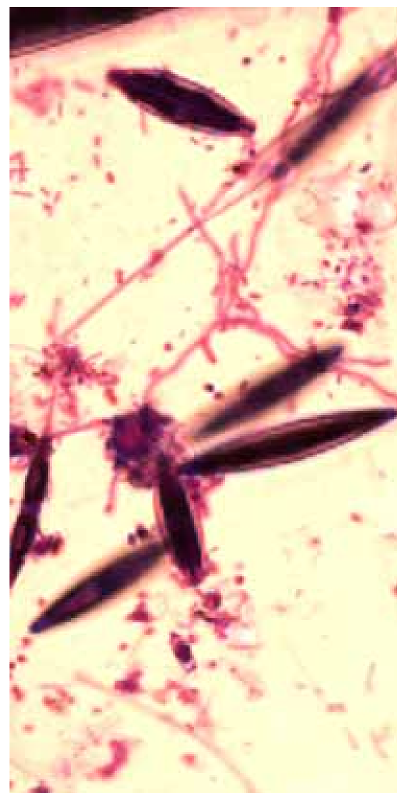


Figure 2: *400× enlargement of marine diatoms stained with a Chrystal violet dye.*

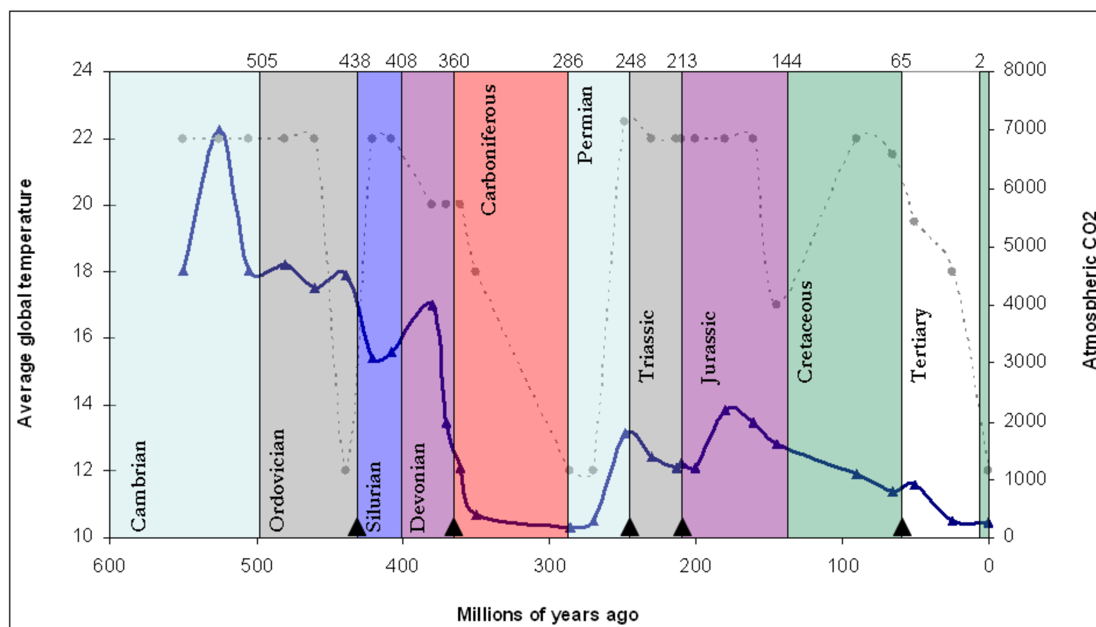


Figure 3: *Overview of geological times (starting with the Cambrian). The solid line depicts the global CO_2 concentration. The dotted line shows the average global temperature. The black triangles at the bottom indicate the 5 major extinction events the Earth has faced. The P-T event being the biggest, where the Earth lost over 80% of all its species. (Image modified after http://www.geocraft.com/WVFossils/Carboniferous_climate.html)*

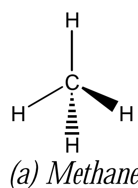
Table 1*: *Compounds which are most abundant in oil*

Paraffins	***Naphtenes***	***Aromats***
paraffins up to $C_{10}H_{22}$		Benzene
isobutane	cyclopentane	toluene
2-methylbutane	Cyclohexane	Ethylbenzene
2,3-dimethylbutane	methylcyclopentane	Xylene
2-methylpentane	1,1-dimethylcyclopentane	1,2,4-trimetylbenzene
3-methylpentane	methylcyclohexane	
2-methylhexane		
3-methylhexane	1,3 dimethylcyclohexane	
2-methylheptane	1,2,4-trimethylcyclohexane	
2,6-dimethylheptane		
2-methyloctane		

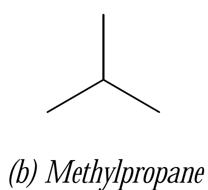
* table taken from 'The properties of petroleum fluids' (McCain, 1990 (second edition))

Classification of hydrocarbons

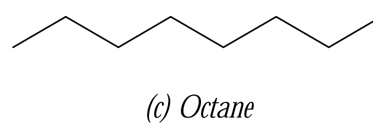
As recognised, hydrocarbon molecules from petroleum have an infinite complexity in their shape and size. Still a classification has been made based on the properties of the various molecules. This classification takes the following aspects into account: 1) The occurrence of double or triple bonds in the molecule. 2) The 3D-structure of the molecule. 3) The overall size of the molecule. Recognized in the so-called 'linear' hydrocarbons are: the paraffin series (alkanes), which only hold saturated C-C and C-H bonds, the olefin and di-olefin series (alkenes), which hold a double bond and multiple double bonds in their molecule respectively and the acetylene series (alkynes) which hold one or more triple bonds in their molecule. Hydrocarbons can also occur as cyclic chains; when hydrocarbons hold cyclic conformations the following series are recognized: the naphtenes (or cycloparaffines), and the aromats. When a hydrocarbon exceeds a certain size, it is accounted as an asphaltene (or bitumen). These molecules resemble coal in their appearance and are even more complex than the other constituents of oil. They are built up out of many aromatic ring structures. Asphaltenes have the charctersistic to dissolve in toluene and precipitate in heptane. Also recognised as a separate class of molecules are the so-called resins. Resins are wax like structures that occur in petroleum. Alkanes characterize themselves by open chains; all the bonds in the molecule are saturated. Due to these saturated sigma-bonds, these molecules are chemically very stable and therefore very inert. They can display isomerism by forming branches. Alkenes and alkynes hold one or more double or triple bonds in their molecule, respectively. This makes them more reactive and scarcer than alkanes. Double and triple bonds allow the addition of H_2 to the molecule. Isomerism occurs through branching and through the position of the double or triple bonds in the molecule. Naphtenes are saturated chains like alkanes but they form closed loops, they also are relatively stable. Aromats can mainly be described as derivatives of benzene. A benzene ring consists of a hexagonal ring which holds a so-called continuous π-bond. This makes benzene inert and highly toxic. Benzene and molecules that are comprised of multiple benzene rings are called aromats because they have a characteristic odour; branching can also occur (Fig. 4, examples). When crude oil is analyzed, often a so-called SARA analysis is performed and a SARA classification is made accordingly. SARA stands for Saturates Aromats Resins Asphaltenes. This analysis is based on a practical separation of the different oil fractions. It is a combination of precipitation reactions and elution chromatography providing required information on these fractions. We mention this classification as it is often used in the oil industry to classify oils. Heavy oil by principle will hold large resin and asphaltene fractions whereas light oil will hold a higher concentration of saturates and aromats. (See for an example (Al-Saffar et al. 2001))



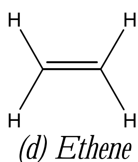
(a) Methane



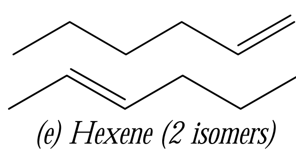
(b) Methylpropane



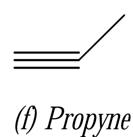
(c) Octane



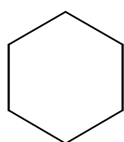
(d) Ethene



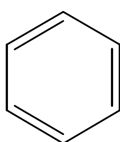
(e) Hexene (2 isomers)



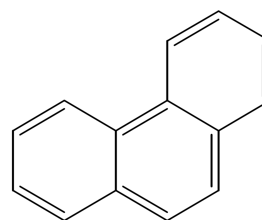
(f) Propyne



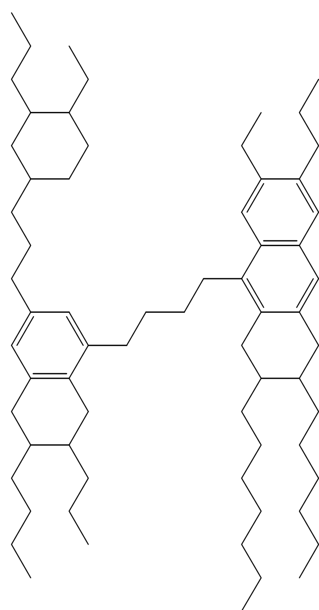
(g) Cyclohexane



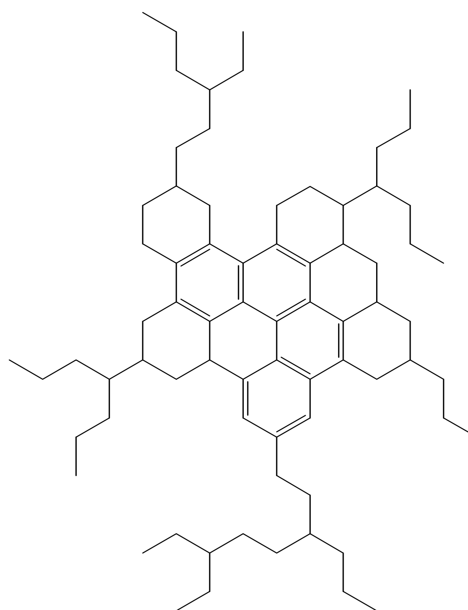
(h) Benzene



(i) Phenantrene



(j) Resin



(k) Asphaltene

Figure 4: *Examples of hydrocarbons (found in petroleum), flat structure formulas (except Fig. 4a)*

Aerobic and anaerobic hydrocarbon conversion by microorganisms

Aerobic oxidation of hydrocarbons by micro-organisms has been known for a century (Söhngen, 1913), but anaerobic oxidation of aliphatic and aromatic hydrocarbons has only been known for the last two decades. Below we describe the most common routes of hydrocarbon degradation; for details we refer to (Widdel & Rabus, 2001). It is well recognized that biodegradation of hydrocarbons requires the activation of the aliphatic chain by disruption of the stable C-H bonds. Under aerobic conditions this is done by the introduction of an O_2-molecule derived oxygen atom into the chain yielding an alcohol, which is modified to an ordinary fatty acid that is subsequently oxidized to CO_2 through β-oxidation. This initial alcohol formation is mediated by a mono-oxygenase enzyme. This enzyme reductively cleaves atmospheric O_2 thereby producing one water molecule (Fig. 5) and an activated (destabilized) substrate (Schlegel, 1997). The hydrogen, yielding the water comes from NADH (NADH is a reduced form of NAD^+ which stands for Nicotinamide Adenine Dinucleotide. This molecule is a biological energy carrier). This substrate activation has mostly been studied in the microorganism *Pseudomonas putida*. The formed alcohol is converted into an aldehyde by an alcohol dehydrogenase. This enzyme removes two hydrogen atoms from the molecule yielding $NADH+H^+$. Subsequently an aldehyde dehydrogenase enzyme incorporates H_2O into the molecule again yielding $NADH+H^+$ and a fatty acid. When regarding the conversion of aromatic molecules to a fatty acid, differences have to be mentioned. First, the aromatic ring needs to be cleaved. The initial step is performed by a dioxygenase enzyme. This enzyme incorporates both oxygen atoms from an O_2 molecule into the ring, yielding commonly the intermediate catechol. Subsequenly catechol is transformed into cis,cis-muconate by again the incorporation of oxygen atoms from an O_2 molecule. Through a series of different intermediates the molecule also ends up in the β-oxidation (Schlegel, 1997). Under anoxic or anaerobic conditions activation of hydrocarbons is more complex. It is currently accepted that the most common anaerobic alkane degradation route involves initial activation of a linear alkane by fumarate addition to the subterminal carbon of the aliphatic chain yielding a substituted succinate, using a glycyl radical as initiator. This glycyl radical is formed in a series of consecutive steps. First a an adenosyl radical is generated by a system which involves a one electron reduction step (Krieger et al. 2001). The formed radical is subsequently converted to a thylyl radical present in the activating protein which activates the aliphatic chain. The route is similar to the well described toluene degradation in which fumarate is added to the methylgroup of toluene. The stronger inertness of the alkane chain is the major difference (Fig. 6). Candidate genes for the proposed activation protein have been studied by Grundmann and Widdel (Grundmann et al. 2008). They identified a gene cluster similar to the toluene activating enzyme

but it displayed a separate line of descent within the glycyl radical enzymes group. This fumarate addition route was also demonstrated by Kniemeyer et al., and described in a recent paper (Kniemeyer et al. 2007), describing the anaerobic degradation of propane by sulphate reducing bacteria. This same activation mechanism of alkanes was also found under denitrifying conditions by (Widdel & Rabus, 2001). The denitrifying strain HxN1 activates n-alkanes yielding methylalkyl succinates providing direct evidence for fumarate addition. With regard to longer n-alkanes however, other findings suggest the existence of a different route for anaerobic n-alkane degradation. A paper by So and Young describes the direct carboxylation at the third carbon of an aliphatic chain as an activation step in which the inorganic carboxyl group is derived from bicarbonate (So et al. 2003). Subsequently the terminal and subterminal carbon atoms are removed yielding a fatty acid one carbon shorter than the original alkane. This route was found in a sulphate reducing bacterium strain Hxd3, earlier described by Aeckersberg et al. (Aeckersberg, 1991) the strain Hxd3 utilized alkanes with a chain length range from C_{12} to C_{20}. Until now, strain Hxd3 remains the only isolate in which an alternative alkane activation route has been displayed. Evidence however for this metabolic pathway, which occurs in consortia of sulphate reducing bacteria, together with the fumarate addition pathway has been obtained thereby suggesting the simultaneous occurrence of these pathways. Both routes did not display activation at the primary carbon of the chain, making the activation of ethane by fumarate addition, which has only primary carbon atoms (Kniemeyer et al. 2007) more interesting. After the initial activation by fumarate addition the formed methylalkyl succinates undergo C-skeleton rearrangement before they are further oxidized. At this moment, the research towards the enzymes involved in these pathways is still in its infancy but ongoing research suggests that similar pathways and enzymes exist among all microorganisms living on hydrocarbons. When regarding the degradability of hydrocarbons, n-alkanes are the most susceptible, followed by simply branched alkanes. Polycyclic alkanes are the most resistant to microbial degradation. This biodegradation in oil reservoirs besides hydrocarbons, requires water and nutrients. This situation is found at the contact interface between oil and water. The remark that hydrocarbon degradation occurs at the oil water boundary, makes all the processes which are related to hydrocarbon degradation essentially surface based processes (Dolfing et al. 2008).

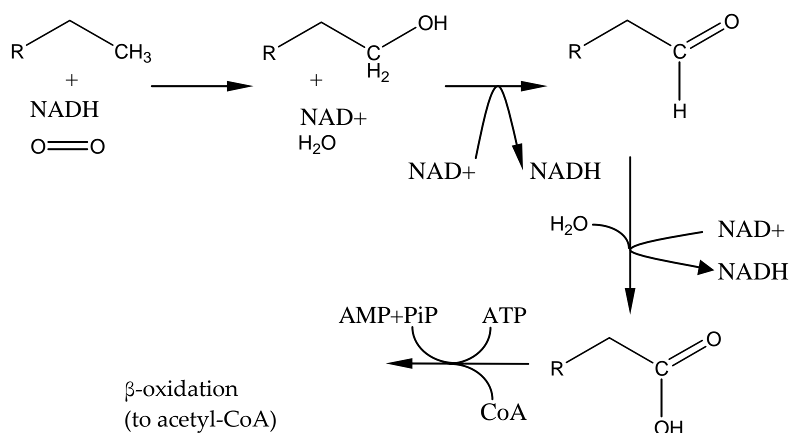


Figure 5: *Aerobic hydrocarbon activation of aliphatic hydrocarbons.*
The aliphatic chain is converted first to an alcohol (monooxygenase enzyme), then to an aldehyde (alcohol dehydrogenase enzyme) and subsequently to a fatty acid (aldehyde dehydrogenase). This fatty acid can be fed directly into the primary metabolism and is metabolised to acetyl-coA units.

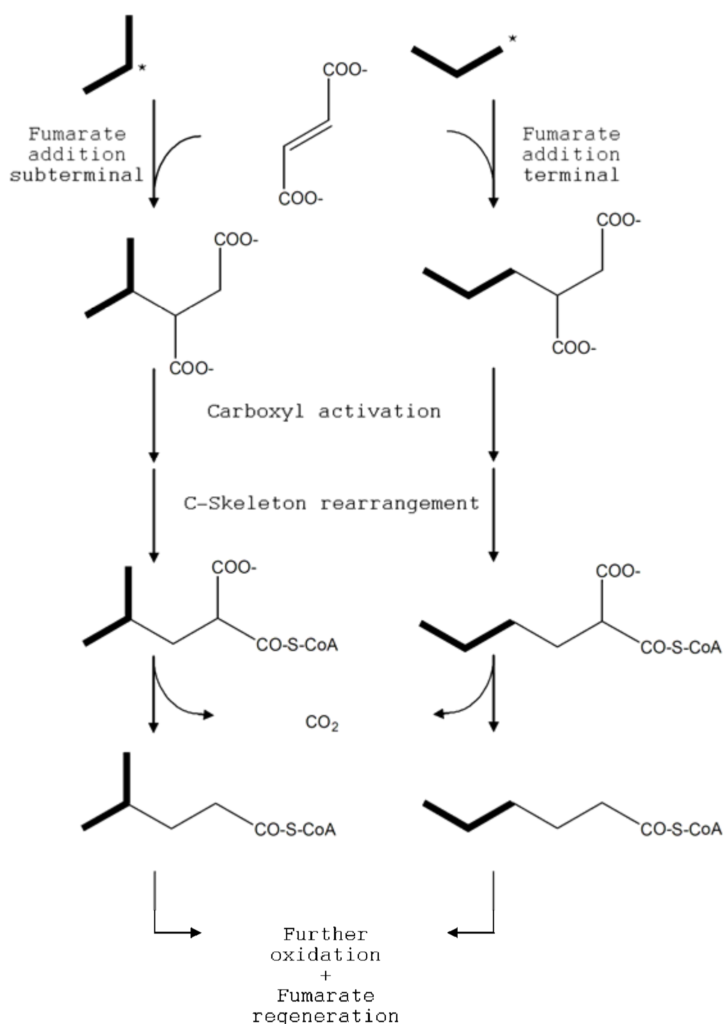


Figure 6: *Anaerobic activation of propane at the secondary and primary carbon atom. First a radical is created and fumarate is added. Then the chain is rearranged and fed into the metabolism.*

Hydrocarbon seeps and oil reservoir environments

Petroleum can enter our biosphere in many different ways; naturally this occurs through so-called hydrocarbon (oil or gas) seeps. They introduce large quantities of carbon into the environment as they are fed by subsurface reservoirs of oil and gas, from which the hydrocarbons migrate through fractured rocks and permeable sediments into the water column. Petroleum also enters the biosphere through human activities, mostly by unwanted discharges and catastrophes with oil tankers and so on. The fact that a lot of excess carbon enters the environment in the form of highly hydrophobic molecules makes these ecosystems surrounding these seeps special. In oil-associated ecosystems there is a physical separation between electron donors (hydrocarbons in petroleum) and electron acceptors that are usually in the water phase (O_2, NO_3^-, SO_4^{2-}, CO_3^{2-}). This is also the case for oil reservoirs. Oil reservoirs are, considered from a microbiological point of view, also special ecosystems. They are deprived of oxygen, which makes them reduced environments. They have different environmental conditions like elevated temperatures and pressures and often high salt concentrations (different from above ground conditions). Energy generation comes solely from chemosynthesis. It has been observed that oil fields holding a temperature of <80 ºC, contain many species of microorganisms. (This however does not rule out the possibility of life in oil reservoirs with a higher temperature.) The petroleum contained in these reservoirs is often to a certain extent biologically degraded (Head et al. 2003). As smaller and simple hydrocarbons are preferred as substrate above heavy oil molecules such as bitumen for example due to their better solubility in water, it is asserted that microbial activity are at the origin of heavy oil deposits since the heavy molecules are left relatively unchanged. Oil reservoirs in general consist of porous reservoir rock, bounded by impermeable rock formations (Fig. 7). In the pores of the porous rock (porous media) petroleum can be found. Usually petroleum has migrated into this porous rock after its formation. Above the oil a gas cap may exist beneath the upper impermeable rock, containing the lightest hydrocarbon fraction. Beneath the oil phase, a water layer may be found. The water layer is referred to as an aquifer. Petroleum reservoirs in general are limited in nutrients, (mainly P-limited). This is confirmed by the fact that cell densities found in brine waters are not very high, on average 10^4 a 10^5 cells per ml are found (Nilsen et al. 1996). Physical constraints enforced by the environment contribute also to these low cell e.g. densities, salinity, temperature and pressure. Temperature is the most discriminating; very few species can live above 110 °C. Many prokaryotes can live at a substantial range of salt concentrations. The temperature is directly associated with the stability of biomolecules. Also reactions run faster at higher temperatures leaving less energy for microorganims who metabolise these reactions.

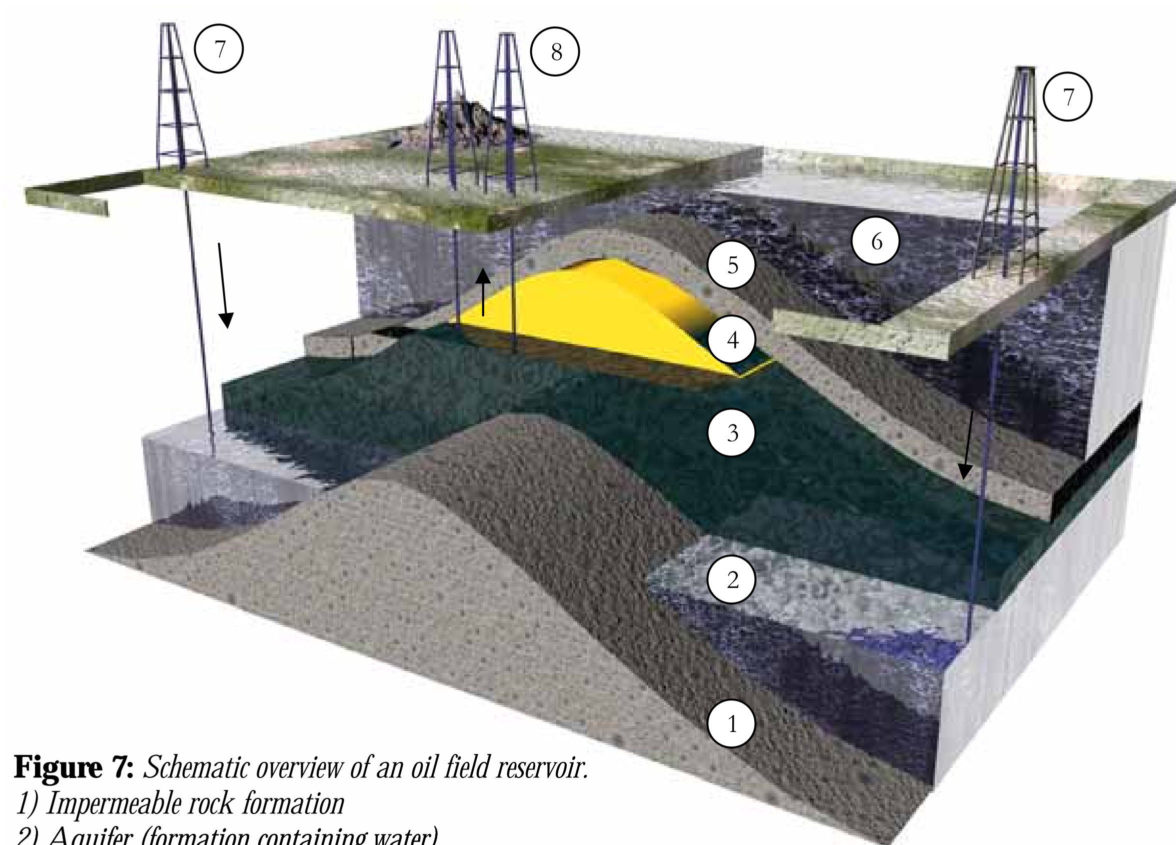


Figure 7: *Schematic overview of an oil field reservoir.*
1) Impermeable rock formation
2) Aquifer (formation containing water)
3) Oil bearing strata
4) Gas cap
5) Impermeable cap rock
6) Water containing strata
7) Water injectors
8) Oil producers

Also other sources of hydrocarbons entering the biosphere can be found, i.e., the so-called gas (predominantly methane) seeps. Methane can interact with water to form the associated gas hydrates. Gas hydrates are solid, ice-like structures containing methane or mixtures of methane and other gases, such as CO_2, ethane, propane and butane. They are formed at high pressure (1-20 MPa) and low temperature (0 – 10 °C), and can be found worldwide at deep-sea spreading zones, convergent plate boundaries, and continental margins (Joye, 2004). The origin of these gases can either be thermogenic or biogenic. Thermogenic gas, mainly C_1-C_5 hydrocarbons, is formed by thermal cracking of fossil organic material (Lorant, 2002). Biogenic gas, predominantly methane, is formed by anaerobic microbial decay of organic matter or of crude oil (Jones et al. 2008) occurring at much lower temperatures (<80 ºC). In contrast to general belief, (Hinrichs et al. 2006) recently showed evidence that also ethane and propane could be produced by microorganisms present in subsurface marine sediments. They proposed that acetate and hydrogen, or acetate, bicarbonate and hydrogen are used as substrates resulting in the production of ethane and propane, respectively.

Microorganisms from hydrocarbon associated environments

Many studies can be found in the literature, that make an inventory of microorganisms that have been isolated from or that have been detected using molecular analysis from oil field reservoirs. Examples are: (Nazina et al. 2006), (Voordouw et al. 1996) and (Telang et al. 1997).

Currently 79 bacterial genera are known to encapsulate one or more members that can utilize hydrocarbons as a carbon source. The ability to degrade hydrocarbons is found in microorganisms throughout the entire prokaryotic tree of life (Fig. 8). Also several species in the field of fungi and algae are known to degrade or transform hydrocarbons. Hydrocarbon degrading microorganisms were first isolated around a century ago (Söhngen, 1913). Molecular techniques like Denaturing Gradient Gel electrophoresis (DGGE) and clone library construction of PCR amplified 16S rRNA genes have yielded a vast amount of information on the predominant organisms found in brine waters and core samples. Species that have been detected largely fall into a few dominant groups: fermentative bacteria, iron reducers, sulphate reducing bacteria and methanogenic archaea. Since the oil industry is experimenting with NO_3^- injection also denitrifying microorganisms are found in the subsurface, see for an example (Gittel et al. 2009). Aerobes and facultative anaerobes have also been detected in oil field samples, but their indigenous nature is questioned. The knowledge of oil field microbiology does however not allow a direct exclusion of the presence of aerobic microorganisms yet. The presence of closely related microorganisms found in distant oil fields does indicate the existence of a widespread anaerobic biosphere in petroleum reservoirs (Magot, 1996).

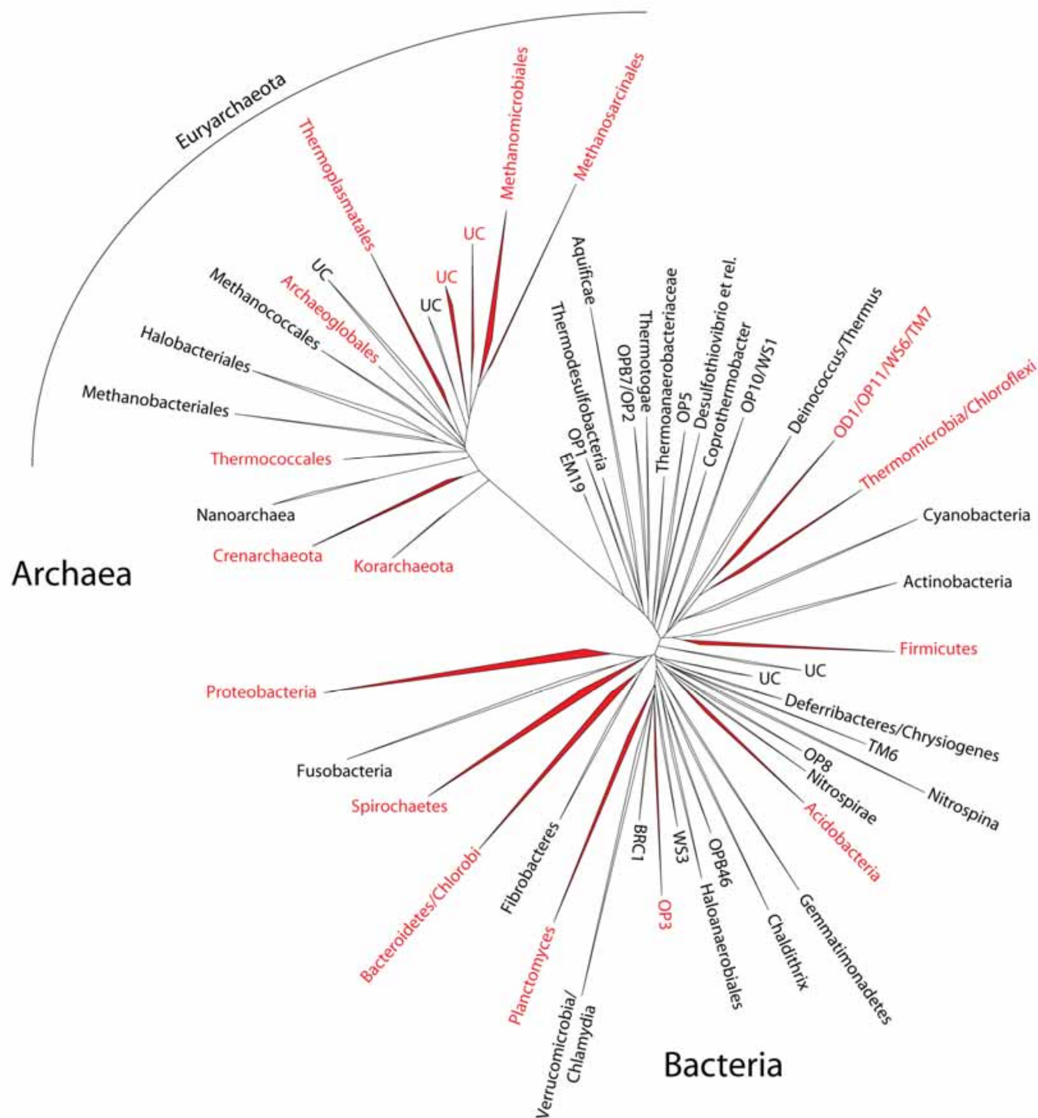


Figure 8: *Phylogenetic tree of bacteria and archaea based on 16S rRNA gene sequence, groups in red include members that can degrade petroleum.*

To give a full overview of all the detected species is outside the scope of this introduction; here we provide a small overview of the dominant prokaryotic groups that are commonly found in oil field environments and of each group we include a species example. The encountered groups are largely determined by the overall metabolic state of the environment. Sulphate reducers are the focal point of the majority of the studies since their effects are detrimental. It is commonly accepted that mesophilic sulphate reducers are mainly responsible for harmful corrosion effects of oil producing facilities while thermophilic sulphate reducers cause *in situ* souring, which can lead to hazardous gas-outbreaks in oil fields. Common mesophilic genera are *Desulfovibrio* and *Desulfobacter*. An example is the halotolerant species *Desulfovibrio longus* (Magot et al. 1992). Thermophilic sulphate reducing genera are *Desulfotomaculum*, *Thermodesulforhabdus*, *Desulfacinum*. An example of a thermophilic species is the complete oxidiser *Thermodesulforohabdus norvegicus* (Beeder

et al. 1995), which was isolated from a North Sea oil reservoir. Most thermophilic bacteria only survive below 82 °C. In addition to the bacterial genera, one archaeal genus contains species that are able to perform sulphate reduction. Hyperthermophilic members of the genus Archaeoglobus have been isolated from oil field environments. One member is *Archaeoglobus fulgidus* (Beeder et al. 1994), also isolated from a North Sea oil reservoir. Another important fraction of detected species in oil fields are fermentative bacteria; their metabolic activities in oil field environments are not well known since they do not contain common substrates for fermenters like carbohydrates. Frequently found are mesophilic members from the genus *Halanaerobium*, considered as being moderate halophiles. An example is *Halanaerobium congolense* (Ravot et al. 1997), isolated from oil field brine water. Thermophilic fermenters are found more often, this is probably due to the high temperatures that prevail in many petroleum reservoirs. Examples of thermophilic and hyperthermophilic genera are *Thermotoga*, *Geotoga* and *Petrotoga*. Most members are thermophilic heterotrophs. An example is the species *Thermotoga subterranea* (Jeanthon et al. 1995). Also the presence of hyperthermophilic Archaea has been reported. Genera examples are *Pyrococcus* and *Thermococcus*. With respect to iron reducers from oil fields, species mainly fall in the *Shewanella* genus. An example of a species belonging to a different genus containing iron reducers is *Deferribacter thermophilus* (Greene et al. 1997), isolated from a waterflooded North sea oil reservoir. It can reduce Fe and Mn in the presence of various electron donors. Also various denitrifyers can inhabit oil reservoirs (Myhr & Torsvik, 2000), Many denitrifyers that use organic acids like acetate as substrate are found. These denitrifyers can either be microaerophilic, facultative anaerobic or anaerobic. Many references in this chapter can be found related to all the mentioned types of microorganisms. The last group of microorganisms addressed are the methanogenic Archaea. The biogenic production of methane has been reported as early as 1983, (Belyaev et al. 1983). Isolation of several methanogenic species from various types of oil reservoirs has been successful. An example is the species *Methanocalculus halotolerans* (Ollivier et al. 1998), this species uses molecular hydrogen for methanogenesis. Since many oil fields hold a high salinity, also the compound methylamine is a well known substrate for many methylotrophic archaea inhabiting oil field reservoirs; a good example is *Methanohalophilus euhalobius* (Davidova et al. 1997). Life thus thrives in oil reservoir environments and oil associated environments. The metabolic activities of microbes in these environments however do not go unnoticed. Metabolic products like H_2S provide the oil industry with severe issues. Life however might also provide the oil industry with opportunities. Both will be addressed in the next paragraphs.

Reservoir souring

Primary recovery of oil field exploitation yields on average only 10-15% of the total oil in the reservoir. Therefore secondary recovery techniques are applied to enhance this recovery. Seawater flooding is frequently applied to displace oil towards producers on off-shore locations. Due to the relatively high content of sulphate (0,0282 mol SO_4^{2-} Kg^{-1} water) in seawater, seawater flooding is often associated with the huge issue of reservoir souring. Reservoir souring is a conceptual name comprising the unwanted production of H_2S by sulphate reducing prokaryotes through oxidation of organic electron donors that can come from the petroleum (Fig. 9). Oil organics are mainly oxidised to acetate and CO_2. Since water injection volumes are large (10000 m^3/day) the amounts of sulphide that can be produced in oil reservoirs can be 1100 kg/day (Hubert & Voordouw, 2007). H_2S reduces the quality of the produced oil and poses a safety concern. H_2S is also a very corrosive compound. It is believed that mainly thermophilic SRPs are responsible for this *in situ* souring process.

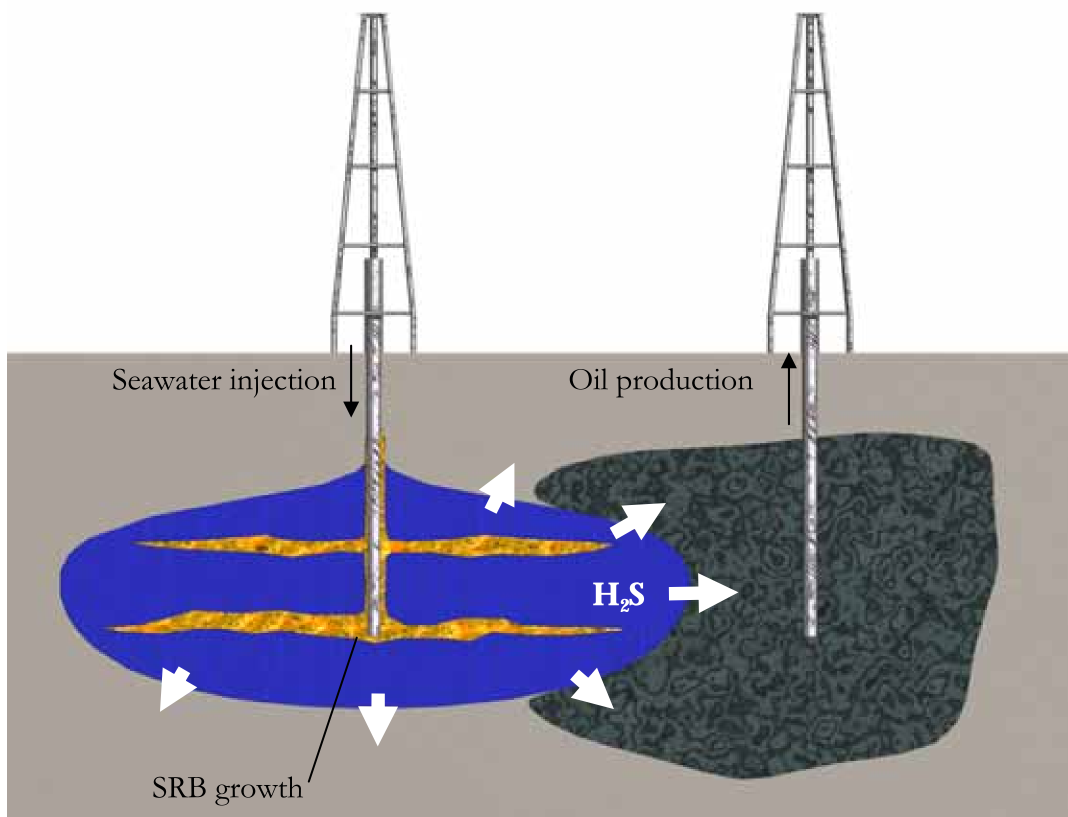


Figure 9: *Reservoir souring schematic, Water is displacing the oil and at the water-oil barrier SO_4^{2-} reduction is taking place. The produced H_2S accumulates in the oil phase.*

Microbial Induced Corrosion (MIC)

Microbial Induced Corrosion poses, next to *in situ* reservoir souring an additional problem throughout the oil industry. In every stage of the process, pipe-lines and tanks corrode and costs are estimated at about $15 billion on yearly bases in the US only. In biocorrosion the biogenic production of H_2S by sulphate reducing prokaryotes plays again a major role. In this case, the electrons come from the steel pipe walls though the dissolution of solid Fe^0 to Fe^{2+} + $2e^-$ and the creation of H_2 from H^+ merging with the freed electrons. The formed H_2 can be used by anaerobic prokaryotes and if again sulphate is present, it will trigger the development of an H_2S producing biofilm (Fig. 10).

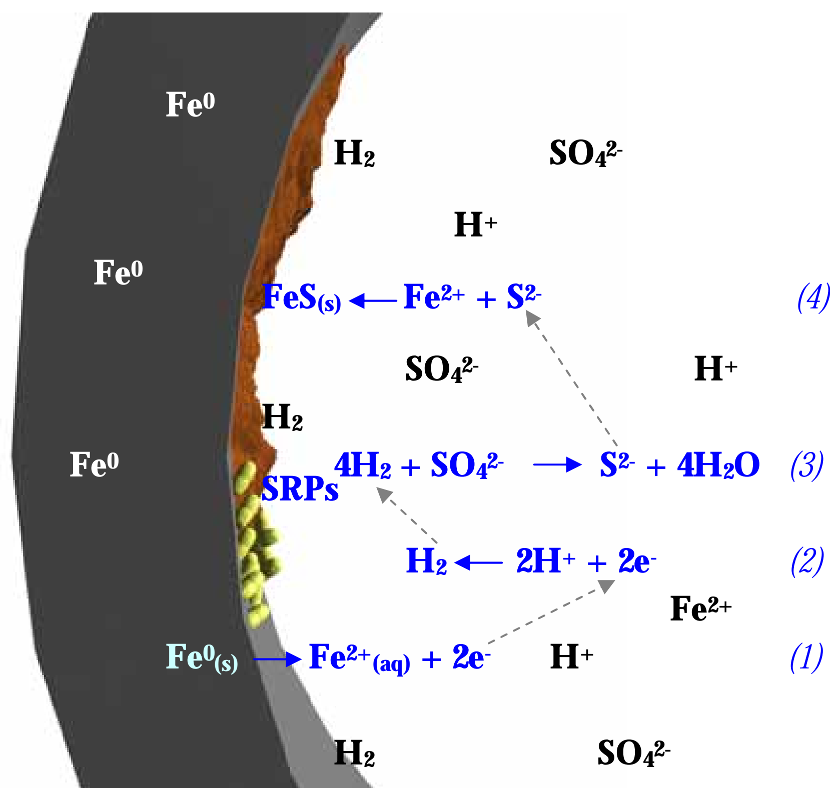


Figure 10: *The commonly proposed process of microbial corrosion (cathodic depolarisation).*
(1) Dissolution of iron.
(2) Conversion of protons to hydrogen by the released electrons
(3) The hydrogen is used by sulphate reducing prokaryotes (SRPs) and H_2S is formed.
(4) Iron and Sulphide precipitate as FeS and form a corrosion deposit on the steel.

One of the current plausible solutions to control souring is nitrate injection (NO_3^-) (Hubert et al. 2005), (Davidova et al. 2001), but often biocides are used as a microbial growth inhibitor. The issue of MIC is addressed in detail in chapter 3 of this thesis and the associated references.

Microbial Enhanced Oil Recovery (MEOR)

To continue on what is said earlier regarding our society being based on oil as the main resource, the world today is facing an increasing concern because the 'easy' oil sources are ending and it is becoming more difficult to retrieve the oil from currently known reservoirs (Bakas, 2007). While using different recovery techniques (water flooding, polymer injection, etc.) on average only 33% of the oil contained in an oil reservoir is retrieved, as mentioned earlier. The remainder stays in the reservoir and this 66% of unused oil holds a major economical value. With the ever increasing demand for energy and resources, new techniques are currently developed in order to retrieve a fraction of this 66% in order to keep oil as a resource affordable. Since the discovery of the existence of microorganisms in oil reservoirs, Microbial Enhanced Oil Recovery (MEOR) is regarded as an option for recovering more oil from reservoirs (Beliaev et al. 2004). The basic concept of MEOR is that microorganisms are used as acting agents to change properties in an oil field in order to make the oil more accessible or displaceable. If one looks in the literature, several methods to use the activities of microorganisms for this particular purpose are presented. They are shortly mentioned below. The variety among reservoir characteristics and the oil found in oil fields are however substantial and it cannot be expected that a single method is applicable for all circumstances. This is in particular so for the oil and reservoir chemistry. We expect that MEOR, if developed, will be only applicable to a limited number of oil fields. Listed below are the major envisioned MEOR mechanisms as they are described in the literature (Bordoloi & Konwar, 2008), (Van Hamme et al. 2003).

Gas production

CO_2 production by microorganisms can be used to repressurize a depleted oil field or to modify the viscosity of the oil the reservoir holds (Chisholm et al. 1990). Such a repressurization can be expected to be effective only if the produced CO_2 would occupy one tenth of a pore volume at high pressures. This implies metabolic conversions on a large scale. If such a large scale conversion is achieved it can be expected to have a positive influence on the oil production. Possible enhancement mechanisms are oil swelling, decrease of interfacial tension, reduction of viscosity and the increase of permeability.

Conversion of inaccessible oil to methane.

Many oil field formation waters contain methanogens (CH_4 producing Archaea). The most common known pathway of methanogenesis is the reduction of CO_2 in combination with H_2 in order to form CH_4. Subjected to oil field conditions however, other substrates like methylamine

or methanol are frequently used (e.g., methylamines, acetic acid, etc). For difficult oil, methane formation may be desirable as it can be used as combustible gas or because it possibly can be used to maintain the reservoir pressure (Gray et al. 2009) (Grigoryan & Voordouw, 2008).

Surfactant, solvent and acid production in order to decrease the viscosity of the oil and to open up the reservoir.

A substantial amount of microorganisms can produce surfactants (molecules with a polar and an apolar region), which possibly can enhance the oil recovery. Mentioned mechanisms are converting trapped oil into emulsion droplets, interfacial tension reduction and detachment of oil films from the rocks. Examples of biosurfactants are acetoin, polysaccharides, glycolipids and phospholipids. Other produced chemicals can cause viscosity reduction of the crude oil, e.g., the production of solvents like n-butanol, acetone and alcohol. Microbes found in the community of an oil field, can produce organic acids by fermentation. These organic acids can readily dissolve $CaCO_3$ and other carbonates and by doing so can enhance permeability within lime stone reservoirs (Tanner et al. 1991). Organic acids and the released CO_2 also decrease the viscosity of the oil. Produced organic acids are commonly acetic acid, butyric acid and lactic acid.

Selective plugging of micro channels allowing a more efficient water flooding.

Residual oil left in reservoirs that is produced by so-called “stripper” wells (an oil well whose production has declined to less than ten barrels a day) can be recovered when highly permeable watered out regions of oil reservoirs can be plugged with biopolymers or biomass. When the high permeable regions of a reservoir are blocked, water is forced through the less permeable regions of a reservoir and thus oil production is enhanced (Cusack et al. 1990).

As stated in the literature, MEOR is the set of proposed technologies to improve oil recovery using the *in situ* activities of microorganisms. If hydrocarbon degradation and oil-associated microorganisms are used in MEOR processes, it will be largely dependent on how fast the micro-organisms are metabolizing, i.e., whether they are significant in the productive life time of the reservoir (~ 10-30 years). Therefore a large part of the success of MEOR depends on enlarging the surface between the oil phase and the water phase, where the microbial activity takes place. The main challenge is having microbes that produce metabolic products that influence the oil or the oil field composition. Although many envisioned methods for MEOR processes can be found in the literature (see earlier), it is nowadays commonly believed by many industrial companies and experts in this field that only selective plugging of high permeable zones in an oil reservoir by biomass (biofilms) is a realistic and viable option, which is not only technically but also economically feasible.

Microorganisms as possible indicators of induced reservoir change (biomonitoring)

A novel idea within the so-called 'smart field' applications is the use of microorganisms present in an oil field as biological markers for field monitoring, not as acting agents as proposed by the various envisioned MEOR processes. The basic concept is that microorganisms present in the subsurface might provide information about the reservoir, which is essentially a black box environment, that can be obtained from their genetic content. This information can then be used for a better oil field exploitation. It is believed that a better knowledge of the reservoir environment allows a practical improved field exploitation of 5 – 10% (Nævdal, 2006). To routinely monitor the biological processes taking place in an oil field, molecular tools will have to be developed that allow fast screening of oil field samples on the presence of microorganisms. Looking at the total amount of biomass present in the subsurface, it has been estimated that there are of the order of 5×10^{30} prokaryote cells in subsurface environments (Whitman et al. 1998). In spite of uncertainties in the estimates, it is generally accepted that deep subsurface biomass may account for at least 90% of the global prokaryotic biomass. Subsurface prokaryotic biomass thus exceeds biomass in ocean and terrestrial environments. As to their impact on petroleum, over 50% of the world's oil reserves are partially biodegraded leaving overall the heavy oil deposits we find today (Head et al. 2003). Since microorganisms are so ubiquitously widespread in these environments, their potential to be used as information carriers is significant. This knowledge could ultimately be used in developing high throughput biological measurement tools that can be used as indicators of reservoir conditions and potentially assess the effects of for instance smart well applications, souring control (NO_3^- injection) and various envisioned MEOR processes (Sen, 2008) The role of microorganisms as biomonitoring agents has long been under debate. Consensus is however being reached on the potential beneficial use of organisms in monitoring applications (Pronk, 2009). Examples are: health monitoring in humans and monitoring of processes in the subsurface. For the last application, also transport behaviour is essential. Eventually it boils down to the question are microbial communities prone to changes induced by human activity in the petroleum reservoir? and if so, has the community a reflection in the observed community in brine waters that are transported to and tapped at the surface?

Scope and outline

The ISAPP program

The research described in this thesis, is carried out within the framework of the ISAPP (Integrated System Approach Petroleum Production) program. (Most of the text below is taken from the website www.isapp.nl). This 5-year research program is an initiative of the Delft University of Technology, Shell Exploration and Production and TNO. The aim of the ISAPP program is to increase hydrocarbon recovery through the application of innovative reservoir development and management technologies. It is envisioned that in the future, hydrocarbon production systems may be more intelligent. These 'smart' hydrocarbon production systems are aiming for faster and higher recoveries from oil and gas fields, cost reductions through automated and unmanned operations, greater flexibility to manage unexpected events that jeopardise production, greenhouse gas emission to be reduced through downhole processing and storage. The main areas of research are:

- Innovative concepts for the hydrocarbons production process 'Smart wells.'
- The development of an integrated 'real-time' dynamic simulation, inversion and validation environment for reservoir, well and processing facilities.
- Laboratory of innovation. The analysis and testing of methods, techniques and work processes to accelerate the process of innovation in the Exploration&Production sector.

The research described in this thesis fits best in the first area of research, in which the microbiology of petroleum systems has been studied to estimate the possible role of microbial communities as an additional information source for smart well applications. Biofilm formation in porous media was studied as a method for enhanced oil recovery.

Aims of this research

This research was carried out at two different departments, i.e., geotechnology and biotechnology, of the Delft University of Technology, the result is a research in which both fields of expertise were combined. The overlying field of research has been subsurface microbiology, in particular of oil field systems. This PhD project has been a pioneering project, looking at the possibilities and options for a better oil recovery within the extensive field of subsurface microbiology. Since this field holds many aspects and lines of research eventually two research questions were formulated within this study.

(1) The first question was: can the microbial community of water samples from an oil field be used as an additional information source regarding reservoir conditions. In Chapter 2, the microbial community of a Dutch oil field and its oil/water separation facility have been studied. The separation facility allowed research towards produced water from one oil field subjected to changing environmental conditions. Samples have been studied by DGGE and Clone libraries. The results hereof are compared to the conditions prevailing in the places where the samples were derived from. In chapter 3 the effect of biocides on the microbial diversity thriving in an oil/water separation facility has been studied in a similar way. Since microorganisms mainly grow as biofilms, the pore water which is sampled might not be representative for the full microbial community. In Chapter 4 therefore the microbial diversity of an oil field core sample has been studied. In Chapter 5 the concept of using the microbial community of a subsurface environment has been investigated for the potential of two different subsurface aquifers in Bangladesh for arsene release when drinking water would be produced from these aquifers.

(2) The second question was: how do microorganisms influence the flow of water or oil through porous rock as it is found in oil fields. Or more precise, how does biofilm develop at the pore level and what are the implications of the presence of biofilms on flow diversion? To answer these questions a micromodel was developed to observe these processes in real time. This development is addressed in Chapter 6. The micromodel is an idealised 2D version of reservoir rock. Development of micromodels is not easy and requires a long path of troubleshooting, redesigning, and new optimizations. Therefore the development of the micromodel is addressed in detail, as is the Particle Image Velocimetry (PIV) technique to observe flow diversion due to biofilm growth. In Chapter 6 and Chapter 6-extended interpretations are given on the effects of biofilms on flow velocities, flowfield disruptions, porosity reductions, and the presence of oil on biofilm formation. In chapter 7, a summary of the obtained results is given and conclusions are drawn. The research described in this thesis ends with recommendations for further research and acknowledgements.

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Well site, Berkel Oil field (Rotterdam, The Netherlands)
October 2007

2

Microbial diversity of an oil-water processing site and its associated oil field:

The possible role of microorganisms as information carriers from oil-associated environments.

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Microbial diversity of an oil-water processing site and its associated oil field: the possible role of microorganisms as information carriers from oil-associated environments.

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Abstract

The phylogenetic diversity of Bacteria and Archaea in water retrieved from a Dutch oil field and different units of the associated oil-water separation site were determined using a combination of two culture-independent methods. Denaturing Gradient Gel Electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments was used as a quick method to scan the microbial diversity in (i) the produced oil-water emulsion, (ii) two different oil-water separator tanks, (iii) a wash tank/O_2-scavenger and (iiii) water taken from an injection well. Subsequently, longer 16S rRNA gene fragments were amplified, cloned and sequenced to determine the diversity in more detail. Comparative sequence analysis showed significant differences in community composition between the sampled environments. It was also demonstrated that each of the sampled communities was statistically different when compared to each other. One of the addressed questions was whether the detected microorganisms could serve as indicators for the environments from which they were retrieved. It was observed that the community found in the production water resembled those reported previously in oil reservoirs, indicating that these ecosystems harbor specific microbial communities. It was shown that small changes, such as a decrease in temperature or the intrusion of oxygen into the system already cause a distinctive shift in these communities. The addition of the alternative electron acceptor SO_3^{2-} to the wash tank in the form of NH_4HSO_3, commonly used in the oil industry to scavenge oxygen, resulted in a complete community change, giving rise to an opportunistic sulphate reducing community. The use of an alternative oxygen scavenger, not containing SO_3^{2-} is therefore advised. The fact that these changes in the community can be linked to changes in their environment might indicate that the same tools can be used for the monitoring of changing conditions in oil reservoirs upon like e.g. water flooding.

Introduction

It is well recognized that microorganisms thrive in oil fields and industrial oil-associated processing sites. Activities of microorganisms in oil-water environments, such as oil reservoirs have been frequently reported. Examples of this activity are the occurrence of heavy oil (Head et al. 2003), and the internal corrosion of oil pipelines (Neria-Gonzalez et al. 2006). Since the discovery of aerobic and anaerobic microorganisms associated with oil-water systems, many species have been detected with the help of molecular techniques. A variety of production waters from different oil fields have been studied (Dahle et al. 2008), (Grabowski et al. 2005), (Nazina et al. 2007). These studies yielded a large variety of microbial communities. Many new microorganisms have been isolated from these environments, belonging to diverse groups including sulphate reducers, fermenting bacteria, iron reducers, acetogens and methanogens (Magot et al. 2000).

Changes in a reservoir are difficult to assess. Since microorganisms are directly influenced by their environment, they might be used as indicators for changes in reservoir conditions. The presence of specific communities could provide information on the characteristics of the oil reservoir itself (e.g., temperature, acidity, salt content, redox, etc). Changes in the microbial community could also be used to indicate changes caused by human activity, such as water flooding. In this way the community composition could be used as a biomonitoring tool, providing information about the conditions and processes that occur down-hole. This information could be a welcome contribution to a better oil recovery in so-called "smart well" applications. Since microorganisms also thrive in surface facilities, where the oil is separated from the water, such a location was picked as a case study environment to investigate the sensitivity of the found communities towards changes in the environmental characteristics like heating and the addition of an external electron acceptor. The different units might provide an insight into the microbial processes in the oil field, when temperature and chemistry are similar between oil reservoirs and units.

The aim of this paper was to make a step towards the possible application of community composition as information source in oil-water-systems. Hereto we made a comprehensive study of the bacterial and archaeal communities present in an oil production well and its associated oil-water separation facility that are located in the western part of the Netherlands. The collected samples were first analyzed by PCR-DGGE in an attempt to obtain a rapid overview of the complexity of the bacterial and archaeal communities and to assess the (dominant) species present in the oil-water emulsion. Subsequently, samples were analyzed in more detail by comparative analysis of 16S rRNA gene sequences obtained by cloning.

Materials and Methods

Description of the oil field and the oil-water processing site

The studied Berkel (designated later as Brk) oil field is located in the Western part of the Netherlands and holds multiple oil production wells and an oil emulsion separation facility, in which the crude oil phase is separated from the water phase (Fig. 1, overview). The field is young in geological terms. The origin of its deposition is estimated to be lower-Cretaceous. It is a shallow marine unconsolidated sandstone deposit found at a depth of approximately 1250 m. The reservoir contains medium heavy crude oil, holding an API gravity of 20°. The porosity of the field on average is 25%, the permeability is between 750 and 1800 mD. The reservoir holds an anticline structure. With respect to oil production, the field is mature. The exploitation of the field started in 1977. The field has a small gas cap. The produced oil on average has a viscosity of 29 cp at reservoir conditions. The produced oil-water emulsion is pumped up by several nodding donkeys (Beam pumps) and is collected at a central point from where it is pumped to a primary oil-water separation tank located at the separation site. The travel time of the liquid from the reservoir to the surface takes 2.5 hours; from the collector to the first separator tank takes an additional 2.5 hours. In the first oil-water separator tank (T101, the 'cold primary' separator) the emulsion is separated based on physical properties only; the bulk of the water is being removed here (the retention time of the water phase is 1 day). After this procedure the remainder of the oil-rich emulsion is pumped towards a second oil-water separator tank (T102, the 'hot secondary' separator) where it is heated to a temperature of approximately 50 ºC to obtain a better separation between the oil and the water, which is removed (the retention time of the water is 3,5 days). The last traces of water are removed in a wash tank (T103). The salinity is lowered through the addition of tap water (the retention time of the water is 6 days). Ammonium bisulphite (NH_4HSO_3) is added to the tap water to scavenge and remove oxygen, since tap water is O_2 saturated. The remaining oil fraction is separated by flotation. The oil fraction is sent from this tank to the refinery. The water from all industrial units is collected at a central water collection point from where it is disposed in a deep subsurface aquifer, beneath the depth from which the oil is retrieved. Therefore, it is designated as a water disposal process. The distance between the production wells, the injection point and the surface facility location (oil-water separator systems) is about half a kilometre. Samples for molecular analysis were taken from the produced oil-water emulsion, the water phase from both oil-water separator tanks, the wash tank and the injection water. Data on the chemical composition of the produced water have been provided by Shell International Exploration & Production (Fig. 1. properties of the surface facility units).

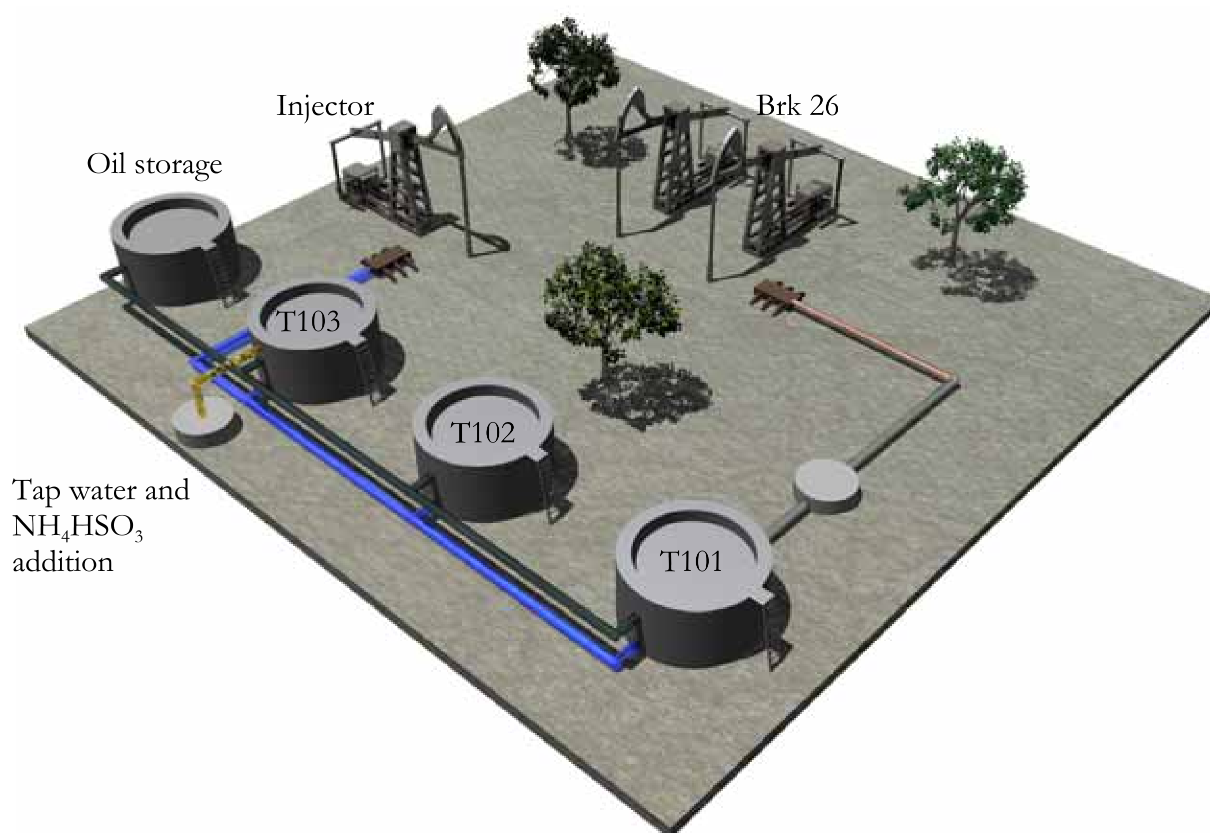

Figure 1: *Overview of the sampling site, picked as a case study model. Samples were taken from the oil well Brk26 (53 °C), the first oil-water separator T101, the second oil-water separator T102, the wash tank T103, and the injector well. In T103, tap water is added to reduce the overall salinity; NH_4HSO_3 is added to the tap water to scavenge the oxygen from the tap water.*

The produced oil-water suspension is first quickly guided through a heat exchanger. T101 is a cold separator (35 °C). Oil is separated based on flotation. T102 is a hot separator. The suspension is heated to 50 °C. T103 is a wash tank. Water is collected from all tanks and is transferred to the well site where it is pumped back into the subsurface.

Sampling procedure and preparation

From each site, 10-liter samples were taken in sterile jerry cans. The jerry cans were completely filled and sealed directly with screw caps to avoid oxygen intrusion. The samples were immediately taken to the laboratory (the time between the sampling and the filtration procedure was by approximation 30 minutes). Water samples from the different tanks were taken close to the oil-water interface. In the laboratory the water samples were filtered using 0.2 μm hollow fiber filters (Spectrumlabs, mediakap-5 hollow fibre filter), in order to concentrate the biomass present in the water. The filtered volume of every sample was 4 l (performed in duplicate). After the filtration procedure, the filters were stored at -20 ºC for further analysis. During filtration, attempts were made to avoid filtering the oil phase. Oil blocked the filters and had a negative influence on the later DNA extraction.

DNA extraction

One filter of each sampled environment was thawed on ice, filter lamella were washed with buffer and approximately 3 ml of the cell suspension released from the filter was collected in sterile Eppendorf tubes and centrifuged for 1 minute at 13,200 rpm. 90% of the supernatant was removed, thereby achieving a 10-times concentration of the biomass and a removal of most potential PCR inhibitors (e.g. residual oil molecules). The pellet was resuspended into the remainder of the supernatant. This suspension was subjected to DNA extraction using the Soil DNA Extraction Kit (Mo Bio Laboratories Inc, Carlsbad) according to the manufacturer's protocol. DNA extraction was also performed on the remaining filter pieces. Subsequently the DNA amount from all extractions was quantified using a Nanodrop 1000 Spectrophotometer (Thermoscientific, The Netherlands). The obtained DNA was used for further PCR amplification. To check for cell lysis after the freeze-thaw cycle also the supernatant was subjected to DNA extraction, but DNA levels were below the detection limit, and no PCR product could be obtained using this as a template, indicating that the vast majority of the DNA was present in the pellet. Also the DNA extraction on the filtered pieces yielded no significant amounts of DNA and no PCR result was obtained.

16S rRNA gene amplification

Partial 16S rRNA gene sequences were amplified from the genomic DNA retrieved from the different Berkel field industrial units, the production water and the injection water. All sampled environments were tested for the presence of bacterial and archaeal 16S rRNA genes. To obtain partial bacterial 16S rRNA gene sequences the universal primer pair 341F+GC and 907R (Schäfer, 2001) was used. In the bacterial PCR a touchdown program was implemented. To obtain partial archaeal 16S rRNA sequences an additional nested approach was used complementary to the direct PCR. In previous studies it was observed that the direct use of the archaeal primer pair Parc519F and Arc915+GC yielded also bacterial sequences (Vissers et al. 2009). Therefore we preceded this PCR amplification with an amplification of the nearly complete archaeal 16S rRNA gene using the primer pair SD-arch-0025-a-S17 and S-*-Univ-1517-a-A-21. Dilutions up to 1/10000 were made from all obtained PCR products, which served as templates for PCR amplification with the primer pair Parc519F - Arc915+GC. The partial archaeal 16S rRNA genes were amplified as described by Coolen et al. (2004). Full (by approximation 1500 bp) 16S rRNA PCR fragments were used as template. The amplification of these fragments was modified after Wilms et al. (2006). In short: A first DNA denaturation step of 5 min at 95 ºC was followed by 34 cycles of 30 sec denaturation at 95 ºC, 40 sec annealing at

58 ºC and 90 sec elongation at 72 ºC. To finalize an extra 10 min elongation at 72 ºC was added to the end of the PCR. The bacterial 16S rRNA gene fragments for clone library construction were obtained by amplification using the universal bacterial primer pair GM3-GM4 as described by Muyzer et al. (1995). For the cloning of the full archaeal 16S rRNA genes, the obtained full PCR products during the first amplification of the nested PCR approach have been used as insert. All PCR amplification reactions were performed in a T1 Thermocycler (Biometra, Goettingen, Germany)

Denaturing Gradient Gel Electrophoresis and clone library construction

DGGE was done according to the method reported by Schäfer & Muyzer (2001). In short: 1 mm thick 6% acrylamide gels with a 20 – 80% urea-formamide gradient were applied for the separation of the partial bacterial 16s rRNA gene fragments. 30-70% gels were used for the separation of archaeal 16S rRNA gene fragments. Gels loaded with bacterial PCR products were run at 100 V for 18 hours. The gels loaded with archaeal PCR products were run at 200 V for 5 hours. Obtained bands were placed 15 µl, 10 mMol Tris buffer, pH 8,5 and kept at 4 ºC for 24 hours. The obtained solution was used as template DNA for re-amplification according to the protocol mentioned above. Clone libraries were constructed using a commercial pCR®4-TOPO cloning kit (Invitrogen) according to the standard manufacturers protocol. Obtained vectors were placed (transformation) into chemically competent *E. coli* cells (delivered with the kit). They were grown on agar plates containing kanamycin. Of each environment 96 clones were selected. Their colonies were picked and re-amplified using the MF-MR primer pair provided with the kit according to the manufacturers instructions.

Cluster analysis of DGGE results

Cluster analyses were performed on all the DGGE gels, using the software package Gelcompar2, v5.0 (Applied Maths, Belgium). Gel images were loaded into the software, gel strips were identified and bands were determined. The gel images were normalised accordingly using internal standards. Cluster analysis was performed using the Pearson correlation. Dendrograms were created using maximum parsimony clustering. The intensity of the bands was not taken into account.

PCR product purification and sequencing

25 µl PCR products obtained from reamplification of the clones or DGGE bands were put in sterile separate vials. 1,6 µl ExoSap-IT enzyme solution (USB Europe) was added to remove the single stranded primers and remainder of the nucleotides. Purification was performed according to the manufacturer's protocol. The purified PCR products were diluted accordingly to obtain a 50 ng/µl PCR products solution, subsequently they were sequenced by a commercial company (Macrogen, Seoul, Korea).

Comparative sequence analysis

To obtain a first indication, obtained (partial) 16S rRNA gene sequences were compared with sequences stored in the GenBank nucleotide database by applying the blast algorithm (Altschul et al. 1990). Sequences were then imported into the ARB SSU rRNA database (Ludwig et al. 2004, see also www.arb-home.de). They were aligned using the automatic alignment tool in the contained in the software package and subsequently checked manually on errors. Phylogenetic trees were generated by application of the Maximum Likelihood (ML) algorithm, FastDNA ML. First, the sequences from the clone library were imported into the ARB database and an ML tree was created. Later the DGGE band sequences were added to this tree. The lengths of all the clones holding sequences with high quality was around 700-800 bp, all the bacterial DGGE bands were around 500 bp, the archaeal DGGE bands are around 400 bp. All obtained (partial) 16S rRNA gene sequences were deposited in the GenBank database. Bacterial DGGE bands, accession numbers (FJ941796 - FJ941826). Archaeal DGGE bands, accession numbers (FJ941438 - FJ941470). Bacterial Clones, accession numbers (FJ941471 - FJ941795). Archaeal clones accession numbers (FJ941116 – FJ941437).

Statistical analysis of the clone libraries

Similarity between the clone libraries was done by applying the available webLIBSHUFF tool. This tool estimates within a 95% confidence limit the similarity of two sets of sequences (Henriksen, 2004). All clone libraries were compared pair wise.

Results

Chemical composition of the production water

The water produced at the oil wells and the water from the first two oil-water separator tanks is hypersaline. It has a NaCl concentration of around 73 g L^{-1} (1,25 Mol L^{-1}), which is over two times the salt content of average seawater. The ions K^{+}, Ca^{2+} and Mg^{2+} are present as minor cations, resp. 0,35, 3,41 and 1,06 g L^{-1}. Ba, Sr and Fe are present in micro-quantities. Inorganic carbon is present as CO_2 0,67 g L^{-1}, and bicarbonate HCO_3^- 0,18 g L^{-1}. The produced water is low in SO_4^{2-} (below the detection limit of 0,1 mg L^{-1}). The field is not seawater flooded. It is however asserted that minute quantities of SO_4^{2-} are present since small quantities of H_2S are detected in the off-gas from the wells (this might also come from organo-sulphur oil component degradation). The water that ends up in the wash tank is diluted with 15 – 20% tap water. NH_4HSO_3 is added up to a concentration of 15 p.p.m. The pH of the produced water on the surface is 6.4. The pH formation water in the field is lower, typically around 5.5., since the water contains dissolved CO_2.

DGGE and clone library analyses

To assess the overall diversity present in all the sampled environments, DGGE was used as a first screening method (Fig. 2). The DGGE analysis targeting the 16S rRNA genes of Bacteria gave a total of 38 distinct bands from which 31 bands gave sequences of satisfactory quality: 10 were from the production water (Brk26), 5 from the primary (cold) separator tank (T101), 7 from the secondary (hot) separator tank (T102) and 9 from the wash tank (T103) (Fig. 1&2a). The injection water displayed similar DGGE patterns as the primary separator tank. The largest part of the water that is produced at the well site is removed in the first oil-water separator tank and consequently represents also the bulk of the injection water (see results cluster analysis, Fig. 2 a&b). Therefore, it was decided to exclude the injection water environment from clone library construction. Also the archaeal community has been analysed with DGGE. For this, we used both a direct and nested PCR approach, since direct amplification of Archaea also yielded deep-branching bacterial 16S rRNA genes. The nested DGGE analysis targeting the 16S rRNA genes of Archaea gave 18 bands with of good sequence quality out of 21: 3 from the produced water (Brk 26), 7 from the primary cold oil-water separator tank (T101), 6 from the secondary oil-water separator tank (T102) and 2 from the wash tank (T103). The direct DGGE analysis resulted in 15 bands with good sequences out of 22 cut bands: 2 from the produced water, 7 from the primary oil-water separator tank, 6 from the secondary oil-water separator tank and 4 from the wash tank (Fig. 1&2b). Clone libraries of the production water and the different tanks (T101, T02 and

T103) gave a more detailed view on the communities (Fig. 3). The results were in-line with those found with the DGGE screening method. From each environment 96 bacterial and 96 archaeal clones were picked. Between 85% and 90% of the bacterial clones and between 70% and 96% of the achaeal clones yielded high quality sequences (see supplementary Table 1). As a quick indication for the coverage of all the clone libraries the method of Good was used (Good, 1953). This method takes the ratio of unique clones ('singletons') into account compared to the number of total investigated clones. The coverage percentage for the bacterial clone libraries varied between 85% and 95%, the coverage of the archaeal clone libraries varied between 96% and 99%. In addition to the differences in diversity, the clone library compositions were compared with each other using the WebLIBSHUFF tool. This analysis showed that all environments were significantly different from each other with respect to both the bacterial and archaeal communities within a 95% confidence interval. The clone libraries were compared pair wise. A p-value below 0.001 indicates a significant difference between the compared environments. All pair wise comparisons of bacterial clone libraries gave p-values below 0.001. Pair wise comparisons of the archaeal clone libraries gave p-values below 0.001, with an exception of the archaeal communities found in the produced water and the primary oil-water separator tank, the XY comparison gave a p-value of 0.61, the YX p-value however was 0.001. X and Y are in this case assigned designations for both compared clone libraries in each pair wise comparison.

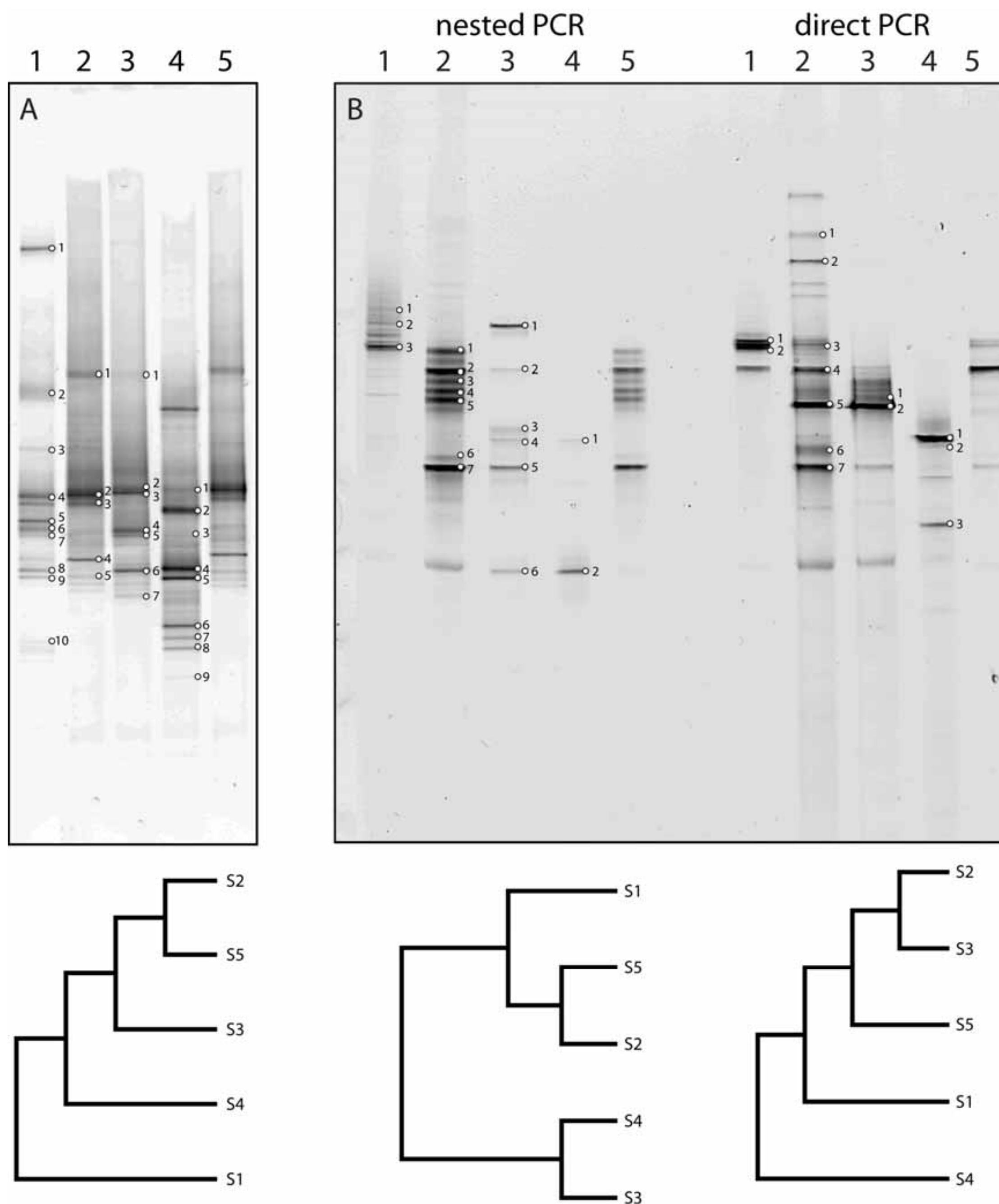

Figure 2: *DGGE analysis of Bacteria (A) and Archaea (B). The numbers above the lanes refer to the different sample sites: 1, Brk26; 2, T101; 3, T102; 4, T103; 5, injector well. Cluster analysis of the different DGGE profiles are below the denaturing gels. Bands indicated by a number were sequenced successfully and used for phylogenetic analysis (see Figs 4 and 5).*

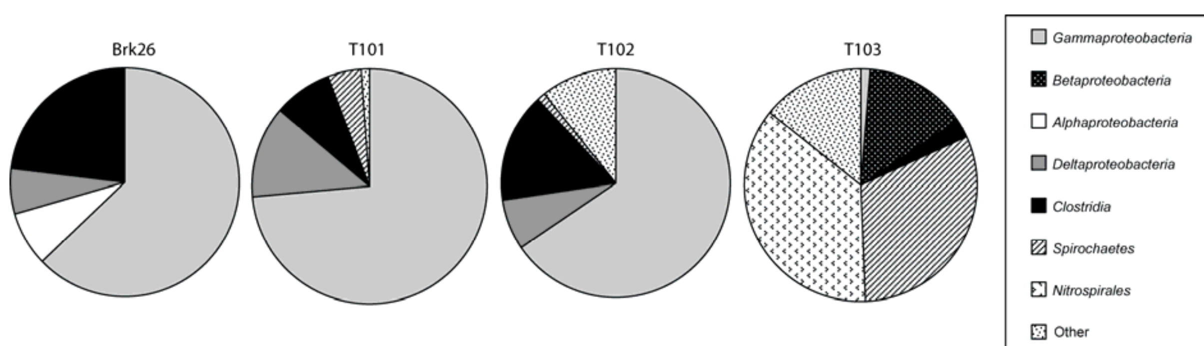

Figure 3: *Phylogenetic distribution of the 16S rRNA sequences obtained from the clone libraries of Bacteria.*

Identification of microorganisms in the production water

Detailed phylogenetic analysis of the sequences retrieved by DGGE and clone library construction yielded a large variety of Bacteria and Archaea (Fig. 4&5). Sequences retrieved from the production water samples displayed similarity to those found in other production water (wellhead) samples taken from oil fields in similar studies (examples below). DGGE bands were associated to the Clostridia, Flavobacteria and Alphaproteobacteria. The sequence Brk26_2 (Fig. 2a) shared a 99% sequence identity with the extremely halophilic clostridium *Halanaerobium congolense* (Ravot et al. 1997) which was isolated from an off-shore Congolese oil field. It was also picked up in the clone library from the production water. The sequence Brk26_10 (Fig. 2a) was 99% related to the species *Anaerobaculum thermoterrenum* (Rees et al. 1997) which was also isolated from the production water of a petroleum reservoir. This sequence was also picked up in the production water clone library. The sequences from Brk26_8 and numerous Brk_26 clones affiliated with the genus *Thermovirga* and displayed relatedness to sequences found in produced water from a high-temperature North Sea oil-field (Dahle et al. 2008). The sequences from the production water clones revealed a wider diversity than the DGGE bands only. The bacterial community in the produced water was dominated by the Gammaproteobacteria (63%, Fig. 3). This division accommodates most of the known halophilic bacteria. The two other major groups in these environments are the Deltaproteobacteria and the Clostridia. Sequences related to the sulphur-cycle bacteria, such as the halophilic SRB species *Desulfohalobium utahense* (Jakobsen et al. 2006) (Great Salt Lake), *Pelobacter carbinolicus* (Lovley et al. 1995) and *Thiomicrospira thermophila* (Takai et al. 2004) (hydrothermal fumarole) were identified. Members of the genus *Marinobacter* were extensively present in the clone library of the production water. The *Marinobacter* sequences were affiliated mostly with sequences found in studies investigating oil-contaminated environments (see for an example the sequence with accession. number EU328021.1). The closest described relatives were *Marinobacter lipolyticus* (Martin et al. 2003) and *Marinobacter hydrocarbonoclasticus* (Gauthier et al. 1992). This last species is well known for its capability to

degrade a substantial variety of hydrocarbons at elevated salinity. It was isolated from oil-polluted seawater and is an obligate aerobe. Members of the genera *Halomonas* and *Idiomarina*, known as versatile heterotrophs developing within a very broad range of salinity, were also found. Some clones showed a 99% match to sequences retrieved from an Alaskan mesothermic petroleum reservoir (Pham et al. 2009). Many of the sequences matched strongly to sequences obtained from oil and salt associated environments, such as salt wells and production well brines, around the world. Examples are an offshore Brazilian basin (Sette et al. 2007), Colombian oil fields and North Sea oil fields like the Ekofisk Oil Reservoir, (published only in GenBank).

With respect to the Archaea: the production water displayed the lowest diversity in the DGGE analysis of PCR products obtained with the direct and the nested PCR approach. All DGGE band sequences were affiliated with sequences found in archaeal communities from hydrothermal fluids at the Yonaguni Knoll IV hydrothermal field (Nunoura & Takai, 2009) (Fig. 2b). The bands from the nested DGGE showed an affiliation to the same study. These sequences were not present in the archaeal clone library. The direct DGGE displayed an additional band that had a 100% similarity to a sequence of *Methanocalculus halotolerans* (Fig. 2b) isolated from oil field production water (Ollivier et al. 1998). With both PCR approaches it was shown that this species was not only present in the production water, but also in the two oil-water separator tanks. The DGGE bands T101_2n-a, T102_2n-a and T101_4d-a (Fig. 2b) and one of the clones from the secondary oil-water separator tank also belonged to the same species. The archaeal clone library from the produced water contained many clones affiliated with sequences found in the hydrothermal sediments of the Guaymas Basin (Dhillon et al. 2003). These sequences were also detected in the oil-water separator tanks. Several clones in the production water matched for 99% with the described species *Methanohalophilus euhalobius* (*Methanococcoides euhalobius*) isolated from oil field production waters (Davidova et al. 1997). This halophilic methanogen was also present in the primary oil-water separator tank, but was not detected in the others tanks. The sequence was also found on DGGE, band T101_1n-a (Fig. 2b). In addition, members of the Methano-microbiales were present in the production water clone library (Fig. 5).

Identification of microorganisms in the oil-water separator tanks

The clone and DGGE band sequences from the primary separator tank overlapped partly with the found sequences in the production water, this can also be deduced from the bacterial cluster analysis (Fig. 2). An example is the sequence of a DGGE band from the primary separator tank is T101_5 that resembles the sequence of Brk26_8 from the production water (Fig. 2a). Like the production water clone library, the primary separator tank bacterial clone library was dominated

by Gammaproteobacteria (74%, Fig. 3) and contained sequences related to *Marinobacter*., specifically to *M. hydrocarbonoclasticus*, *M. bryozoorum* (Romanenko et al. 2005), *M. koreensis* (Kim et al. 2006) and *M. gudaonensis* (Gu et al. 2007). *M. bryozoorum* is a PAH-degrader isolated from the deep sea; *M. gudaonensis* was isolated from oil-polluted saline soil in a Chinese oil field. Sequences related to the genus *Marinobacter* also shared a close association with sequences found in a similar study on the Chinese Qinghai oil field. DGGE band T101_2 also showed a strong similarity with these sequences. Band T101_3 was affiliated to the genus *Thermotoga* (Fig. 2a), it was not picked up in the primary separator tank clone library. Clones related to members of the genus *Halomonas* were also detected. Similar to the production water clone library, sequences were found related to the species *Pelobacter carbinolicus*, *H. congolense* and several *Thermovirga* members.
In the secondary separator tank, the bacterial sequences detected by DGGE and in clone libraries, displayed an overlap with those found in the production water. It included moderately halophilic genera *Marinobacter* and *Halomonas*. Sequences related to two unique species were found that were not detected in the two previous environments: *Desulfotomaculum geothermicum* (100%), isolated previously from geothermal groundwater (Daumas et al. 1988); and members of the genus *Flexistipes*, although the sequence identity was only 94% with the described species *Flexistipes sinusarabici* (Fiala G, 1990). The appearance of sequences related to the genus *Marinobacterium*, was of special interest. Members of this genus were not detected in the production water. The observed sequences were related to the species *Mb. halophilum* (Chang et al. 2007) isolated from the Yellow Sea, *Mb. georgiense* (Gonzalez et al. 1997) isolated from a marine enrichment, *Mb. stanieri* (Satomi et al. 2002) and *Mb. litorale* (*Insulimonas litoralis*) retrieved also from the Yellow Sea (Kim et al. 2007), which have been described as strictly aerobic. Also observed is the appearance of Spirochaeta members. A notable difference the absence of Alpha-proteobacteria in both separator tanks, since they were detected in the production water.

The archaeal diversity in the primary oil-water separator tank was higher than in the production water. Many of the sequences found in the production water were also found in the primary oil-water separator tank. As mentioned earlier, dominant bands from the direct and nested DGGE matched with *M. halotolerans*. The sequences from a second dominant band found with both DGGE techniques in the oil-water separator was closely related (99%) to an uncultured archaeon from a deep subsurface shale (GenBank EF117481). This archaeon was also detected in the secondary oil-water separator tank. The clone library from the primary oil-water separator tank was dominated by members of the genus *Methanolobus*. The sequences shared a 99% sequence similarity with sequences retrieved from the Qinghai oil field. The bacterial community also holds sequences associated to this habitat. The closest described archaeon (97%)

is *Methanolobus zinderii* isolated from a deep subsurface coal seam (GenBank EU711413). The same sequences were also obtained by the nested DGGE approach (bands T101_3n-a and T101_4n-a). This dominant archaeon was not present in the production water. As mentioned earlier, the clone library from the primary oil-water separator tank also contained sequences closely related to the species *M. euhalobius.* A few sequences were found that were related to sequences from tar sand. The closest related described species was *Methanosaeta harundinacea.*

The clone library from the secondary oil-water separator tank resembled the clone library from the primary oil-water separator tank, except that sequences related to *M. euhalobius* could not be detected in the secondary separator. The cluster analysis of the nested DGGE in this case does not show a good grouping, since one environment (S4) displays only one band (Fig. 2b) The sequence from the DGGE band T102_6n-a had a 99% match to an uncultured sequence found in the Ekofisk Oil Reservoir, which also contained sequences found in the bacterial community studied in the present work. It was 93% related to the described species *Ferroglobus placidus* (Hafenbradl et al. 1996). This species was not detected in the clone library.

Identification of microorganisms in the wash tank

The bacterial community in the wash tank is dominated by *Thermodesulfovibrio yellowstonii*, which belongs to the phylum Nitrospirales. It was isolated from thermal vent water in Yellowstone National Park. (Henry et al. 1994) and it was by far the most dominant sequence in the clone library (36 % from the total) It is known for its sulphate reducing capabilities at high temperatures. DGGE band T103_3 shares a 99% sequence identity with this species (Fig. 2a). Also a substantial increase of the Spirochaeta (31 %) was observed. Furthermore, members of the Betaproteobacteria (14%) were detected only in the wash tank. The Gammaproteobacteria displayed a nearly complete disappearance. Members of the Deltaproteobacteria were no longer detectable (Fig. 3). DGGE band T103_6 matches (95%) with a described isolate from a study towards marine sulphate reducing bacteria able to oxidise short chain alkanes anaerobically (Kniemeyer et al. 2007); it was not detected in the clone library. DGGE band T103_1 (Fig. 2a) and the wash tank clone library also detected members from the phylum Aquificales accommodating a deep lineage of extremely thermophilic bacteria. The found sequences had up to 98% sequence identity to sequences retrieved from hot spring microbial mats (Skirnisdottir et al. 2000). DGGE band T103_2 was affiliated to an unknown member of the Spirochaetales and was also dominant in the wash tank clone library (Fig. 2a). It shared 98% identity to sequences obtained from an anaerobic toluene degrading aquifer microbial community in a tar oil contaminated plume (Winderl et al. 2008). DGGE band T103_9 (Fig. 2a) sequence was 98%

similar to sequences of thermophiles in deep subsurface geothermal environments (Kimura et al. 2006). It was not detected in the clone library. On the other hand, the wash tank clones however did identify members from the Bacteroidetes and Thermotogales families.

On the nested archaeal DGGE, only two bands were distinctly visible (T103_1n-a and T103_2n-a, Fig. 2b). T103_1n-a was similar to the most dominant sequence present in the clone library and shared a 100% sequence match with the methylotrophic methanogen *Methanomethylovorans thermophila* isolated from a methanol-fed thermophilic bioreactor (Jiang et al. 2005). T103_2n-a clustered with an uncultured archaeon and was similar to the sequence from the band T102_6n-a (Fig. 2b). Only a single additional sequences was detected in the clone library which displayed 99% sequence similarity with the acetoclastic methanogen *Methanothrix thermophila* (*Methanosaeta thermophila*) isolated form a thermopillic anaerobic digestor (Kamagata et al. 1992). The found sequences were also affiliated to the sequences retrieved from a high temperature gas field in Japan (Mochimaru et al. 2007).

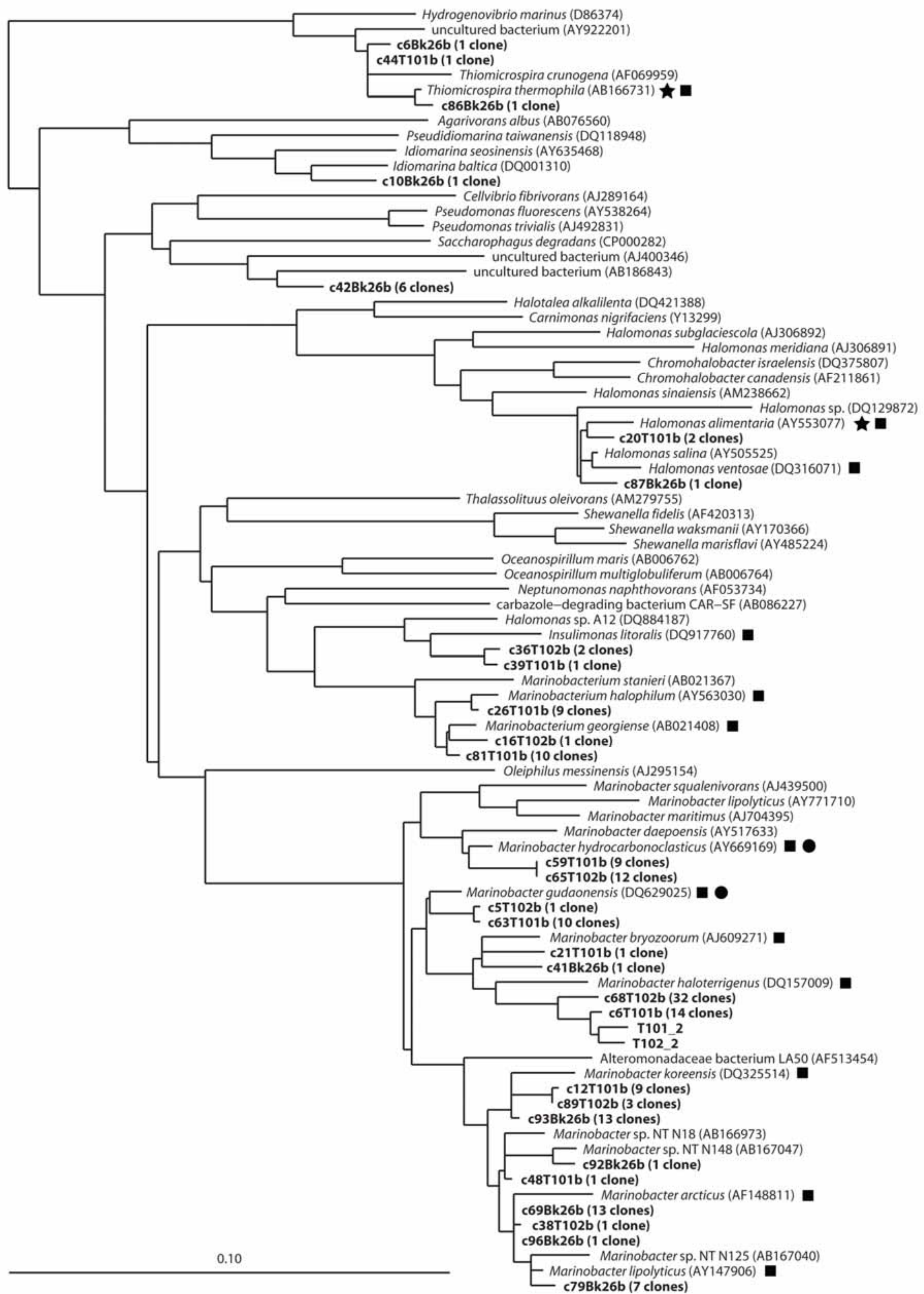

Figure 4a: *Phylogenetic analysis of the bacterial 16S rRNA sequences, Gammaproteobacteria: Sequences determined in this study are printed bold. Names indicate sequences obtained from clone library (starting with a 'c') or from DGGE, and from which sample site (i.e., Brk26, T101, T102, T103). The number of clones with the same sequence is written between parentheses. A star behind the sequence name indicates thermophily; a square indicates salt-loving; a dot indicates the isolation from oil associated environment. The bar indicates 10% sequence differences.*

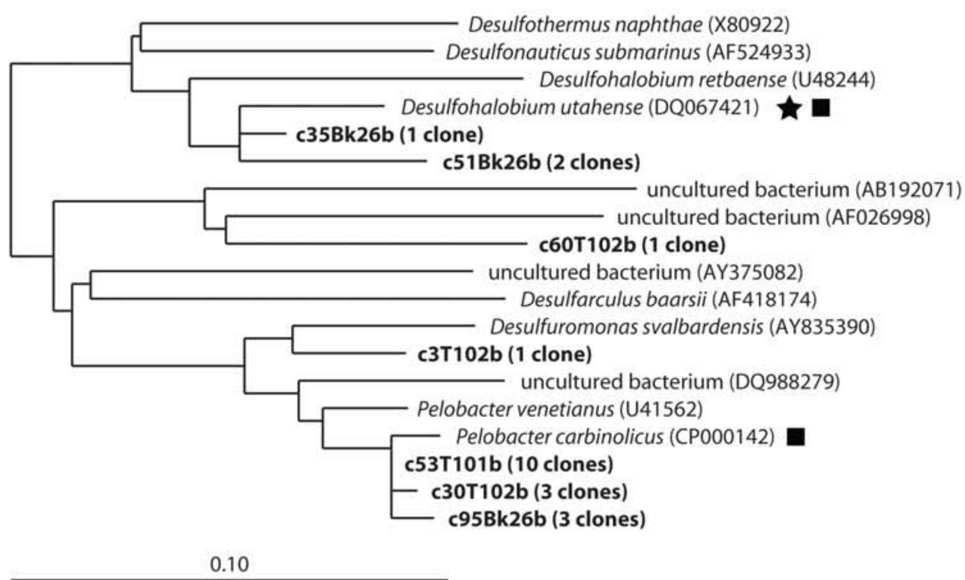

Figure 4b: *Phylogenetic analysis of the bacterial 16S rRNA sequences, Deltaproteobacteria.*

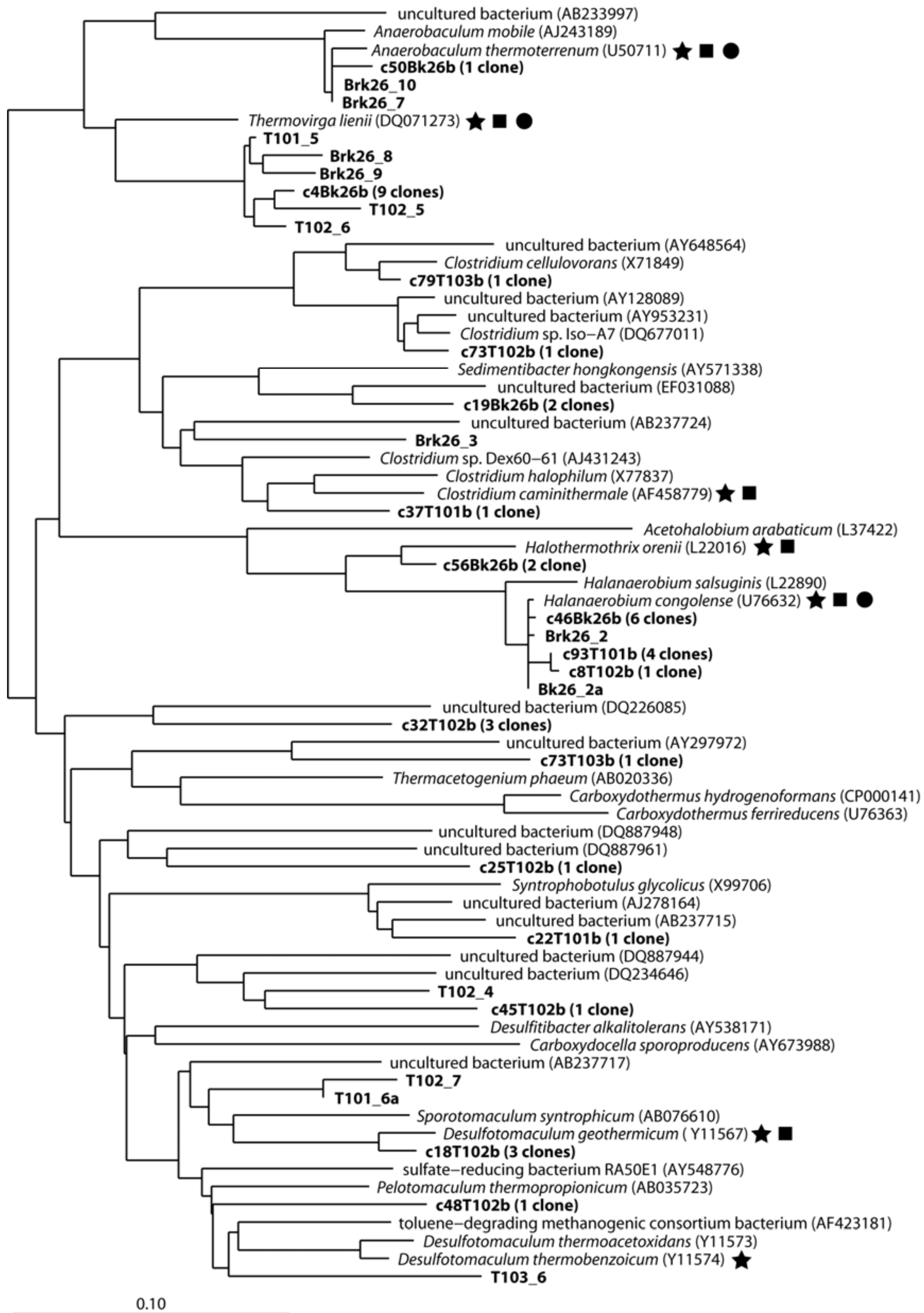

Figure 4c: *Phylogenetic analysis of the bacterial 16S rRNA sequences, Clostridia.*

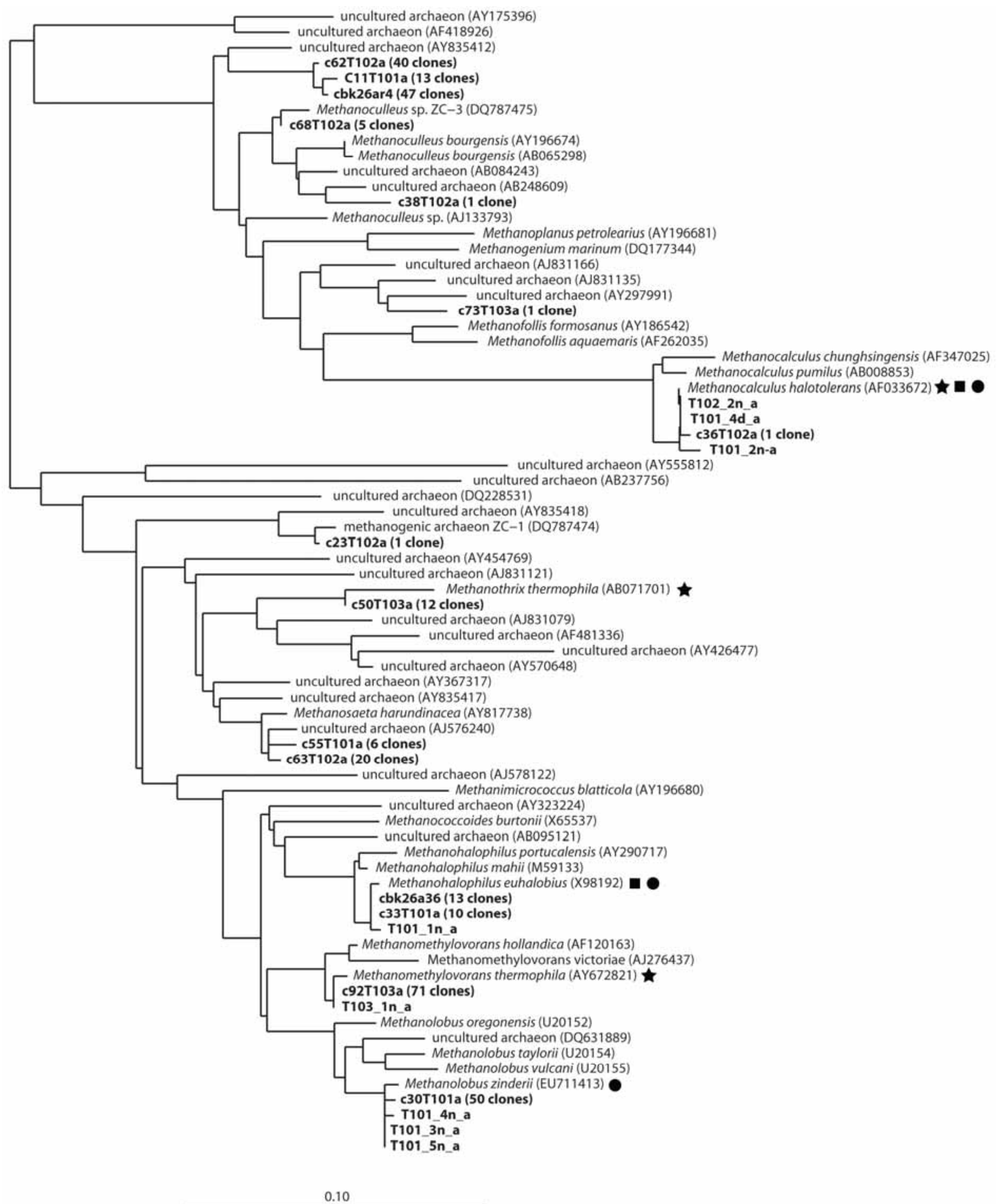

Figure 5: *Phylogenetic analysis of the archaeal 16S rRNA sequences.*
Sequences determined in this study are printed bold. Names indicate sequences obtained from clone library (starting with a 'c') or from DGGE, and from which sample site (i.e., Brk26, T101, T102, T103). The number of clones with the same sequence is written between parentheses. A star behind the sequence name indicate thermophily; a square indicates salt-loving; a dot indicates the isolation from oil associated environment. The bar indicates 10% sequence differences.

Discussion

The presence of similar microorganisms in production water from oil-fields.

Analysis of the microbial diversity from DGGE and clone libraries yielded several sequences commonly found in oil field environments (Pham et al. 2009), (Dahle et al. 2008). Their frequent occurrence indicates that they are a part of specific communities associated with these environments, and at least some of these species can therefore be seen as indicators for an ecosystem, which contain fossil hydrocarbons and high amounts of salt. Phylogenetic grouping of our sequences with sequences of described organisms isolated from oil fields substantiates this and indicates similar physiologies. Since the investigated oil field in this study is not extensively water flooded, it is to be expected that at least part of the observed microorganisms are indigenous to the field. There is also no sulphate introduced into the field which is the case in many studied oil fields. Detected were sequences related to the moderately thermophilic and halophilic citrate-fermenting *A. thermoterrenum*, and the extremely halophilic fermentative bacterium *H. congolense*. These two species are both isolated from oil field production waters and share a profile that fits the field characteristics with respect to salt concentration, pH and temperature optimum. *H. congolense* belongs to a specific group of obligately anaerobic halophilic bacteria that have a salt-in strategy (similar to Haloarchaea) using inorganic K^+ ions to maintain their osmotic balance, which is quite uncommon since only two groups of bacteria hold this feature. It has fermentative capabilities but is also known to reduce thiosulphate and elemental sulphur which could indicate the presence of low quantities of sulphur compounds in the habitat. This is reinforced by the detection of sequences related to the halophilic Deltaproteobacterium *D. utahense* which is a known sulphate/thiosulphate reducer. With respect to the detected archaeal species, sequences were found related to *M. halotolerans* (dominant in the DGGE profile, Fig. 2b) and *M. euhalobius* (dominant in the clone library). These Archaea also share properties that match their environmental conditions. Both Archaea have been isolated from oil field production waters and both are halophilic. *M. halotolerans* requires acetate for growth when growing with H_2+CO_2 and formate. *M. euhalobius* is a methylotrophic methanogen. It also has a calcium requirement. Sequences related to another archaeon extensively present in the clone library was the filamentous organism *M. harundinacea*, which is specialized on acetoclastic methanogenesis (Ma et al. 2006). All the above mentioned species have characteristics that are complementary to the environmental conditions of the investigated oil field. The detected clones match with species isolated from oil field production waters, which is an indication that these clones have a similar physiology.

Presence of members from the genus *Marinobacter* distinctively indicates the presence of species not directly linked to the oil field, as is the detection of sequences related to the microaerophile *T. thermophila*. Most *Marinobacter* species are aerobic although some members can denitrify. As oil fields are deprived of oxygen, the presence of these *Marinobacter* as indigenous species in the field is dubious at best. They are however frequently found in many other studies towards the microbial diversity of oil field production waters (see results). They are extremely salt tolerant hydrocarbon degraders and are often detected in oil contaminated sites. This clearly displays their affinity with this type of ecosystem (Yakimov et al. 2007). Judging the overall community found in the production water, it seems that the halophilic species are a mixture of microorganisms indigenous to oil fields (anaerobes) and microorganisms that are associated to the surface facilities (e.g. piping system of the well, aerobes). Some species also are indicative of the presence of small amounts of oxidized sulphur compounds. Since the community is indeed specific for oil fields and oil associated environments, some species can be used as indicators of such a system in future research.

Temperature-induced community changes.

When comparing the microbial community in the primary separator tank and the production water, it is observed that there is a substantial overlap between the two communities. This is logical since the tank is only used for separation of the collected water/oil mixture, no chemicals are added and no aeration is applied in the tank. There are however notable differences. The main difference is the occurrence of *Marinobacterium* species in the first tank. Most members of the genus *Marinobacterium* are strictly aerobic, with the exception of *M. litorale* (also detected) that can denitrify. All the detected *Marinobacterium* members have a lower temperature range and a lower optimum growth temperature (30 - 40 ºC), with respect to the species detected in the production water. They are halophilic and are commonly associated with seawater and can be associated with marine oil related ecosystems (Yakimov et al. 2005). Despite this, there is probably no association between the oil field and the occurrence of the *Marinobacterium* species, considering their strict aerobic nature and maximum growth temperatures that are below the field temperature. They have however characteristics that match the environment of the primary cold oil-water separator tank. The appearance of sequences related to strict aerobes like *Mb. halophilum* and *Mb. georgiense* and the disappearance of sequences related to some strict anaerobes like *A. thermoterrenum* points in the direction of an increased oxygen intrusion and a decrease in temperature. Another difference is the disappearance of the Alphaproteobacteria, a cause for this observation cannot be given. It is likely that due to the pumping the oil water mixture gets

aerated, inducing microbial activity. It should be noted that even small amounts of oxygen lead to substantial increase in bacterial population (the presence of 1 milligram of oxygen can support the aerobic growth of 1 billion cells given no other limitations). The sensitive PCR methods can therefore already indicate small variations in the system when comparing the first tank and the production water. This implies the possibility that biologically there is a large shift while chemically there is almost no difference to be detected.

The archaeal community from the production water is comparable to the primary oil-water separator tank. A clear difference is the occurrence of *Methanolobus*, specifically sequences related to the species *M. zinderii* (*Methanolobus* sp. SD1), which is a methylotroph isolated from a subsurface coal seam, The proliferation of this archaeon is most likely also an effect of the decreasing temperature. The microbial community detected in the primary oil water separator tank (35 ºC) was similar to the secondary oil water separator tank (50 ºC). It is highly unlikely that all these species indeed can survive at these elevated temperatures. The fact that these species are still detectable is probably caused by the fact that despite their inactivation, the dead cells are still detectable by the DNA analysis in the water phase as it enters the secondary hot oil-water separator tank from the cold one. The secondary oil-water separator tank does display sequences from or related to two bacterial species not detected in the previous environments. One is the clostridium *Desulfotomaculum thermosapovorans*, which is a thermophilic sulphate reducer utilizing long chain fatty acids. Also sequences related to the *Flexistipes* genus were detected.

Negative effects of ammonium bisulphite addition.

Before reinjection of the produced water in a deeper subsurface layer, the oxygen mainly introduced via the tap water that is used to reduce the salt concentration, is scavenged from the system by addition of ammonium bisulphite (NH_4HSO_3) in the wash tank. In the oil industry this is a common practice to prevent heavy corrosion of the injection wells by a combination of O_2, NaCl and elevated temperatures down hole. Ammonium bisulphite is used specifically for its quick reactivity with oxygen, which is often required if the water is to be completely oxygen free within a short time frame. From a production chemist point of view this is more efficient than the use of slower reacting compounds such as sodium bisulphite or other oxygen scavengers. From a microbiological point of view this exercise seems illogical. The addition of ammonium bisulphite means the introduction of an activated form of sulphate in combination with the addition of a nitrogen source which undoubtedly leads to an establishment of an opportunistic sulphate reducing community. Such a community, indeed, has been detected in the wash tank from the surface facility. The community in the wash tank consisted predominantly of specific

thermophilic sulphate reducing bacteria. Most dominant in the bacterial clone library were sequences related to *T. yellowstonii*, this species can utilize sulphate, thiosulphate and sulphite with various organic acids and alcohols as electron-donors. The species belongs to a deep lineage branching near the division between Bacteria and Archaea. Other detected deep-lineage bacteria included *Sulfurihydrogenibium azorense* and the members of Aquificales. They are thermophiles which can grow well in the presence of sulphur compunds utilizing them both as electron donors and acceptors. Their presence indicate elevated temperature plus availability of inorganic sulphur compounds.

Sequences affiliated to two specific Archaea were detected in the water from the wash tank, namely *Methanomethylovorans thermophila* and *Methanothrix thermophila*. The first can only utilise methanol and methylamines, the second can only use acetate. Presence of sulphate reducing bacteria selects for these types of methanogens. They use substrates that are poorly used by sulphate reducing Bacteria allowing them to survive in this sulphidogenic environment. Overall, the microorganisms detected in the wash tank are related to sulphur associated hydrothermal vent systems. It is observed that the NH_4HSO_3 in combination with fermentation end products already present in the water gives the perfect environment for a specific thermophilic sulphate reducing community. That H_2S is indeed produced is proven by the fact that it can be found in the off-gas measurements of tank T103. H_2S levels are found up to 250 ppm. This NH_4HSO_3 addition seems to be in contradiction with many large scale investigations towards the prevention of reservoir souring and Microbial Induced Corrosion (MIC). For example it is reported that the pipeline after this wash tank is indeed subjected to heavy corrosion. This addition is therefore not advised and it is encouraged to search for a good alternative oxygen scavenger, which does not contain SO_3^{2-} or ammonia.

Combined use of different techniques in community analysis

The DGGE technique is widely used in the oil industry to judge the microbial community present in oil-associated environments. It was shown that DGGE indeed gives a good first overview of the microbial diversity present in the environments of interest. The observed differences are likely due to primer biases and mismatches causing preferential amplification of some of the present species (Suzuki & Giovannoni, 1996). The clone libraries gave a more detailed overview of the community in respect to e.g. individual populations ('singletons'). An example of such a singleton is a sequence from the production water related to *Halomonas salina* (*Deleya salina*) (Valderrama et al. 1998). Other techniques such as Tag-sequencing (Huse et al. 2007) and metagenomics (Singh et al. 2009) will provide even more details on these ecosystems.

In this study, we described the microbial diversity of oil field production water and the associated surface facility separation units using a combination of two culture independent methods. Summarizing the results from the DGGE and clone library, it was concluded that the communities found although diverse, match with the characteristics of the specific conditions, such as temperature, available electron acceptor and salinity. The fact that these changes in the community can be linked to changes in their environment has the potential to indicate changing conditions in an oil reservoir upon e.g. water flooding. However, the results in this paper have been deduced from 16S rRNA gene sequences and do not give full certainty on the metabolic properties of the species, which should be considered in future studies. The communities in the production water and in the two oil-water separator tanks, although all found to be different, displayed an association with other oil-related ecosystems. This is a first step in the use of microorganisms as information carriers of reservoir conditions. The addition of ammonium bisulphite in the wash tank led to a substantial enrichment of sulphate reducers. From a souring and corrosion point of view it is advised to look for alternative oxygen scavengers that does not contain SO_3^{2-} and ammonia.

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Supplementary material

Table 1: Affiliation of sequences found with relevant known/described species or sequences.

Clostridia

Organism	Similarity (Max identity (to found clones)	Abundance in clone library (%) / Found on DGGE (yes+/no-)	Affiliation	Environment (Source)	Metabolic properties
Anaerobaculum thermoterrenum	99%	Brk26 (1%) / + T101 (0%) /- T102 (0%) /- T103 (0%) /-	Clostridia	Oil field production water	Obligate anaerobe, Halophilic, ferments citrate, S° and thiosulphate are reduced to H_2S
Uncultured Thermovirga	96%	Brk26 (11%)/ + T101 (0%) /- T102 (0%) /- T103 (0%) /-	Clostridia	High temperature North sea oil field	**
Halanaerobium congolense	99%	Brk26 (8%) /+ T101 (5%) /- T102 (1%) /- T103 (0%) /-	Clostridia	Oil field production water	Obligate anaerobe, Obligately halophilic, fermentative capabilities, S° and thiosulphate are reduced to H_2S
Desulfotomaculum geothermicum	100%	Brk26 (0%)/- T101 (0%) /- T102 (4%) /- T103 (0%) /-	Clostridia	Geothermal ground water	Anaerobe, fatty acid degrading, sulphate reducing bacteria.

Deltaproteobacteria

Organism	Similarity (to found clones)	Abundance in clone library (%) / Found on DGGE (yes+/no-)	Affiliation	Environment (Source)	Metabolic properties
Uncultured *Pelobacter*	99%	Brk26 (4%) /- T101(11%) /- T102 (4%) /- T103 (0%) /-	Deltaproteobacteria	High-temperature North Sea oil-field	**
Desulfohalobium utahense	99%	Brk26 (4%) /- T101 (0%) /- T102 (0%) /- T103 (0%) /-	Deltaproteobacteria	Great salt lake	Moderately halophilic, Fatty acid utilizing, sulphate and thiosulphate reducing bacterium

Gammaproteobacteria

Organism	Similarity (to found clones)	Abundance in clone library (%) / Found on DGGE (yes+/no-)	Affiliation	Environment (Source)	Metabolic properties
Marinobacterium georgiense	99%	Brk26 (0%) /- T101 (11%)/- T102 (1%) /- T103 (0%) /-	Gammaproteobacteria	Marine environment	Aerobe, Halophilic utilizes aromatic compounds
Marinobacterium litorale	97%	Brk26 (0%) /- T101 (1%) /- T102 (2%) /- T103 (0%) /-	Gammaproteobacteria	Yellow sea costal water	Facultative anaerobe, Halopillic
Marinobacterium halophilum	97%	Brk26 (0%) /- T101 (10%)/- T102 (0%) /- T103 (0%) /-	Gammaproteobacteria	Yellow sea	Aerobe, Halophilic,
Marinobacter hydrocarbonoclasticus	99%	Brk26 (1%)/- T101 (10%) /- T102 (14%) /- T103 (0%) /-	Gammaproteobacteria	Oil polluted seawater near a petroleum refinery	Facultative anaerobe, extreme halotolerant, degrades a large variety of alphatic and aromatic HCs
Marinobacter gudaonensis	97%	Brk26 (0%)/- T101 (11%)/-	Gammaproteobacteria	Oil polluted saline soil from	Facultative anaerobe,

		T102 (1%) /- T103 (0%) /-		the Shengli Oilfield	Halophilic
Marinobacter haloterrigenus	97%	Brk26 (0%)/- T101 (16%)/+ T102 (38%)/+ T103 (0%) /-	Gammaproteobacteria	Saline wetland	**
Marinobacter koreensis	99%	Brk26 (12%)/- T101 (3%) /- T102 (15%)/- T103 (0%) /-	Gammaproteobacteria	Korean sea sand	Facultative anaerobe, Halophilic,
Marinobacter arcticus	96%	Brk26 (16%)/- T101 (0%)/- T102 (1%)/- T103 (0%)/-	Gammaproteobacteria	Seawater	Utilizes aromatic hydrocarbons and acetate

Other Bacteria

Organism	Similarity (to found clones)	Abundance in clone library (%) / Found on DGGE (yes+/no-)	Affiliation	Environment (Source)	Metabolic properties
Uncultured *Thermotogales* bacterium	99%	Brk26 (0%) /- T101 (0%) /+ T102 (0%) /+ T103 (0%) /+	Thermotogales	Yellowstone geothermal ecosystem	**
Uncultured Aquificales bacterium	100%	Brk26 (0%)/- T101 (0%)/- T102 (0%)/- T103 (5%)/-	Aquificales	Subsurface gold mine	**
Thermodesulfovibrio yellowstonii	100%	Brk26 (0%)/- T101 (0%)/- T102 (0%)/- T103 (39%) /+	Nitrospirales	Yellowstone thermal vent water	Thermophilic sulphate reducing Bacterium
Uncultured *spirochete*	99%	Brk26 (0%)/- T101(5%)/- T102 (1%)/- T103 (34%)/-	Spirochaetales Also on T103 DGGE gel	chlorinated ethene-degrading culture	**

Archaea

Organism	Similarity	Abundance in clone library (%) / Found on DGGE (yes+/no-)	Affiliation	Environment	Metabolic properties
Methanocalculus halotolerans	100%	Brk26 (0%)/+ T101 (0%)/+ T102 (1%) /+ T103 (0%) /-	Methanomicrobia	Oil field production water	Halotolerant Uses H_2,CO_2 and formate, requires acetate.
Uncultured archaeon	97%	Brk26 (71%)/- T101 (14%)/- T102 (51%)/- T103 (0%) /-	Environmental sample	hydrothermal sediments of the Guaymas Basin	**
Methanothrix thermophila	99%	Brk26 (0%)/- T101 (0%)/- T102 (0%)/- T103 (14%)/-	Methanomicrobia	a thermopillic anaerobic digestor	Thermophilic Acetate is the sole substrate
Methanosaeta harundinacea	99%	Brk26 90%)/- T101 (6%)/- T102 (26%)/- T103 (0%)/-	Methanomicrobia	Anaerobic sludge reactor	Acetate is the sole substrate used
Methanohalophilus euhalobius	99%	Brk26 (20%)/- T101 (11%)/+ T102 (0%) /- T103 (0%) /-	Methanomicrobia	Oil field production water	Halophilic uses methylamines and methanol
Methanomethylovorans thermophila	100%	Brk26 (0%)/- T101 (0%)/- T102 (0%)/- T103 (86%)/+	Methanomicrobia	Anaerobic methanol fed reactor	Low salt tolerance Uses methanol and methylated compounds
Methanolobus zinderii	99%	Brk26 (0%)/- T101 (53%)/+ T102 (0%)/- T103 (0%)/-	Methanomicrobia	deep subsurface coal seem	**

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The effect of biocide on the present microbial community thriving in an oil-water separation surface facility.

Geert M. van der Kraan, Suzanne van der Velde, Gerard Muyzer, Johannes Bruining, & Mark C.M. van Loosdrecht

Abstract

The aim of this study was to analyze the influence of a biocide treatment on the microbial community in different units of an oil-water separation plant located near Rotterdam (the Netherlands) using 16S rRNA gene fragment amplification, denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR). Biocide treatment of this site was performed in order to prevent growth of detrimental microbial populations. Bacterial and Archaeal communities were both analyzed. A direct and nested PCR approach was used for the latter. Phylogenetic analysis revealed members of four bacterial (Firmicutes, Deltaproteobacteria, Gammaproteobacteria, Deferribacteres) and two archaeal classes (Euryarchaeota and Crenarchaeota). A significant microbial diversity in the oil-water environments was found. No bacterial sequences were found in the nested approach, making the nested approach specific for Archaea. Comparison between the different DGGE patterns showed no significant changes due to the biocide treatment, but qPCR revealed a decrease of a 95 % gene copy numbers in the samples that were taken during the treatment indicating an overall unbiased effect of the biocide on the community. Although at first hand a decrease of 95% seems a good reduction, it represents only a decrease of one order of magnitude indicating an effect that is not long lasting.

Introduction

Microbial Induced Corrosion (MIC) poses serious issues throughout the oil industry. In every stage of the process, pipe-lines and tanks corrode and costs are estimated at about $15 billion on yearly bases in the US only (Brondel et al. 1994). Since it is economically unrealistic to totally prevent corrosion, commonly efforts are taken to control the corrosion rate. This study focused on the microbial diversity in an oil-water separation facility. In such systems both biogenic aerobic and anaerobic corrosion are of relevance.

An important issue caused by microorganisms contributing to this corrosion is the production of hydrogen sulphide (H_2S) by sulphate reducing prokaryotes. H_2S formation causes so-called 'souring,' it is a corrosive agent and it poses a serious personnel safety hazard (Iverson, 1987). Microbes form biofilms on steel, iron (and many other) surfaces. These biofilms are mainly anaerobic at the metal surface and therefore electrochemical corrosion occurs. Iron is then degenerated and hydrogen is formed. Sulphate Reducing Prokaryotes (SRPs) are able to consume this hydrogen and this has influence on the equilibrium of the chemical decay of the metal surface (Muyzer & Stams, 2008). Videla et al. (2005) gave an extensive historical background of the field of MIC. In spite of the elaborated interest in the field of bio-corrosion during the 1960s, an adequate understanding was not reached unit the late 70s. Before this time, experiments in this area were carried out in laboratories with pure strains of microorganisms. Therefore important interactions between organisms and (metal) surfaces, such as biofilm formation, were neglected. During the 80s, research was expanded as a result of the increase of focus from different industries. By the end of the 1990s several techniques had been developed to analyze and clarify the role of biofilms in the corrosion process. Various methods for prevention and corrosion control were developed. Common practice in the oil industry is the use of biocides. At the beginning of this century environmental awareness became a more important issue, therefore new biocides were developed. One of these new biocides is THPS. THPS (tetrakis-hydroximethyl phosphonium sulphate), is a non-oxidizing biocide with low environmental toxicity and is widely used for a variety of water treatment applications in the oil-industry. This biocide is efficient on a wide range of Bacteria, Fungi and Algae and has the ability to dissolve (FeS) ferrous sulphide (Talbot et al. 2002).

A literature survey yielded some studies in which THPS treatments were compared with conventional bacterial treatments and demonstrated that downhole injection of THPS decelerate the grow rate of SRB and therefore negatively influenced the rate of H_2S production (Robert E. Talbot et al. 2000). A trial accomplished by Larsen et al. showed a short term effect of this biocide. Observed was a reduction in H_2S production with an addition of 400 p.p.m. THPS for 7-

10 hours. An overall diminishing trend was not obtained. After this trial they showed a long term downward trend in H_2S production over a 6 months period with addition of 200 p.p.m. THPS for 72 hours. A significant drop in microbial activity in the injection water system with a lower concentration (125 p.p.m.) was demonstrated as well. Nowadays molecular techniques are particularly promising in research towards bio-corrosion. These modern techniques offer the potential to identify dominant bacteria in a given system and regard the possible changes in overall population caused by biocides.

The aim of this research was to evaluate the effect of THPS treatment on the microbial community in the different units of the Rotterdam oil-water separation surface facility. The plant was treated with THPS (SIBAC™, SISCHEM B.V., The Netherlands) during 5 hours (reaching a concentrations up to 500 p.p.m.) and samples were taken before, during and as a control, after this treatment. The influence of the treatment was analyzed based on DNA from produced water samples, which was extracted from collected oil-water suspension samples. Amplification of 16S rRNA fragments was performed on Bacteria and Archaea and was followed by Denaturing Gradient Gel Electrophoresis (DGGE) and quantitative (q)PCR. This chapter displays the results of the different analysis and provides a quick characterization of the microbial community and contributes to the understanding of the effect of THPS.

Materials & Methods

Site description

The Rotterdam oil field is Located in the western part of The Netherlands and holds multiple production wells and an oil-water separation surface facility. Samples were taken before, during and after biocide treatment (control), from various units of an oil- water separation facility and the production water (Fig. 1). The production water was also sampled and was used as a reference. First the oil-water mix is pumped up from the production wells, RTD13 in our case. This is also referred to as production water (PW) or brine water. Then the mix enters a deemulsifier (V010 B), here no samples were taken. In this tank, foam formation is prevented. The mix subsequently enters the separation tank V011 were the oil-water mixture is separated. The oil is transferred to a storage tank (T202) and is later on transported to the oil refinery in Pernis. The separated water enters the water injection buffer tank (T301). In this tank the trace oil goes back into the deemulsifier V010b. The water is then transported to a pump (P202) were ammonium bisulphite (NH_4HSO_3) is added as an oxygen scavenger. Next the solids are removed in centrifuges (S310) and the water is reinjected into the production well via several injection

wells (RTD16, was sampled in our case). The water is reinjected into the reservoir to keep the field pressurised. The liquid retention time of the complete surface facility is around 3 hours.

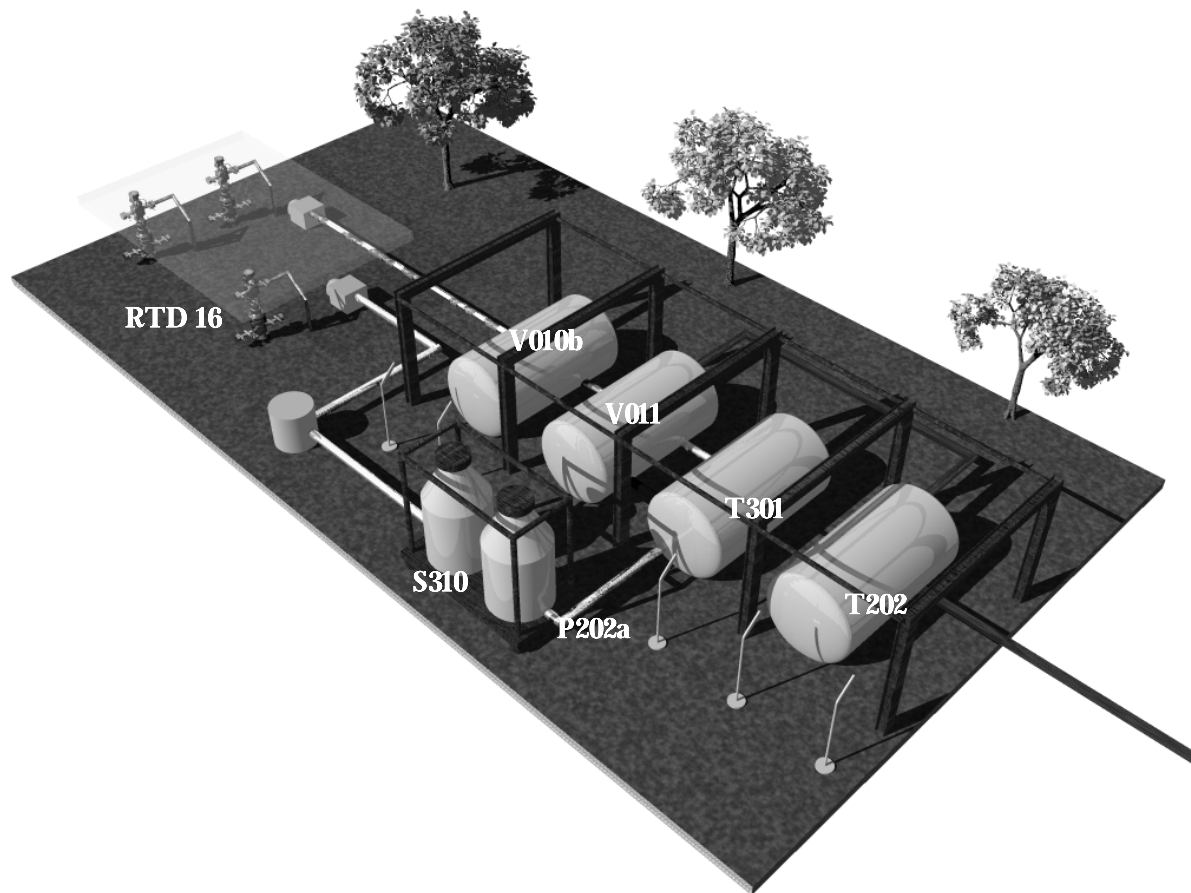

Figure 1: *Overview of the sampling site. Water samples were taken from an oil well (RTD13), the first oil water separator (V011), centrifuges (S310), a water pump (P202) and an injector (RTD 16).*

Sampling procedure ad preparation

From each site, 10-liter samples were taken in sterile jerry cans. The jerry cans were completely filled and sealed directly with screw caps to avoid oxygen intrusion. The samples were after collection, directly taken to the laboratory (the time between the sampling and the filtration procedure was by approximation 30 minutes). In the laboratory the water samples were filtered using 0.2 µm hollow fibre filters (Spectrumlabs, mediakap-5 hollow fibre filter), in order to concentrate the biomass present in the water. The filtered volume of every sample was 4 litres (performed in duplicate). After the filtration procedure, the filters were stored at -20 ºC for further analysis. During filtration, attempts were made to avoid filtering the oil phase. Oil blocked the filters and prevented later DNA extraction procedures. The freeze thaw cycle did not cause any significant cell lysis as was previously described by van der Kraan et al. (2010)

DNA extraction

One filter of every sampled environment was thawed on ice, filter lamella were treated with buffer and approximately 3 ml of the cell suspension released from the filter was collected in sterile Eppendorf tubes and centrifuged for 1 minute at 16100 g. 90% of the supernatant was removed, thereby achieving a 10-times concentration of the biomass and a removal of most potential PCR inhibitors (e.g. residual oil). The pellet was resuspended into the remainder of the supernatant. This suspension was subjected to DNA extraction using the Soil DNA Extraction Kit (Mo Bio Laboratories Inc, Carlsbad) according to the manufacturer's protocol. Subsequently the DNA amount from all extractions was quantified using a Nanodrop 1000 Spectrophotometer (Thermoscientific, The Netherlands). The obtained DNA was used for later analysis. The supernatant was subjected to DNA extraction as a control for cell lysis after the freeze thaw cycle, but DNA levels were below the detection limit, and no PCR product could be obtained using this as a template, indicating that the majority of the DNA was present in the pellet.

16S rRNA gene amplification

Amplification of 16S rRNA gene fragments was performed on the extracted DNA samples using a T1 thermocycler (Biometra, Göttingen, Germany). Amplification of bacterial 16S rRNA gene fragments was conducted by a touchdown program to reduce formation of nonspecific amplification. Thereto the primer pair BAC341F+GC and BAC907Rm was used (Schäfer, 2001). Archaeal 16S rRNA gene fragments were obtained using a nested and a direct approach. Previous studies showed that direct use of the archaeal primer pair Parch519F and Arch915+GC yielded also bacterial sequences (Vissers et al. 2009). Therefore, in addition a nested PCR was performed to circumvent this issue. In this nested approach amplification of the almost complete 16S rRNA gene was carried out using the primer pair SD-arch-0025-a-S17 and S-*-Univ-1517-a-A-21. Dilutions up to 1/10000 were made from all obtained PCR products, which served as templates for PCR amplification with the primer pair Parc519F - Arc915+GC (Coolen et al. 2004). The reactions were checked for bacterial and archaeal 16S rRNA fragments on a 1,5% agarose gel (45 minutes at 100V). All samples gave a positive result with the exception of centrifuges (S310) during and S310 after for archaeal amplification. The long fragments, which were used in the nested approach, were amplified according to PCR regime described by (van der Kraan et al. 2010). For the samples which were amplified in the nested approach, 30 amplification cycles were used in the direct approach.

Denaturing Gradient Gel Electrophoresis (DGGE).

DGGE was performed as described by Schäfer and Muyzer (Schäfer, 2001) on the amplified 16S rRNA fragments, using a acrylamide/bisacrylamide (37.5:1) gel with a urea/ formamide gradient. The "bacterial" gel contained a gradient of 20-80%. For the "archaeal" gels a 30%-70% gradient was used. Bacterial gels were run at 60ºC and 100V for 18 hours on a BIO RAD Dcode system and stained with Gel Green (Biotum, USA). Images were taken using a blue light safe imager in a C-box doc system using Genesnap software (Syngene). The same procedure was followed for the archaeal gels only they were run for 5 hours on 200V. DNA fragments were excised from the gel using a sterile blade and incubated in 20 µl, 10mMol Tris buffer (pH 8,5). The samples were incubated overnight at 4 ºC. The obtained solution was used as a template for a reamplification using primers that hold no GC-clamp. The samples were checked for a PCR product of the correct size on a 1,5% agarose gel as described earlier.

PCR product purification and sequencing

After reamplification, of the obtained PCR products (25 µl) were purified with ExoSap-IT enzyme solution (USB Europe) to remove single stranded primers and remainder of the nucleotides, according to the manufactures protocol. (The tubes were placed in a T1 thermocycler for 30 min at 37°C followed by 15 min at 80°C. Samples were diluted accordingly (50 ng μl^{-1} PCR product) and were sent for sequencing to a commercial company (Macrogen, Seoul, Korea)).

Comparative sequence analysis

To obtain a first indication, the obtained 16S rRNA gene fragment sequences were compared with sequences stored in the Genbank nucleotide database by applying the blast algorithm (Altschul et al. 1990). Sequences were then imported into the ARB SSU rRNA database (Ludwig et al. 2004) see also www.arb-home.de). They were aligned using the automatic alignment tool in the contained in the software package and subsequently checked manually on errors. Phylogenetic trees were generated by application of the Maximum Likelihood (ML) algorithm, FastDNA ML. First an ML tree was created with sequences from the closest related sequences; the obtained sequences were added to that tree.

Quantitative PCR

Quantitative PCR was performed on samples from the V011 oil-water separator using an iCycler IQ5 real time PCR instrument (BIO RAD, Hercules, California). The mixture contained 10.0 µl IQ Sybrgreen supermix (BIO RAD), 9.1 µl DNA-RNA free water (Qiagen), 0.16 µl BSA, 0.15 µl of each primer (Bac341F, 50 µM / Bac907rM(rA+rC), 50 µM) and 0.4 µl template. The amplification was performed according to the following program:

A denaturating step at 95 ºC for 5 minutes, followed by 40 cycles of 30 seconds at 95 ºC, 40 seconds at 57 ºC, 40 seconds at 72 ºC and 25 seconds at 80 ºC. This was followed by a final extension at 72 ºC for 7 minutes and a subsequently a meltdown control which started at 60 ºC for 10 seconds that was increased every cycle by 0.3 ºC, until a final temperature of 90 ºC was reached.

Results

Chemical composition of the production water

The water produced from the reservoir is hypersaline and mainly holds NaCl approximately 1,5 mol L^{-1}. It also contains the cations K^+, Ca^+, Mg^+ in small quantities (rep 9, 96 and 35 mM L^{-1}). Minute quantities of Iron, Ba and Sr were also found. biarbonate was present 5,5 mM L^{-1}. The reservoir is low on sulphate (resp. 6 µM L^{-1}). The measured reservoir pH varied between 5 and 7. The down-hole pH of the reservoir will be closer to the measured minimum pH, since brine water contains dissolved CO_2.

Microbial community analysis of the bacteria

The influence of THPS on the microbial community in the different oil-water separation facilities was examined by PCR-DGGE analysis of 16S rRNA gene fragments (Fig. 2. DGGE results for bacterial and archaeal analysis, Fig. 2A bacterial gel, fig 2B and 2C archaeal gels, Fig. 3. phylogenetic results). The DNA samples exacted from the producer was used for comparison as a reference. After sequencing, 29 out of 36 sequences resulted in a high quality sequence. Bands were associated with the genera *Caminicella*, *Pelobacter*, *Marinobacter*, the Desulfobacteraceae family and various uncultured bacteria.

Eight bands, some in different positions in the same lane, showed 100% sequence similarity with an uncultured *Caminicella* species detected in a high temperature North Sea oilfield (Dahle et al. 2008). One organism can thus be seen at different positions in the same lane. Three other bands showed a lower similarity with this organism. An uncultured *Pelobacter* sp. (DQ647158), which was found in the same North Sea oilfield, showed 99-100% sequence similarity with band M9

and D12 respectively. Therefore we can assume that the bands located at the same height belong to this bacterium. Bands B2 (separator before) and K2 (injector before) showed 99% similarity with an uncultured bacterium from a Colombian oilfield (Hernandez, J. et al. unpublished results, 2008). This band can also be found in lane C (separator during treatment) and L (injector during treatment) but it is not observed in the other samples. A 99% similarity with *Marinobacter* sp. SKA S8 found in halocline water from Antartic meromictic lakes (Matsuzaki, M. et al. unpublished 2006, AB252063) was found in the separator before the biocide treatment (B9). The band at position B10 is highly comparable with *Desulfohalobium utahense* found in an extreme hypersaline sediment of the Great Salt Lake in Utah (Kjeldsen et al. 2007). An uncultured Desulfobacteraceae found in stratified lakes with oxygen-sulfide interface (Casamayor, E.O. et al. unpublished results, AM749876) was found in the separator before and during biocide treatment (band C8, 97% sequence similarity). The band at position E12 in the pump before biocide treatment showed some similarity with an uncultured *Pelobacter* sp. found in crude oil contaminated soil (Yu, S.L. et al. unpublished results, EU328012). A band at the same position in the centrifuges (before sample H7) have an affiliation with an uncultured bacterium detected in a deep sea microbial mat from the northeastern Japanese Sea (Sato, T. et al. unpublished results, AB426436). No higher similarity was found for bands at these positions. Overall DGGE patterns displayed similar banded patterns. In the producer no intense bands are present at the lower part of the gel. The separator (V011) showed an intense band similar to *Marinobacter* sp. SKA S8 (B9). In the other lanes no intense bands are present at the same height. Analysis with the ARB software package revealed relatedness of this sequence with *Marinobacter koreensis* (Kim et al. 2006). Bands at position G6 (pump, after treatment) and D9 (separator after treatment) are also related to this species. *Desulfohalobium utahense* (B10, separator before treatment) and an uncultured Desulfobacteraceae (C8, separator after treatment) can be seen at the same position in the gel and no other samples showed a band at this position. In pump p202 an uncultured *Pelobacter* sp. was found with BLAST. In the ARB database the sequence fell close to an uncultured Delta-proteobacterium of a deep-sea station of Pacific Nodule Province (Xu, M.X. et al. unpublished 2003, DQ394960). This band, at position E12, is intense before biocide treatment compared to the other bands. During the treatment the intensity had reduced and after the treatment hardly any band can be seen. The centrifuges showed differences at position H3 in the gel. This band can also be found at position F1, which gave no sequencing result, and at position B1. The BLAST algorithm linked sample B1 (separator, before) to an uncultured *Caminicella* (98% similarity) and to uncultured Clostridiaceae bacterium (96% similarity), both found in the same high temperature North Sea oilfield. Using the ARB software a resemblance with *Caminicella*

sporogenes, isolated from a hydrothermal vent (Alain et al. 2002) and an uncultured *Clostridium* for this sequence was found. At this position an intense band can be detected before biocide treatment. During the treatment the intensity decreases and after the treatment no band is visible.

Microbial community analysis of the archaea

In addition to the bacterial community, the archaeal community in the different units of the oil-water separation plant has also been investigated; a nested and a direct PCR-DGGE approach were both used. This resulted in 41 high quality sequences in total yielding 33 archaeal and 8 bacterial sequences. Bacterial sequences were found only using the direct DGGE approach. Interestingly only one of them had a resemblance with the different bacteria found using the bacterial 16S rRNA-DGGE primers. With the nested approach no bacteria were detected, but some Archaea which were detected with the direct approach were not found in the nested approach. (Fig. 2B and 2C, DGGE results, Table 1B detailed analysis of the sequences). Bands were associated with the genus *Methanocalculus*, and several species belonging to the Halanaerobiaceae and various different uncultured Archaea. The bacterial genera *Flexistipes* and *Pelobacter* were found in addition. Seven bands showed 99% similarity with the species *Methanocalculus halotolerans* (Ollivier et al. 1998) found in an oil-producing well, 3 other bands showed similarity of 91 – 97% with this organism.

Two different uncultured Archaea retrieved from a deep surface shale (Nusslein, K. et al. unpublished results EF117481 & EF117423) were associated with 12 bands in the archaeal gel. Five other bands showed similarity with two different uncultured Archaea from Meromictic Artic lakes (Pouliot et al. 2009). The species *Flexistipes sinusarabici* from Atlantis II Deep brines of the Red Sea (Fiala G, 1990) had an affiliation with sequences sequenced from 3 different bands. A 99% sequence similarity with an uncultured *Pelobacter* sp. detected in produced water from a high temperature North Sea oil-field was found in band F7 (Separator before biocide treatment in direct approach). This organism showed close relatedness with the DGGE band sequences M9 and D12. A sequence from the direct approach of the producer (before biocide treatment, band B3) showed relatedness to a halanaerobiacaea bacterium from an extreme hypersaline Salt Lake in Utah. An uncultured euryachachaeote from a hypersaline microbial mat (Robertson et al. 2009). No significant changes are found between the environments and the different stages (before, during and after). The bands at position C12, F11 and U11 gave no sequence results. A notable band can be found at position L1 in the pump in the direct approach. Unfortunately the band yielded an unusable sequence. This band (L1) can also be found in the centrifuges before the treatment (O1) and in the injector before and after the treatment (both in the direct approach). A

94% similarity (and 96% query coverage) was found with Hanlanaerobiaceae sp. S191. Band O1 and band U1 showed an affinity with an uncultured archaeon from a deep surface shale.

The two uncultured Archaea from the meromictic high artic lakes were found in all environments, except the pump, in the directed approach only. During biocide treatment the bands of these organisms became less intense compared to the other bands in the same lanes.

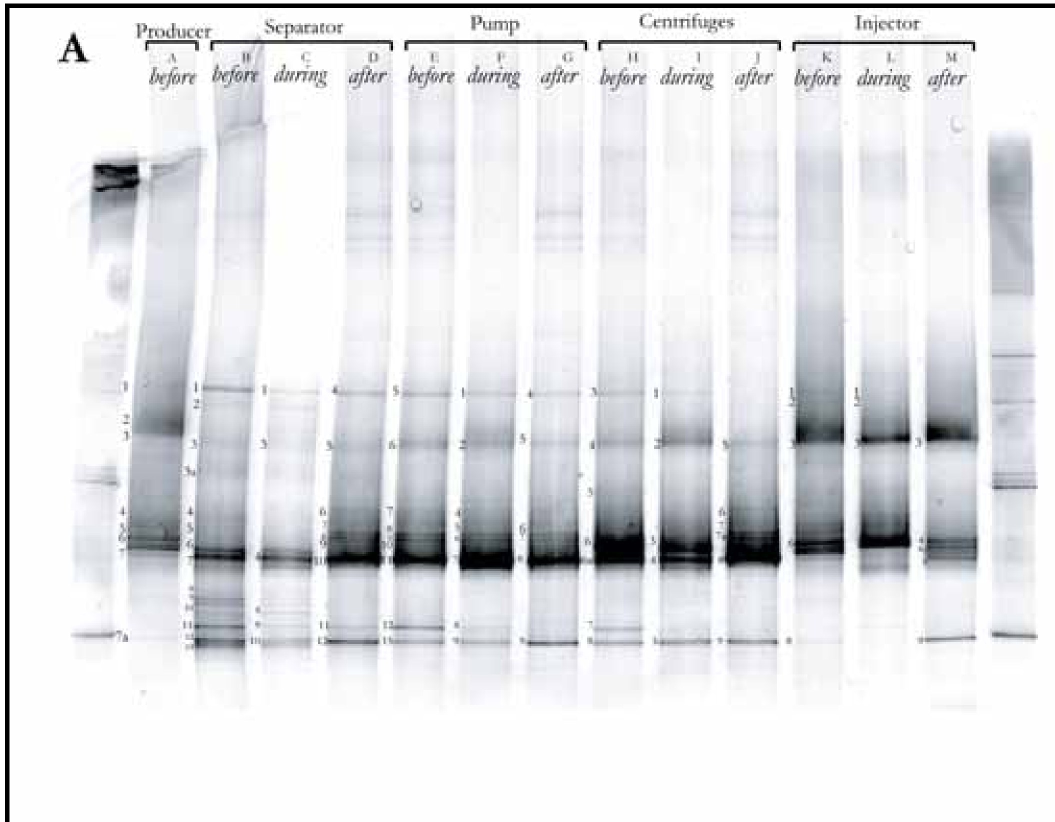

Figure 2a: *DGGE of bacterial 16S rRNA gene fragments from different units of an oil- water separation site before, during and after biocide treatment.*

[A] *Lane A: RTD13(before); lane B: V011 (before); lane C: V011 (during); lane D: V011 (after); lane E: P202a (before); lane F: P202a (during); lane G: P202a (after); lane H: S310 (before); lane I: S310 (during); lane J: S310 (after); lane K: RTD16 (before); lane L: RTD16(during); lane M: RTD16 (after)*

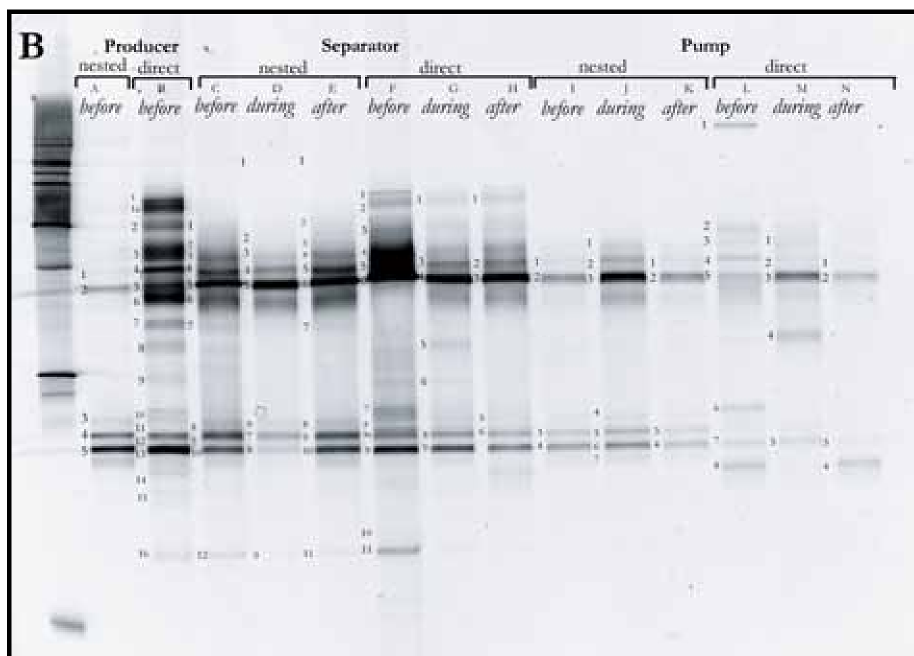

Figure 2b: *DGGE of 16S rRNA gene fragments from different units of an oil- water separation site before, during and after biocide treatment.*
[B] *Archaeal 16S rRNA gene fragments in nested an direct approach Lane A: nested RTD13(before); lane B: direct RTD13(before); lane C: nestedV011 (before); lane D: nestedV011 (during); lane E: nestedV011 (after); F: directV011 (before); lane G: directV011 (during); lane H: directV011 (after); lane I: nestedP202a (before); lane J: nestedP202a (during); lane K: nestedP202a (after); Lane L: directP202a (before); lane M: directP202a (during); lane N: directP202a (after)*

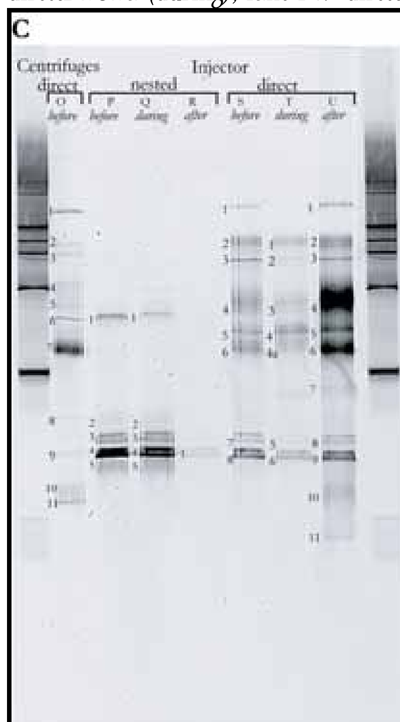

Figure 2b (continued): *DGGE of 16S rRNA gene fragments from different units of an oil- water separation site before, during and after biocide treatment.* ***[C]*** *Archaeal 16S rRNA gene fragments in nested an direct approach. Lane O: directS310 (after); lane P: nestedRTD16 (before); lane Q: nestedRTD16 (during); lane R: nestedRTD16 (after); lane S: directRTD16 (before); lane T: directRTD16 (during); lane U: directRTD16 (after).*

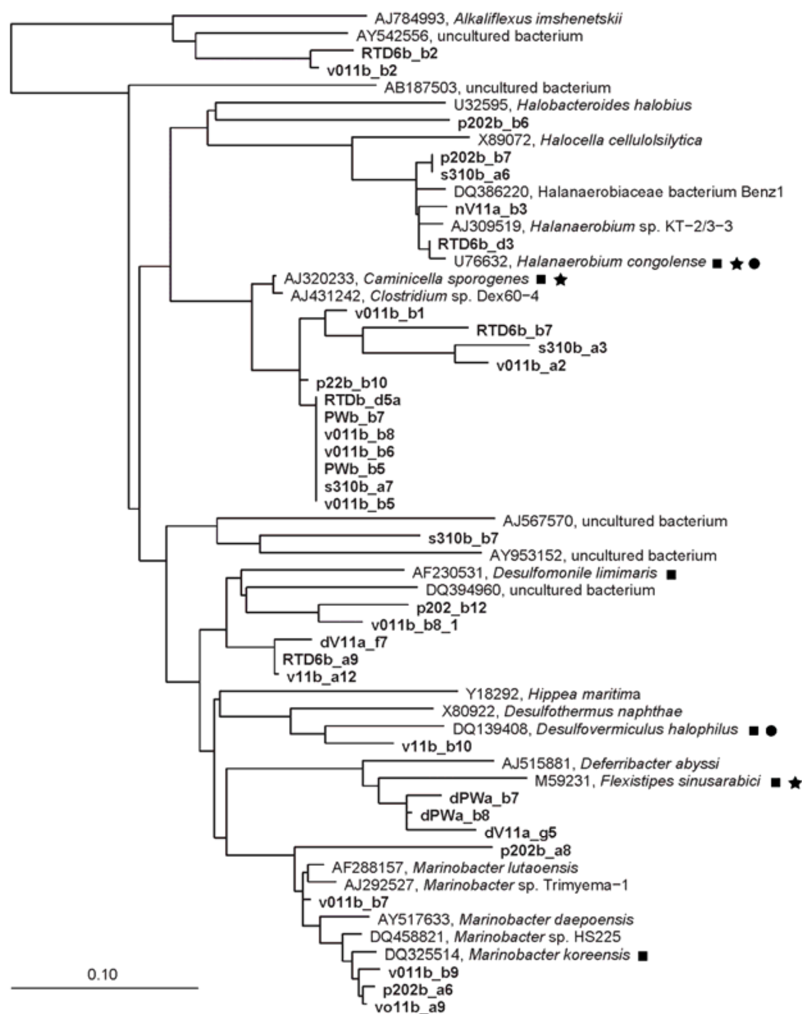

Figure 3a: *Phylogenetic analysis of the bacterial 16S rRNA gene sequences retrieved from the DGGE analysis. A star behind the sequence name indicate thermophily; a square indicates salt-loving; a dot indicates the isolation from oil associated environment. The bar indicates 10% sequence differences.*

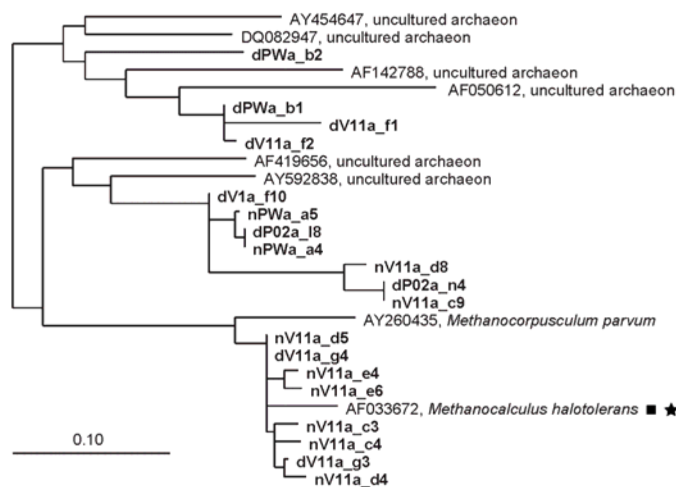

Figure 3b: *Phylogenetic analysis of the archaeal 16S rRNA gene sequences retrieved from the DGGE analysis.*

Quantitative PCR

A quantitative PCR was performed to quantify the amount of gene copies present before, during and after the biocide treatment. For this experiment samples from the oil-water separator (V011) were used. (Fig. 5. comparison of the qPCR results of the two stages, before-during and before-after). The difference of the DNA copies in the samples that were taken before the treatment, were compared with the samples during and after the treatment. The different amount of copies between before and during was 111 and between before and after 16. This was corrected for the sample volume that was filtered (4 litre/filter) and led to 28 copies L^{-1} between before and during and 4 copies L^{-1} between before and after.

Discussion

Overall diversity study

The sequences found in this study, belonged to four bacterial and two archaeal classes: Firmicutes, Deltaproteobacteria, Gammaproteobacteria, Deferribacteres, Euryachaeota and Crenarchaeota. This revealed a substantial microbial diversity in the oil-water samples and correspond to the most common organisms found in oil fields (Magot et al. 2000). Sequence analysis revealed similarities with organisms found commonly in oil field environments. In addition organisms from other extreme situations, like hydrothermal vents, meromictic lakes, deep sea and hypersaline environments, were identified.

Not every band on the DGGE gel represented a different organism. An uncultured *Caminicella* sp. was found at different locations in the gel. These different positions can be clarified due to an artefact of the DGGE technique and can be explained by for example an organism with more than one promoter site, which can display different amplified sequences. Reasons for the occurrence of these 'so-called' shadowbands is given by (Janse et al. 2004). Furthermore considerable changes could be found between the directed and the nested approach. All bacteria were found with the direct PCR-DGGE approach, but this approach revealed archaeal sequences which were not detected with the nested approach as well. This revealed that the nested approach is ineffective to amplify all Archaea in the samples and implies that one molecular technique is not sufficient to give a complete overview of a community.

A notable change is the presence of an uncultured *Pelobacter* sp. in the injector after the treatment. This band can be found in all environments except during the biocide treatment in the injector environment. A possible explanation for this might be that DNA sequences of another organism can be present in higher concentrations and therefore reduce amplification of lower DNA concentrations. Another notable change can be seen in the band at positions E12 and H7. This

band can be detected in the separator in all environments. In the other environments this band is only visible before the treatment, but in the injector a band was only detected in the sample which was taken during the treatment. The latter can be due to an artefact, but the absence in the other samples during the treatment can be dedicated to the effect of the THPS or possibly a stronger amplification of the other bands in the same samples.

The overall effect of the TPHS treatment

With the exception of some small changes (as discussed) no remarkable changes can be seen due to the THPS treatment. Some bands become less intense compared to other bands during the treatment. Nevertheless no notable changes in band patterns can be seen between before and after the treatment.

The flow through the different units is probably so quick, that the biocide is rinsed out of the system before it shows an specific effect on separate groups of organisms. On the other hand, the different units can be seen as bioreactors because of the favourable conditions they provide for specific groups. Therefore a short injection of THPS is most likely not enough to have a long-term effect on the community. A prolonged treatment is probably required to get an eventual sufficient reduction of microbial growth.

A better method for long-term H_2S reduction might be the injection of nitrate (Davidova et al. 2001), (Bødtker et al. 2008). This biocompetitive strategy is increasingly used by the oil-industry and stimulates the growth of competitive bacterial populations. Therefore SRB are effectively displaced by biocompetitive exclusion. The use of nitrate in order to control SRB and hydrogen sulfide showed an effect in laboratory experiments and several field studies. Davidova et al. (2001) suggested that nitrate injection will decrease the amount of H_2S reinjected into reservoirs during the disposal of oil-field production water (Davidova et al. 2001). PCR-DGGE studies showed existence of a dominant population in a water-oil tank system on an offshore platform in Brazil (Jurelevicius et al. 2008). They showed that controlling sulphide production by nitrate treatment could reduce the quantity of chemical biocides required to control microbial activities.

The effect of the biocide on the overall population (determined by qPCR)

Quantitative qPCR showed that the total amount of DNA in the samples taken during biocide treatment was quantitatively lower than before and after the treatment. Although the amounts of DNA in the samples taken after biocide treatment were higher than during the treatment, it didn´t reach the values of the samples before the treatment. The absolute amounts of DNA in the samples that were taken at the different stages could not be measured, but since the changes

in DNA amounts are investigated this is not an addressed issue. The difference between the copy numbers per liter was calculated. In this study only one unit of the facility (separator V011) was used to get an insight in the quantitative differences between the samples taken before, during and after the treatment. This led to the conclusion that THPS treatment has an effect on the amount of DNA, and therefore the amount of organisms, in the oil-water separation facility. However, when the treatment stops amounts of DNA will most probably rise again. More qPCR experiments need to be done to get a more precise insight in the effect of THPS on the whole facility.

Conclusions

The effect of biocide treatment on the microbial community in an industrial oil-water separation facility was analyzed based on PCR-DGGE and qPCR techniques. This qPCR was done to see quantitative changes in amounts of DNA in the different stages (before, during and after the treatment).

The organisms that matched with the sequences from both DGGE analyses showed are commonly found in oil reservoirs. The biocide treatment overall yielded no significant changes in DGGE patterns. Quantitative qPCR showed a decrease in DNA due to THPS treatment, up to 28 copies L^{-1}, although the samples that were taken after the treatment showed 4 copies L^{-1} differences with samples before the treatment. It was therefore concluded that the biocide had an overall nonspecific effect on the complete microbial community, indicating that the biocide had no selective effect on specific groups of microbes. Since the effect of the biocide only showed a decrease of one order of magnitude on the detected DNA amounts it can be regarded as a short lasting effect, therefore multiple treatments are required on a regular basis. It is advised to search for a better alternative to reduce the growth of unwanted microorganisms like NO_3^- injection.

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4

Microbial diversity of an oil field core sample recovered from the Rabi oil field (Gabon, Africa)

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Abstract

Our laboratory received a frozen unconsolidated sandstone core from the Rabi oil field located in Gabon (Africa). Subsegments were taken from four different spatial positions in the core. A PCR-DGGE analysis was performed on PCR amplified 16S rRNA gene fragments obtained from the different subsegments, in order to get a rapid overview of the microbial diversity. In addition a subsegment of the outer core was taken as a contamination control, checked was if the outer area of the core had a different community composition with respect to the core interior. From the DGGE profile it was clear, that only minor differences between the different subsegments could be seen, providing an indication that the outer region of the sample was not contaminated. All of the obtained sequences showed close affiliations with sequences from described species that had been previously isolated from petroleum reservoirs and (hyper) saline environments. These microorganisms could be grouped into three phyla: Proteobacteria, Firmicutes and Halobacteria. These three phyla belong to the six bacterial phyla and the only archaeal phyla that are known to accommodate halophilic organisms. Based on the properties of the closest related described species, which overall match to the environmental conditions of the core ecosystem, the organisms from which the sequences were obtained likely have similar physiologies and can therefore be regarded as indigenous to the core environment.

A notable observation was that no sequence was obtained that had an affiliation to methanogens, SRB (Sulphate-Reducing Bacteria) or IRB (Iron-Reducing Bacteria), which are common inhabitants of oil reservoirs. This lack could be justified by looking at the bioenergetics of the metabolic processes of these micro-organisms. Living at extreme salt concentrations is energy demanding and from studies found in the literature it is clear the metabolic processes of methanogenesis and sulphate reduction likely do not generate enough energy at these elevated salinities in the core ecosystem to sustain these types of microorganisms.

Introduction

Research towards the microbial diversity of oil fields is largely dominated by studies towards oil field production waters, since production water sampling is relatively easy and cheap (see previous chapters). Such studies have yielded valuable insights; various groups of diverse microorganisms (with respect to physiological and metabolic activities, and to phylogenetic affiliations) have been recovered and identified from oil reservoir environments. The microorganisms living in oil reservoirs are able to sustain an underground deep biosphere, under often harsh conditions (high temperature, low pH, elevated salt concentrations, high pressure, and low nutrient concentrations). Diverse micro-organisms have the ability to utilize

hydrocarbons (Heider et al. 1998). The microbial diversity of oil field production waters however gives only part of the story, since many microorganisms are attached to surfaces, e.g. the porous matrix of the oil reservoir rock, and only the suspended free cells are commonly produced along with production water. The relative amount of surface is large therefore research on both oil field core material and formation waters gives a more direct and complete view of the indigenous organisms present in a subsurface environment. However, so far only a small number of cores have been analyzed, due to the high costs involved in taking such as sample. In addition the retrieval of a core sample usually involves the cooperation of an oil company. As a cause of this involved effort in sampling and the high costs involved, only a small number of articles can be found in the literature on studies regarding oil reservoir cores retrieved during the drilling of new exploration or production wells. Belyaev et al. (1983) studied oil-bearing rock cores, collected from the Bondyuzhskoe oil field at a depth of 1675 meters. In this study, a new methanogenic bacterium was found; *Methanobacterium* sp. strain Ivanov. In 1996, Azadpour et al. studied 13 core samples from North-American oil reservoirs, regarding the presence of SRBs to find indigenous organisms responsible for the H_2S production. Core sample depths ranged from 245 to 4420 meters. Next to the 13 samples, production waters of 11 of these sites were examined for presence of SRB. Interestingly, all eleven production waters contained SRB, while none of the studied cores yielded H_2S production. The authors suggest that introduction of SRB in petroleum reservoirs could be an introduced problem due to human activity, and finding ways to avoid SRBs from entering petroleum reservoirs during drilling and secondary recovery operations could prevent corrosion problems. More recently, Spark et al. (2000) studied the presence of indigenous microorganisms in nine reservoir oil field rocks in the North Sea and Irish Basin areas. The oilfields varied in depth from 1067 to 4575 meters, with *in situ* temperatures up to 150 degrees °C. Next to the cores, the live drilling mud was examined. Interestingly, the authors showed by 16S rRNA gene sequence analysis that the cores contained none of the bacterial types observed in the live drilling mud, indicating the indigenous nature of the microorganisms observed in the core samples. Moreover, this is direct evidence that production waters are more likely to contain exogenous microorganisms than core samples do. The issue of contamination during sampling oil reservoirs has been addressed earlier (Magot et al. 2000).

Criteria for the indigenous origin of species in a core sample

Determining whether a microorganism is indigenous in an oil reservoir is essential before any conclusions can be made, regarding its role in the ecosystem under investigation. Contamination of oil-reservoir materials obtained during sampling is an issue that constantly concerns petroleum

microbiologists due to the number of possible sources of contamination upon sampling (e.g. drilling, well equipment operations and waterflooding). In oil reservoirs, it is most probable that part of the indigenous community thrives in biofilms attached to the surfaces of the porous rock. Magot (2005) suggests two main criteria to determine the indigenous nature of microbial strains obtained from petroleum reservoirs and petroleum production waters: (1) comparing the isolate's growth optima to the *in situ* conditions in the petroleum reservoir, and (2) comparing the global distribution of the strain's phylotype in oil reservoir samples worldwide. While useful, the optimum temperature of a microorganism is not necessarily an accurate reflection of the *in situ* temperature. Due to the heterogeneity of soils, the parameters governing growth are not uniformly divided across the soil. Thermophilic isolates with much lower temperature optima than their environment and thermotolerant isolates with low temperature optima have been reported from high-temperature ecosystems (Takai et al. 2004), (Vetriani et al. 2004).
Similarly, some halophilic and halotolerant microorganisms recovered from salt crystals have a relatively low salt tolerance (Vreeland et al. 2002), (Mormile et al. 2003), (Arahal et al. 2007).
A more reasonable approach may be considering range (minimum and maximum growth limits) or the ability to survive for prolonged periods of time at the *in situ* reservoir condition. In addition, the global presence of specific microbial lineages in geographically isolated oil reservoirs can be taken into account as a good indication of their indigenous nature. A critical remark on this criterion is that it could theoretically exclude novel groups that are indigenous to a specific oil reservoir where specialized niches exist. Due to the difficulties in obtaining contaminant-free oil reservoir samples, personal judgment still is a critical factor in determining the origin of isolates and 16S rRNA gene sequences encountered in oil reservoirs. Methods have been developed to collect representative samples, whilst maintaining *in situ* conditions and protecting them from contamination (Griffin et al. 1997). However, using these techniques poses huge restraints in terms of personnel requirements, budget and field campaign scheduling. This is the reason why these techniques have never been actually implemented and standardised in the oil industry (Magot, 2005).

Microbial communities in hypersaline environments

Hypersaline environments comprise hypersaline waters and soils. Frequently oil field environments can be placed into this category. Waters are considered hypersaline when the salt concentration is higher than that of seawater (35 g L^{-1}) (Rodriguez-Valera, 1988), whereas soils are considered hypersaline when they contain more than 0.2% (w/v) soluble salt (Ventosa et al. 2008). Organisms that are able to survive in saline environments are classed in five groups, based

on the salt concentration for optimal growth (Kushner & Kamekura, 1988). Halophiles are found in all three branches of life. Within the Bacteria, halophiles are known belonging to the phyla Cyanobacteria, Proteobacteria, Firmicutes Actinobacteria, Spirochaetes and Bacteroidetes. Within the Archaea most salt-requiring microorganisms are found in the class Halobacteria. Compared to hypersaline aquatic environments, very little information exists about the diversity of halophilic and halotolerant micro-organisms isolated from saline soils (Ventosa et al. 1998).

Soils and sediments are among the most diverse microbial ecosystems and are estimated to contain an order of magnitude more different prokaryotic species than aquatic ecosystems do, based on the higher spatial heterogeneity (Curtis et al. 2002). The diversity of hypersaline soils is more similar to non-saline soils than to hypersaline waters. An extensive overview of microorganisms found in hypersaline soils is given in the book 'Microbiology of Extreme Soils' (Ventosa et al. 2008)

Only a few articles were published on the microbial communities in high-saline oil fields. However, halotolerant and/or halophilic organisms found in oil contaminated soils examples are: (Obrazstova et al. 1988), (Bhupathiraju et al. 1994), (Beliakova et al. 2006).

An example of a study towards a hypersaline oil field is given by Yuehui et al. (2008) a molecular analysis was performed on the production water of the Qinghai hypersaline petroleum reservoir (80-160 g L^{-1} NaCl).

Aim of the research

The aim of this study was to screen the microbial diversity of the core sample and in addition, provide a contribution to knowledge on the downhole microbial diversity of hypersaline oil fields. Thereto a PCR-DGGE analysis was used to scan the microbial diversity in a core sample of the Rabi oilfield (Gabon, Africa). The field is a hypersaline petroleum reservoir holding mesophilic temperatures (43 °C). The combination of hypersalinity and oil presence provided us with a unique sample, which could give us new insights into the microbial community present in this special ecosystem. Based on the gene sequence analysis an indication was given towards the indigenous nature of the detected species. In addition, the difference in microbial communities at different cross-sectional positions was compared. Since studies on oil field core samples are scarce, the information obtained in this study is a welcome contribution within this field of research.

Materials and Methods

Sample description (provided by Shell Exploration & Production)

The core sample was taken directly from the Rabi oil field located in Gabon, Africa. It was retrieved from a depth of 1154 meters. The ambient temperature and pressure were 43°C and 120 bar respectively. The environment of the core is salt saturated, indicating a salt concentration of approximately 300 g L^{-1}.

DNA extraction

Subsegments of the core were taken from four different spatial positions (Fig. 1). The core pieces were cut using a chisel and subsequently crushed using a sterile a mortar and a pestle while submerged into liquid N_2. The chisel, mortar and pestle were sterilized before usage using autoclavation at 160 ºC for 4 hours. DNA was extracted from the crushed core pieces in duplo (by approximation 0,75 g per segment) by application of a soil DNA extraction kit (MoBio Ultraclean Soil DNA Isolation Kit) according to the manufacturer's instructions. Extracted DNA was quantified on a Nanodrop 1000 Spectrophotometer (Thermo Scientific, The Netherlands). The obtained DNA was used for further PCR amplifications.

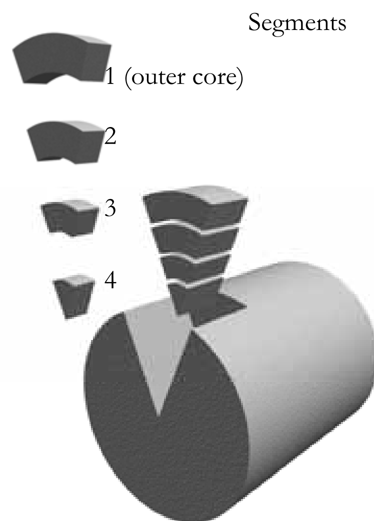

Figure 1a: *Image of the core sample* **b:** *Spatial sampling of the core (schematic)*

16S rRNA gene amplification

Partial 16S rRNA gene sequence fragments were amplified from the genomic DNA retrieved from different core subsegments. For the amplification of bacterial 16S rRNA gene fragments the bacterial primer pair BAC-341F+GC and BAC-907Rm was used (Schäfer & Muyzer, 2001). During the bacterial PCR a touchdown program was implemented. Partial archaeal 16S rRNA

gene sequences were amplified using the primer pair Parch-519fm and ARC-915R+GC as described by Coolen et al. (2004). PCR products were checked on 1.5% (w/v) agarose gels run at 100V for 45 minutes. For an overview of the different applied amplification techniques, see (van der Kraan et al. 2010).

Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed on bacterial and archaeal partial 16S rRNA gene fragments as described by (Schäfer, 2001). In brief: 1 mm thick, 6% polyacrylamide gels holding a urea-formamide gradient of 20%-80% were used to separate bacterial PCR products. Gels loaded with bacterial PCR products were run at 100V for 18 hours. Archaeal PCR products were run on a gel holding a 30-70% urea-formamide gradient at 200 V for 5 hours respectively. Gels were stained with a Gel Green solution (Biotum, USA), and were photographed with a blue light safe imager in a C-box doc system using Genesnap software (Syngene). Bands were cut from the gels using a sterile surgical knife and subsequently transferred to separate sterile 1.5 ml eppendorfs. 20µl of sterile 10 Tris buffer 10 mMol at pH 8.5 was added. The bands were maintained overnight at 4 ºC and were reamplified using the earlier described PCR program using GC-clamp free primers.

PCR product purification and sequencing

1.6µl ExoSAP-IT (USB, Europe) solution was added to 25 µl PCR product of each reamplified DGGE band to remove single stranded primers and the remainder of the nucleotides. Purification was performed according to the manufacturers' instructions. Treated PCR products were checked for purity and concentration on a 1.5% agarose gel and were diluted accordingly to obtain a 50 ng μL^{-1} product solution. The obtained samples were sent to a commercial company for sequencing (Macrogen, Seoul, Korea).

Comparative sequence analysis

To obtain a first indication, the partial 16S rRNA gene sequences were compared with sequences stored in the GenBank database using the BLAST algorithm (Altschul et al. 1990). Sequences were subsequently imported into the ARB SSU rRNA database and aligned using the automatic alignment tool of the software package and manually checked for errors (Ludwig et al. 2004). Phylogenetic trees were generated by application of the Maximum Likelihood (ML) algorithm, FastDNA ML. First, closest relatives were found and selected in the database and an ML tree was created. The DGGE band sequences were later added to this tree. All the bacterial DGGE bands were around 500 bp. in length, the archaeal DGGE bands were around 400 bp.

Results

DGGE analysis of 16S rDNA gene sequences

The DNA extracted from the Rabi core consisting of hypersaline unconsolidated sandstone, was subjected to a PCR-DGGE analysis. The DGGE was performed in duplo on four subsegments (Fig. 1b.) The bacterial DGGE yielded 30 bands from which 28 bands yielded sequences with satisfactory quality (93%). The archaeal DGGE yielded 22 bands with 17 bands holding sequences with satisfactory quality (77%). Sections 1 to 3 showed similar microbial communities, while section 4 (most inner part of the core sample) showed faint additional bands in the DGGE profiles. These bands yielded unusable sequences.

Identification of microorganisms found in the core.

All of the obtained sequences showed similarity in their 16S rRNA gene sequences to micro-organisms previously isolated or detected from petroleum reservoirs and (hyper)saline environments. The microorganisms belonged to the different genera: *Halanaerobium*, *Halomonas*, *Chromohalobacter*, *Orenia* (Bacteria) and *Haloferax* (Archaea). These genera (Protebacteria, Firmicutes & Halobacteriales) are part of the six bacterial phyla and the only archaeal phyla (Euryarchaeota) that are known to accommodate halophilic organisms. The archaeal DGGE showed in addition bacterial sequences, and thus was not archaea-specific. This has also been found in other studies, (Vissers et al. 2009), (van der Kraan et al. 2010). The results of the bacterial DGGE (Fig. 2a) are summarized in Table 1a. The bands at the position of A1 (A1, to H1) showed a 100% sequence similarity to the species *Halanaerobium praevalens* which was isolated from a hypersaline sediment (>20% w/v) of the Great Salt Lake, Utah (Arahal & Ventosa, 2006). The bands at the position of A3 and A4 (A3, A4, to H3,H4) were most similar to the species *Chromohalobacter israelensis* isolated from a Tunisian solar saltern (Arahal et al. 2001), holding sequence similarities between 99-100%. Bands E2 and H2 were most similar to an Uncultured bacterium clone. Band G3 held a sequence 100% similar to a sequence from a *Paracoccus* sp. 10-1-100 (100% similarity) isolated from the desert of Xingjiang, China (and was in addition 99% similar to the species *Albidovulum inexpectatum* isolated from a marine hot spring (Albuquerque et al. 2002)). Band G4 showed an affiliation with the species *Halomonas elongata*, isolated from a Solar Saltern (Vreeland et al. 1980). The sequence similarity was only 95%. The results of the archaeal DGGE (Fig. 2b) are summarized in table 1b.

The bands at position I1 (I1 to P1) showed a 96% sequence similarity to the species *Orenia salinaria* (Moune et al. 2000), isolated from a Mediterranean anaerobic saltern. *O.salinaria* does not belong to the archaeal kingdom, which shows that the used primers are not archaea-specific.

Bands J3 and L4 also affiliate to *O. salinaria*. This species thus displayed multiple bands on this DGGE gel. Bands J2, K2 and L2 showed a 98% sequence similarity to the species *Halanaerobium kushneri*, isolated from a hypersaline oil brine. Band N3 and P2 in addition showed an affiliation to the *Halanaerobium* genus, Band L3 showed a 99% serquence similarity to the Haloarchaeon MSNC 16(6). This is an alkane-degrading strain belonging to the *Haloferax* genus, retrieved from an uncontaminated hypersaline pond in Camargue, France. Band K3 showed an affiliation to an organism from the same genus, *Haloferax* sp. FC28_21. Since both bands are situated at the same height on the DGGE band, they are assumed to be the same organism. The bands G2, G5, K4, M2, N2, N4 and P3 yielded unusable sequences.

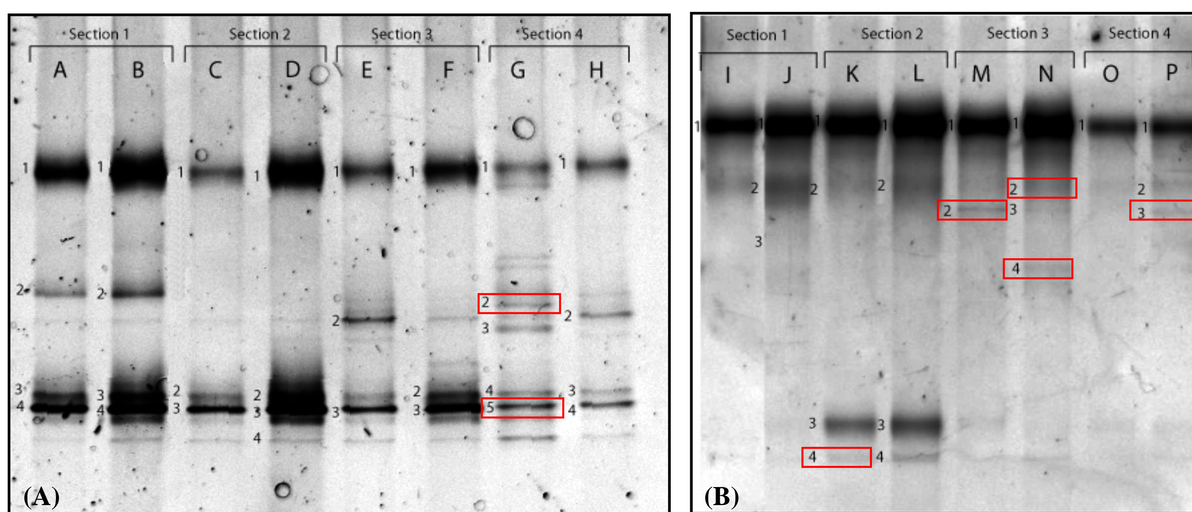

Figure 2: *DGGE analysis of 16S rRNA gene fragments (A) Bacteria and (B) Archaea. Lane pairs correspond to the different subsegments taken from the core (section 1 being the outer area of the core. Boxes indicate bands that were not successfully sequenced.)*

Table 1: *Closest relatives of the obtained 16S rRNA gene sequences and isolation source.*

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

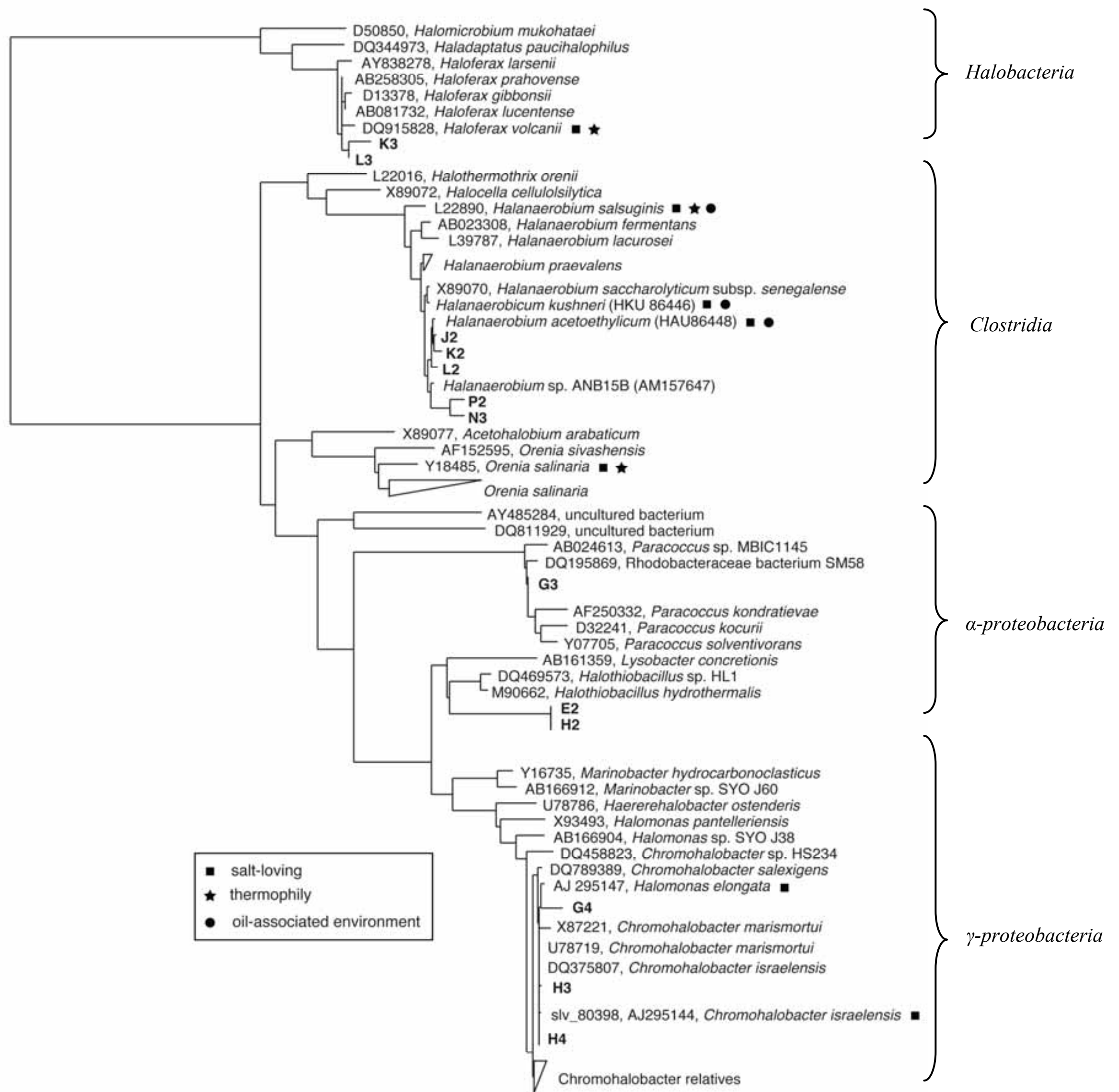

Figure 3: *Phylogenetic analysis based of the partial bacterial and archaeal 16S rRNA gene sequences retrieved from both DGGE gels (Fig. 2). Sequences obtained in this study are shown in boldface. Sequence accession numbers are shown between parentheses. The white triangles indicate compressed sections of the tree, with multiple sequences grouped together. Next to the tree, the classes are shown the microorganisms in the phylogenetic tree belong to. DGGE sequences are indicated by the lane name (A to P) followed by the band number as shown in the DGGE gels (Fig. 2).*

Discussion

Microbial diversity of the Rabi core: comparison of described relatives to the core ecosystem.

Determining whether a microorganism is indigenous to an oil reservoir is essential in understanding its role in the environment. To determine whether a microorganism is indigenous to a petroleum reservoir, the following two criteria can be used as already mentioned (adapted from Magot et al 2000): (1) comparing the isolate's growth ranges to the *in situ* conditions in the petroleum reservoir, and (2) comparing the global distribution of the strain's phylotype in oil reservoir samples worldwide. In this study, a core was analyzed, taken from a depth of 1154 meter in the Rabi oil field, situated in Gabon. DNA was extracted and subsequently two PCR-DGGE analyses (Bacteria & Archaea) were performed. The Rabi core displayed, based on 16S rRNA gene comparison, not a wide diversity. By combining the sequence results obtained by comparison with the GenBank database and the results of the phylogenetic analysis by the ARB software package (Fig. 3), it was found that mostly sequences related to fermentative organisms were detected. A set of close relatives was chosen that showed high similarities to the obtained sequences in this study. This set was compared to the ecosystem of the core regarding growth conditions (minimum, optimal and maximum) as a function of temperature (°C) and salinity (% w/v). All of closest relatives were isolated from petroleum reservoirs and/or from (hyper)saline environments. The given descriptions of these closest relatives served only as an indication of the microorganisms present in the Rabi core, and do not give the real microbial community. These indicative organisms are in addition, used to provide an estimation of the indigenous nature of the obtained sequences. 8 bacterial and 1 archaeal species were compared on the basis of temperature range and NaCl tolerance range. Of the bacteria, three belonged to the phylum proteobacteria, and five to the phylum firmicutes. The eight bacteria were *H. praevalens*, *C. israelensis, Halomonas halodenitrificans* (formerly *Paracoccus halodenitrificans*), *H. elongata, O. salinaria, Halanaerobium kusneri, Halanaerobium salsuginis and Halanaerobium acetoethylicum. The only archaea was Haloferax volcanii.* The species *H. praevalens, H. kushneri, H. salsuginis, H. acetoethylicum* and *O. salinaria* belong to the phylum firmicutes (class Clostridia, order Halanaerobiales, family Halobacteroidacea). The Halanaerobiales is a phylogenetically coherent group consisting entirely of halophiles and consists of obligate anaerobes that live by fermentation of sugars or amino acids. These microorganisms are stainable as gram-negative bacteria and are physically gram-negative, while they are phylogenetically related to the gram-positive bacteria. Characterized haloanaerobes isolated from oil field reservoirs/brines mainly belong to this genus, including *H. acetoethylicum* (Rengpipat et al. 1988), *H. congolense* (Ravot et al. 1997) and *H. salsuginis* (Bhupathiraju et al. 1994). An extensive review on the order Halanaerobiales was published by (Oren, 2008).

The species *C. israelensis, H. elongata* and *H. halodenitrificans* belong to the Gammaproteobacteria. The phylum Proteobacteria is one of the six phyla in the kingdom of bacteria that houses halophilic microorganisms. The *Halomonas* genus comprises slightly or moderately halophilic, chemo-organotrophic, gram-negative rods that are widely distributed throughout hypersaline environments (Arahal & Ventosa, 2005) *H. volcanii* belongs to the phylum Euryarchaeota (class Halobacteria order Halobacteriales family Halobacteriaceae) in the archaeal kingdom, and was originally isolated from the Dead Sea (Mullakhanbhai & Larsen, 1975) *H. volcanii* is an chemoorganotrophic aerobic organism. Another archeaon in the genus of *H. volcanii, Haloferax* sp. D1227, an extreme halophile isolated from soil contaminated with highly saline oil brines, is the only reported archaeon utilizing aromatic compounds as the sole carbon and energy source. *H. volcanii* and *Haloferax* sp. D1227 show 98% sequence similarity to sequence L3. The order Halobacteriales is the only known order in the kingdom of the archaea that is known to contain halophilic archaea. Microorganisms in this order are found in hypersaline environments in which salt concentrations exceed 150-200 g L^{-1}. An extensive review on information about the order Halobacteriales was published by (Oren, 2006).

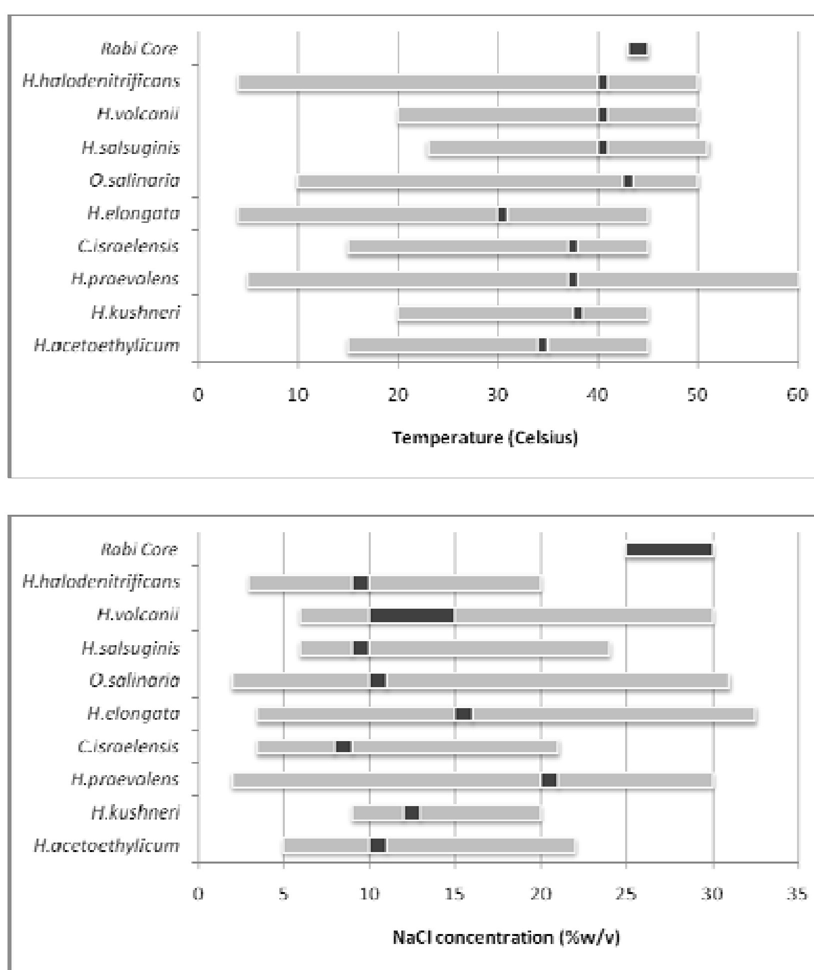

Figure 4: *Graphs showing the range (grey bars) and optimal temperature and NaCl (black) concentration for the various closest relatives (information was gleaned from the literature).*

Table 2: *Comparison of the chosen described closest relatives to the core ecosystem.*

Scientific name	Temp Range (°C)	Temp opt (°C)	Salinity range (% w/v)	Salinity opt (% w/v)	Anaerobic growth	Ref.
Halomonas halodenitrificans	4 – 40	40	3 – 20	9	F.A.*	(Miller et al. 1994)
Haloferax volcanii	20 – 50	40	6 – 30	10 – 15	NO**	(Mullakhanbhai & Larsen, 1975, Oren, 2006)
Halanaerobium salsuginis	23 – 51	40	6 – 24	9	YES	(Bhupathiraju et al. 1994)
Orenia salinaria	10 – 50	42,5	2 – 30	7,50	YES	(Moune et al. 2000)
Halomonas elongata	4 – 45	30	3,5 - 32,5	15	F.A.	(Vreeland et al. 1980)
Chromohalobacter israelensis	15 – 45	37	3,5 – 20	8	F.A.	(Huval et al. 1995)
Halanaerobium praevalens	5 – 60	37	2 – 30	12,5	YES	(Zeikus et al. 1983)
Halanaerobium kushneri	20 – 45	37,5	9 – 18	12	YES	(Bhupathiraju et al. 1999)
Halanaerobium acetoethylicum	15 – 45	34	5 – 22	10	YES	(Rengpipat et al. 1988)

** F.A. is used as abbreviation for facultative anaerobic. Organisms that are F.A. can grow aerobically, and are also able to grow anaerobically (e.g. by reducing nitrate to nitrite – denitrification)*
*** This organism was reported as obligate aerobe. It is capable of reducing nitrate to nitrate, but it cannot grow anaerobically (Mullakhanbhai & Larsen, 1975). However, anaerobic growth on nitrate has been reported (Franzmann* et al. *1988).*

In Table 2 this comparison is displayed regarding the temperature range and optimum, and the salinity range and optimum for the chosen set of microbes (Fig. 4.), as well as the references on which the table was created. At the *in situ* temperatures at the depth the Rabi core was taken from, all closest relatives should be able to sustain growth. Growth optima were even close to the *in situ* temperature.

The growth conditions at increasing NaCl concentrations gave a more speculative indication. Only four closet relatives (i.e. *H. volcanii*, *O. salinaria*, *H. praevalens* and *H. elongata*) should be able to sustain growth at the salt-saturated conditions in the Rabi field. However, as was put forward by Magot et al. (2000), that optimum growth conditions of organisms should not necessarily reflect the actual conditions in the reservoir itself, due to soil heterogeneities. If this soil heterogeneity is taken into account, a classification can be made based on halotolerant and halophilic organisms. Halotolerant can sustain growth, but do not necessarily need NaCl to sustain growth. Halophilic organisms cannot sustain growth without NaCl, and thus clearly depend on NaCl for their survival. Halophilic organisms as defined as follows: microorganisms that grow optimally at salt concentrations of 50g L^{-1} (equivalent to 0.85M) or higher, and tolerate 100 g L^{-1} salt (equivalent to 1.7M NaCl) at least (Oren, 2008). Using this definition, five additional organisms were classified as being halophilic (i.e. requiring NaCl for growth), being *H.*

salsuginis, *C. israelensis*, *H. kushneri*, *H. acetoethylicum* and *H. halodenitrificans*. Next to the first four organisms that initially should be able to sustain themselves based on their salt requirement also closest relatives could be able to survive the core environment

The second criterion determining the indigenous nature of microorganisms in oil reservoirs, is to compare the global distribution of the strain's phylotype in oil reservoirs worldwide. This distribution can also be applied on hypersaline environments worldwide. The most ideal situation would be when certain organisms were found in hypersaline petroleum reservoirs. Up to date, not many research has focused on this topic. *Halanaerobium* species are common inhabitants of (hypersaline) petroleum reservoirs. *H. kushneri, H. salsuginis* and *H. acetoethylicum* were originally isolated from hyper-saline oil brines, showing similarity to the Rabi petroleum reservoir. The organisms found were mainly isolated from hypersaline environments. Due to the extreme salinity of the examined core, the global distribution of the organisms in hypersaline environments is assumed to be more important than the global distribution in (low-saline) oil fields. All organisms that showed most identity to the found sequences, and all compared indicative microorganisms have been isolated from diverse hypersaline environments.

According to the criteria as postulated by Magot et al, these microorganisms could possibly be addressed as indigenous to the reservoir with the exception of the relative *H. volcanii*, with has no consensus on anaerobic growth.

Microbial diversity at increasing salt concentrations and the absence of SRBs and IRBs in the core.

Microbial life can exist at salt concentrations up to NaCl saturation. The presence of increasing salt concentrations places an increasing stress on the microorganisms. In theory, two different strategies are used by microorganisms to maintain their cytoplasm at least isoosmotic with their surroundings. The first is the 'high salt-in' strategy, whereby accumulation of K^+ and Cl^- ions is used to maintain an osmotic balance. This strategy is energetically relatively inexpensive, but it requires extensive adaptation of the cellular machinery to high salt concentrations. This mechanism is mainly used by the Halobacteriales and by the Halanaerobiales. The other strategy of haloadaptation is based on the biosynthesis and/or accumulation of organic osmotic solutes, whereby cells exclude salt from their cytoplasm as much as possible. The energetic price the cells have to pay for this method is significant, since the biosynthesis of these solutes is energetically expensive. In an article by Oren et al, it has been postulated that from a salt concentration of about 100-150 g L^{-1} onwards, metabolic diversity becomes limited, see (Oren, 2001). Life at high salt concentrations is energetically expensive, and therefore the factors that determine whether a

certain type of microorganisms can sustain at high salt concentrations are based on the bioenergetics in their dissimilatory metabolism and the mode of osmotic adaptation used.

One interesting microbial process, harnessed by the Methanogens, is methanogenesis, whereby CO_2 is reduced by hydrogen, is already absent at salt concentrations of over 120 g L^{-1}. This is mainly to the low amount of energy gained using this process (-34.0 kJ mol^{-1} substrate). This might explain the absence of methanogens in the salt-saturated Rabi core, which are common inhabitants of petroleum reservoirs. Another interesting microbial process harnessed by the SRB's is sulphate reduction. The upper salt limit of this process is 130 g L^{-1} for complete oxidizers and 240 g L^{-1} for incomplete oxidizers. At higher salt concentrations, sulphate reduction does not provide for enough energy to support both biosynthesis of osmotic solutes and growth. This might explain the general absence of SRB in the Rabi field core. Another interesting theory suggests that SRB are introduced by water flooding, and they only appear in analyzed production waters, whereas they are missing in actual cores (Azadpour et al. 1996). The findings in this study support this hypothesis. The absence of IRBs can be contributed to the same bioenergetic effect: reduction of iron compounds does possibly not provide for enough energy to sustain cell growth in hypersaline environments.

Presence of fermentative and denitrifying organisms

Aerobic respiration, denitrification and fermentation can provide enough energy to sustain microbial growth up to very high salt concentrations. Microorganisms belonging to the *Halobacteriaceae, Halomonadaceae and Halobacteroidacea* are known to sustain themselves in very high salt concentrations. The halobacteriaceae and the Halomonadaceae are aerobic heterotrophs, many of which have a potential for anaerobic growth by means of denitrification. The *Halobacteroidacea* are anaerobic fermentative bacteria. Whereas the *Halobacteriaceae* and the *Halobacteroidacea* use the high-salt in strategy to survive in high salt concentrations, the *halomonadacea* use the biosynthesis of osmotic solutes. This also explains the fact that members of the halomonadaceae use aerobic respiration to provide for energy, since this is one of the only microbial processes that provides enough energy to produce the osmotic solutes needed to keep the NaCl out of the cell at very high salt concentrations. Although anaerobic fermentation does not provide that high levels of energy, it is still sufficient for the Halanaerobiales due to the high-salt in strategy being employed.

Metabolism vs. the indigenous nature of the obtained sequences.

It has been put forward by *Magot et al*, (2000) that only strict anaerobes can be considered as truly indigenous to oil reservoirs. Therefore, in low-salinity, low-temperature oil fields, the presence of aerobic bacteria is generally an indicator of contamination. However, concerning high-saline oil fields, this relation does not necessarily have to hold, due to the more extreme conditions in these environments. Additionally, our knowledge of the microbiology of oil fields is still insufficient for to exclude aerobic bacteria as exogenous contaminants of oil fields. All the organisms that were found were related to anaerobes or facultative anaerobes, with the exception of *H. volcanii.* However, Franzmann et al. reported positive anaerobic growth on nitrate for this organism (Franzmann, Stackebrandt et al. 1988). Members of the Halanaerobiales are found in many oil related environments, and due to their extreme NaCl-tolerance, and their ability to anaerobically ferment sugars present in the reservoir, these organisms can be marked as being truly indigenous to the reservoir. Organisms found belonging to the Halomonadaceae, although being aerobic, can also grow anaerobically by the process of denitrification (Arahal and Ventosa 2006) although it is expected that NO_3^- has long been depleted by bacterial activity. Members of the Halobacteriaceae use the high-salt in strategy, whereas members of the Halomonadaceae use the low-salt in strategy. The Halobacteriaceae therefore have to obtain less energy from metabolic processes than the halomonadaceae. Furthermore, a *Haloferax* sp. (*Haloferax* sp. D1227) was isolated that is able to utilize aromatic compounds as the sole carbon and energy source (Fu and Oriel, 1998). Combining the more extreme nature of the Rabi field, these organisms can also be justified as being indigenous to the reservoir.

Conclusions

The microbial diversity of the hypersaline Rabi petroleum reservoir (Gabon, Africa) was analyzed using PCR-DGGE. No major differences were found in the DGGE profiles indicating that the outer area of the core was not contaminated. Sequences belonged to the genera *Halanaerobium*, *Halomonas*, *Chromohalobacter, Orenia* and *Haloferax.*

The general absence of sequences related to SRB and IRB suggest that the hypersalinity of the Rabi Oil field excludes the presence of these microbial classes, based on their energy yield.

A set of nine described closest relatives was chosen based on their sequence alignment to serve as indicative microorganisms and as a control for a possible contamination. Growth ranges of these described relatives were compared related to temperature (°C) and salinity (% w/v). Also their metabolism was compared to that of the core ecosystem. According to the criteria set by Magot

et al in 2000, it was concluded that all the described species related to sequences found in this study should be able to sustain growth at the *in situ* temperatures of the Rabi oil field, which indicates that the species from which the sequences in this study were obtained most likely have similar physiologies and can be classified as indigenous to the core ecosystem.

- The closest relatives *H. volcanii, O. salinaria, H. praevalens* and *H. elongata* are able to live at salt-saturated conditions, and the closest relatives *H. salsuginis, H. acetoethylicum, C. israelensis, H. kushneri* and *H. halodenitrificans* are classified as halophiles (i.e. requiring NaCl for growth)
- The Halanaerobiales species (*O. salinaria, H. salsuginis, H. acetoethylicum, H. praevalens* and *H. kushneri*) which were closely related to the found sequences could therefore be classified as being indigenous to the reservoir, based on their high salt tolerance, their capability of anaerobic fermentation, and their indicated presence at high-saline oil fields.
- The microbes holding sequences related to species *C. israelensis, H. elongata and H. halodenitrificans* can possibly be classified as being indigenous to the reservoir, based on the high salt tolerance, and the ability to utilize the process of anaerobic denitrification as a source of energy.
- The relative *H. volcanii*, with no consensus on anaerobic growth, could possibly be classified as being indigenous, due to the extreme salt tolerance, the high-salt in strategy being employed, and the isolation of a *Haloferax* isolate utilizing aromatic carbon sources as the sole carbon and energy source.

With respect to the distribution of the closest described relatives, some of them were originally isolated from hyper-saline oil brines, showing similarity to the Rabi petroleum reservoir. Many were overall isolated from hypersaline environments.

It should be noted however that phenotypic information based on 16S rRNA gene comparison is only an indication and does not give a proof. Therefore, the sequences that are related to e.g. *H. volcanii* can still be indigenous to the reservoir since the organism itself might not be capable to display aerobic respiration. The same holds for the related species *C.israelensis, H.elongata and H. halodenitrificans.*

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5

Community analysis from non-hydrocarbon subsurface environments

Characterization of geochemical constituents and bacterial populations associated with As mobilization in deep and shallow tube wells in Bangladesh

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Characterization of geochemical constituents and bacterial populations associated with As mobilization in deep and shallow tube wells in Bangladesh.

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Abstract

While millions of people drink arsenic contaminated tube well water across Bangladesh, there is of recent no scientific explanation which is able to either comprehensively explain arsenic mobilization or to predict the spatial distribution of affected wells. Rather, mitigation strategies have focused on the sinking of deep tube wells into the currently arsenic free Pleistocene aquifer. In this study, Bangladesh shallow tube wells identified as contaminated and uncontaminated, as well as deep tube wells, were analyzed for geochemical and *in situ* microbiological composition. Whereas arsenic was detected in all Holocene aquifer wells, no arsenic was found in wells accessing the Pleistocene aquifer. Bacterial genera, including Comamonadaceae, *Acidovorax*, *Acinetobacter*, and *Hydrogenophaga*, associated with tolerance of high arsenic concentrations rather than dissimilatory Fe(III) or As(V), reduction were identified in shallow tube wells, indicating that mobilization may not occur at depth, but is rather due to drawdown of contaminated water. Deep tube wells contained microbes indicative of aerobic conditions, including the genera *Aquabacterium*, *Limnobacter*, and *Roseomonas*. It is concluded that through drawdown of arsenic or organic matter, further utilization of the Pleistocene aquifer could result in contamination similar to that observed in the Holocene aquifer.

Introduction

In order to combat the occurrence of diseases associated with the consumption of untreated surface water, shallow tube wells were and continue to be installed throughout Southeast Asia as a source of pathogen free drinking water (Caldwell et al. 2003). Owing to their low cost, minimal maintenance, and convenience, it is currently estimated that in Bangladesh over 97% of the rural population utilizes 6-11 million government funded and privately sunk wells (Yu, Harvey & Harvey, 2003); (Jakariya et al. 2007). However, elevated As concentrations have been found; it is estimated that over 100,000 people have developed skin lesions due to drinking As contaminated water and that, without mitigation, excess deaths of 3000 per year should be expected (Yu, Harvey & Harvey, 2003).

Arsenic occurs naturally in Bangladesh sediments due to weathering of arsenopyrite from the Himalayas and subsequent deposition by the Ganges-Brahmaputra-Meghna River system (Acharyya et al. 2000). Solid phase As is found coprecipitated in or coadsorbed on a number of minerals in concentrations consistently below 10 μg/g in both the Holocene and Pleistocene aquifers (Swartz et al. 2004); (Akai et al. 2004). In contrast, the concentration of dissolved As shows high depth variations and no clear pattern of spatial distribution (Swartz et al. 2004).

Several geochemical explanations for As release have been proposed (Acharyya et al. 1999); (Chowdhury et al. 1999). However, correlations between contamination and reducing conditions has led to the broadly accepted hypothesis that dissimilatory Fe oxyhydroxide reduction leads to the release of adsorbed and coprecipitated As (Nickson et al. 1998&2000); BGS and DPHE (2001); (McArthur et al. 2001); (Harvey et al. 2002); (Dowling, et al. 2002). Respiration on organic carbon present in the aquifer (McArthur et al. 2001&2004), or infiltrating from the surface (Harvey et al. 2002) leads to anoxic conditions, under which other electron acceptors, such as Fe and As, are employed.

Although it appears that microbial activity leads to reducing conditions associated with As mobilization, to date investigations of bacterial communities have focused only on analyzing population shifts in incubation experiments utilizing environmental samples from contaminated sediments in Southeast Asia. Anoxic incubations of sediments from Bangladesh and West Bengal, India with electron donors yielded increased aqueous As concentrations, and molecular analysis indicated a shift in the bacteria community towards the Fe(III)-reducers Geobacteraceae (Akai et al. 2004); (van Geen et al. 2004); (Islam et al. 2004). Similarly, the presence of organisms possessing the *arrA* gene encoding for As respiration has been confirmed by molecular analysis in incubation experiments with As contaminated Cambodian sediments amended with acetate (Lear et al. 2007).

Whereas previous research supports the biogeochemical basis for As mobilization, such incubation experiments fail to identify the responsible *in situ* microbial populations. Additionally, as Fe(II) concentrations do not show a consistent correlation with As in either field studies (Swartz et al. 2004); (Zheng et al. 2004 & 2005)) or incubation experiments (van Geen et al. (2004); (Islam et al. 2004); (Gault et al. 2005), it has yet to be concluded that Fe(III) reduction is responsible for As mobilization.

As a decisive explanation of and solution to shallow tube well contamination has yet to be presented, recent mitigation strategies have led to the sinking of tens of thousands of thus far uncontaminated deep tube wells (Ahmed et al. 2004 & 2006). It is assumed that the absence of organic carbon impedes microbial As mobilization in wells utilizing the oxic Pleistocene sediments, where increased weathering during the last glaciation has led to a higher concentration of iron oxides onto which As can absorb (McArthur et al. 2004). However, somewhat conflicting associations between Fe and As and assertions that organic carbon may not limit microbial mediated As mobilization argue against the aforementioned explanations (van Geen, et al. 2004).

Although dissimilatory Fe reduction may play a role in As mobilization, no theory as yet is able to predict the location of contaminated wells or explain their inconsistent distribution. Rather, it is clear that As mobilization is a complex interplay of microbial mediated reactions and geochemical processes sensitive to site specific hydrology and sediment composition. In contrast to previous microbiological investigations of microbial population shifts, this study intends to identify *in situ* bacteria communities and geochemical constituents associated with As mobilization. Through analysis of water samples obtained from contaminated and uncontaminated shallow tube wells at close proximity to one another, this work aims to investigate As mobilization in wells with similar sediment composition and hydrology. In addition to identifying bacteria populations and chemical compositions associated with elevated As concentrations, geochemical and microbiological analysis of the insufficiently investigated deep aquifer aims to provide confirmation of conditions under which contamination is not observed. The results presented in this chapter provide documentation of the chemical and microbial characteristics of water in As affected and unaffected wells in Bangladesh.

Materials & Methods

Site description and sample collection

Water samples were collected in April and May 2008 from deep tube wells (DTW) and shallow tube wells (STW) at five villages in Bangladesh: four locations in the Munshiganj district (samples DTW4, STW5, DTW6, STW7, DTW8, STW9, STW10, DTW11, STW12, STW13) and one location in the Jessore district (samples DTW1, STW2, STW3) (see Supplementary Data Table 1 for upzilla and village names). As this study did not include sediment sampling, information on lithology is gleaned from the literature. Swartz et al. and Polizzotto et al. provide a thorough core description for Munshiganj to a depth of 165 m (Swartz et al. 2004); (Polizzotto et al. 2006). Briefly, a 3.5 m thick clay layer covers the Holocene aquifer, which is composed of grey and greyish-green sands with interspersed silty-clay layers, to a depth of 119 m. The Holocene aquifer is separated from the Pleistocene aquifer, starting at 150 m depth, by 30 m of greenish clay. The As content in the solid phase is below 3 μg/g throughout the core (Swartz et al. 2004). Core analysis for Jessore district shows reducing sandy sediments to a depth of 61 m with muddy layers encountered at a depth of 3-9 m and in dispersed patches between 33 and 46 m (Akai et al. 2004). Solid phase As concentrations for the mud and sand layers are 7-16 μg/g and 2-5 μg/g, respectively.

Additionally, the British Geological Survey (BGS) has extensively investigated an area near Faridpur, which lies between the two sites investigated in this study (BGS and DPHE (2001). The upper aquifer from 15 m to 44 m is composed of grey sand deposits with wood fragments observed between 25 m and 44 m. A few meters of silty clay layers exists at 45 m, followed by grey sand and gravel deposits to a depth 134 m, the position of the sea level low stand of the last glaciation. Sediments from 134 m to 155 m are grey brown sands deposited prior to the last glaciation.

At each site, water was collected from a deep tube well (n=5, installed by the Arsenic Mitigation Research Foundation (AMRF)) and the nearest shallow tube well marked as As contaminated by red paint (n=5, within 20 m of each deep tube well). When available, water from a shallow tube well identified (with green paint) as having As concentration below the BDWS standard was also collected (n=3, two wells (STW4, STW13) were within 20 m of the deep tube well, a third (STW10) was 400 m away). After inquiring into age, depth, and usage, the tube well was flushed by pumping until electrode measurements steadied (temperature, conductivity, pH and oxidation-reduction potential). Water samples from each well were collected and filtered (0.2 μm filter) or acidified, following the procedure described below:

50 mL sample without headspace was collected for alkalinity analysis, which was performed within 10 hours (Hach Digital Titrator Test Kit);

3 HDPE screw cap bottles (Nalgene) were filled with 60 mL filtered sample, acidified to 1% (w/v) with suprapure HNO_3, and stored at 4 °C for trace metals and elements quantification;

5 mL filtered sample for dissolved organic carbon analysis (DOC) was collected in a glass tube and stored at -20 ºC;

3 filtered samples of 2 mL each were collected in HDPE microcentrifuge tubes, acidified to 1% (v/v) with suprapure HCl, and stored at -20 ºC for PO_4^{3-} and Si quantification;

3 filtered samples of 2 mL each were collected in HDPE microcentrifuge tubes without acid and stored at -20 ºC for NO_3^-, NO_2^-, and NH_4^+ quantification;

60 mL unfiltered sample was prepared for As speciation analysis as described previously (Karori et al. 2006) and stored at 4 ºC. Briefly, the sample was acidified to a final concentration of 0.01 M HAc and 0.5 mM EDTA, allowed to incubate for a few minutes, and then poured through a 10 mL chloride resin mini-column (Dowex 1X8). The final 40 mL of the sample from the column was analyzed for As concentration, which is reported here as As(III).

5 L sample collected in a polycarbonate jug was filtered by gravity within 10 hours through a 0.2 µm hollow fiber filter (Spectrum labs, mediakap-5 hollow fiber filter) to concentrate the biomass. The filter was wrapped in parafilm, placed in a 50 mL sterile tube, and stored at -20 ºC until use for DNA extraction.

Laboratory analytical methods

Samples were analyzed for dissolved constituents in May 2008 at Utrecht University Geolab (Faculty of Geosciences, Utrecht, The Netherlands) using conventional methods. Trace metals and elements, including total As and As speciation analysis, as well as Al, B, Ba, Ca, Fe, K, Li, Mg, Mn, Na, P, S, Si, and Sr were performed using inductively coupled-plasma optical emission spectroscopy (ICP-OES, Spectro CIROS CCD, Kleve, Germany). NH_4^+ and PO_4^{3-} were quantified by photometry at 660 nm and 880 nm, respectively (Bran and Luebbe autoanalyzer AA3, Europe). F, Cl, Br, NO_2^-, NO_3^-, and SO_4^{2-} concentrations were determined using ion chromatography (Dionex IonPac AS14, Benelux). DOC was measured by combustion on a TOC-5050A analyzer (Shimadzu,'s-Hertogenbosch, The Netherlands).

Molecular techniques

DNA extraction. DNA was extracted from the biomass collected on fiber filters at the University of Dhaka. Filters (n=13) were first thawed on ice and then cut open in a sterile environment over a sterile petri dish. Both the water retained within the filter as well as the filter fibers themselves were retained in sterile tubes. DNA extraction was performed on a mixture of approximately 1 mL liquid and ¼ of the filter fibers using a soil DNA extraction kit (MoBio Laboratories Inc, Carlsbad) according to the manufacturer's protocol. DNA was quantified on a Nanodrop 1000 Spectrophotometer (Thermo Scientific, The Netherlands).

PCR. Purified DNA was transported on ice to Delft University of Technology for further analysis. Partial 16S rRNA gene sequences were amplified using the universal bacterial primer pairs 341F+GC and 907R (Schäfer & Muyzer, 2001). A 50 μL PCR mixture containing Taq PCR master mix (Qiagen), DNA-RNA free water (Qiagen), 1 μM of each primer, and approximately 80 ng template DNA was run on a T1 thermocycler (Biometra, Goettingen, Germany) following a touchdown program (Schäfer & Muyzer, 2001). PCR product was checked on a 1.5% agarose gel run at 100 V for 45 minutes. PCR products were obtained for all wells except STW13.

Denaturing Gradient Gel Eletrophoresis (DGGE). DGGE was performed on the bacterial 16S rRNA fragment using a 1 mm thick, 6% polyacrylamide gel with a urea-formamide gradient of 20-80%, as described previously (Schäfer & Muyzer, 2001). Gels were run at 60 ºC, 100 V, 43 mA for 16 hours on a BIO RAD Dcode system, stained with Gel Green (Biotum, USA), and photographed using a blue light safe imager in a C-box doc system with accompanying Genesnap software (Syngene). Bands were cut using a sterile blade and incubated for 48 hours at 4 ºC in 15 μL 10 mM tris buffer (pH 8.5). The bands were reamplified using the solution as a template in the aforementioned PCR regimen and GC clamp free primers. Purification of 25 μL PCR product was performed using 1.6 μL Exo-Sap-IT (USB, Europe) according to the manufacturer's recommendations (30 minutes at 37 ºC, 15 minutes at 80 ºC). The final product was first checked for purity and concentration on a 1.5% agarose gel, diluted to 50 ng/μL and then sent away for commercial sequencing (Macrogen, Seoul, South Korea).

Clone libraries. Clone libraries for STW4, DTW5, and STW10 were constructed using pCR®4-TOPO cloning kit (Invitrogen), as described by the manufacturer. *E. coli* colonies, grown on kanamycin plates to select for vectors containing an insert, were picked (95 per sample) and reamplified using the universal M13 primer pair (Invitrogen). The product was purified, quantified, and sequenced as described above for DGGE products.

Sequence analysis. Sequences were screened for purity and chopped to remove primers. The resulting sequences were compared to those in GenBank using BLAST (Zhang et al. 2000);

www.ncbi.nlm.nih.gov/BLAST). The sequin program was used to submit sequences for accession numbers (www.ncbi.nlm.gov/sequin). DGGE bands have accession numbers FJ196237 to FJ196259 and FJ232946. Clones have accession numbers FJ204929 to FJ205138. Additionally, sequences were loaded into ARB software, which was used for alignment and in the creation of a phylogenetic tree utilizing the neighbour-joining algorithm (Ludwig et al. 2004); www.arb-home.de). Finally, clone library coverage was determined using webLIBSHUFF, which estimates within a 95% confidence level the similarity of two sets of sequences (Henriksen, 2004); http://libshuff.mib.uga.edu).

Results

Chemical composition of wells

To assess the geochemical conditions in shallow and deep tube wells, water samples were analyzed for chemical constituents. All tube wells accessing the Holocene aquifer contained As concentrations above the BDWS of 50 μg/L, including those marked as being within safe limits during the testing campaign from 1999-2005. As concentrations were between 72 and 432 μg/L. (For the entire geochemical data set, see Supplementary Data, Table 1.) Dissolved As existed predominantly in the reduced species, As(III), making up on average 80% of the total concentration. Such partitioning between As(V) and As(III) species is consistent across all shallow tube wells (R^2=0.836 and 0.988, respectively; Fig. 1). As was not found in any deep tube well.

The concentrations of dissolved ions indicate a difference in geochemical conditions between shallow and deep tube wells (Fig. 2). Higher average NH_4^+ and PO_4^{3-} concentrations are found in shallow tube wells and show a correlation to As (R^2=0.709 and 0.558, respectively; Fig. 2 and 3). When the three data points from the geologically dissimilar Jessore are removed, this linear relationship is significantly more robust, with R^2=0.964 for NH_4^+ and 0.706 for PO_4^{3-}. NO_3^- was identified in DTW11, but was absent in all STW. Over ten times more dissolved Fe is found in samples from the Holocene aquifer compared to the Pleistocene aquifer (Fig. 2); however, no correlation with As is found (R^2=0.302). The higher concentrations of constituents associated with reducing conditions and microbial activity may be related to higher DOC concentrations observed in shallow tube wells, 6.40 mg C/L, as opposed to deep tube wells, 1.96 mg C/L (Fig. 2). DOC measurements for Munshiganj are overall lower but of the same order of magnitude as those reported by Swartz et al. (2004). However, no correlation between organic carbon content and As concentrations is observed in shallow tube wells (R^2=0.026).

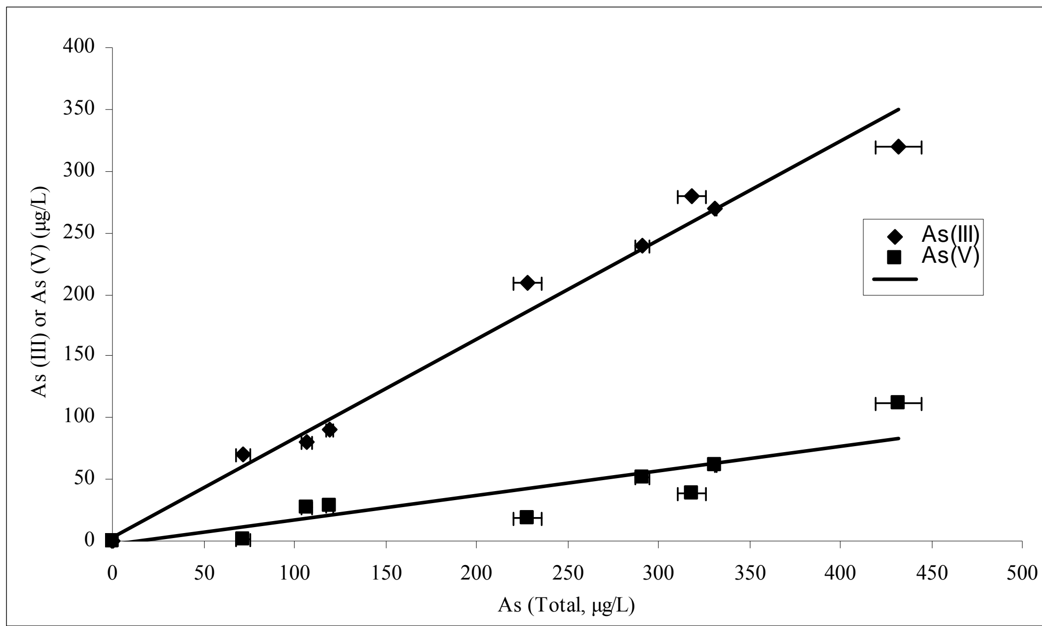

Figure 1: *As(III) (♦) and As(V) (■) versus total As. Error bars, for total As only, are one standard deviation.* R^2*=0.988 for As(III) and* R^2*=0.836 for As(V).*

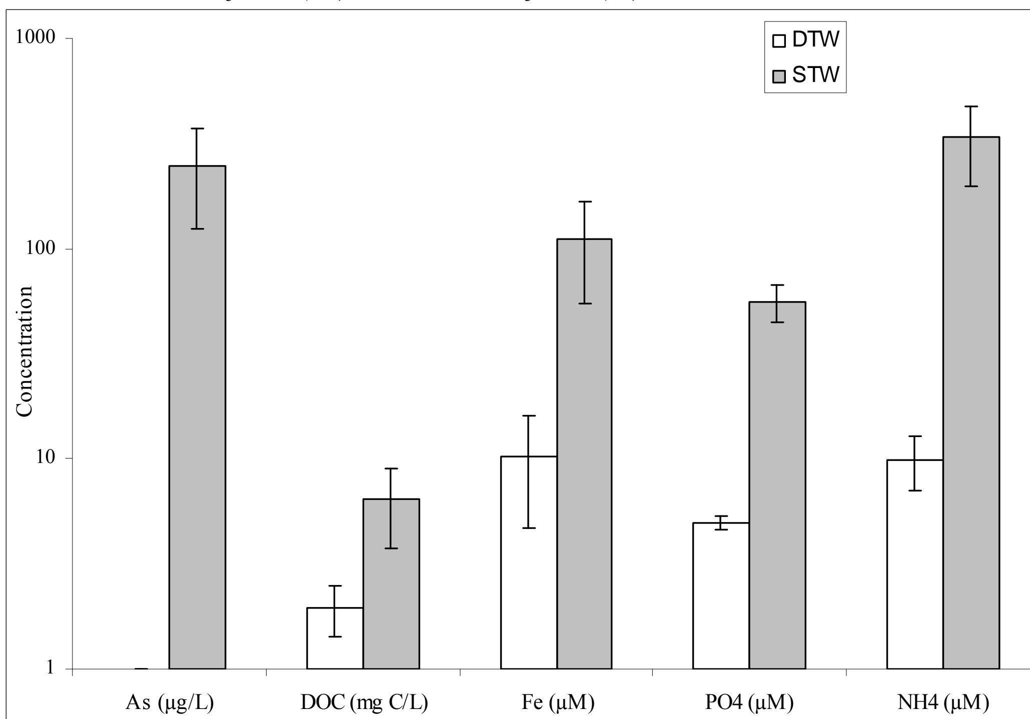

Figure 2: *Average dissolved constituents for deep tube wells (white) and shallow tube wells (grey). Note that the Y axis is logarithmic. Error bars are one standard deviation.*

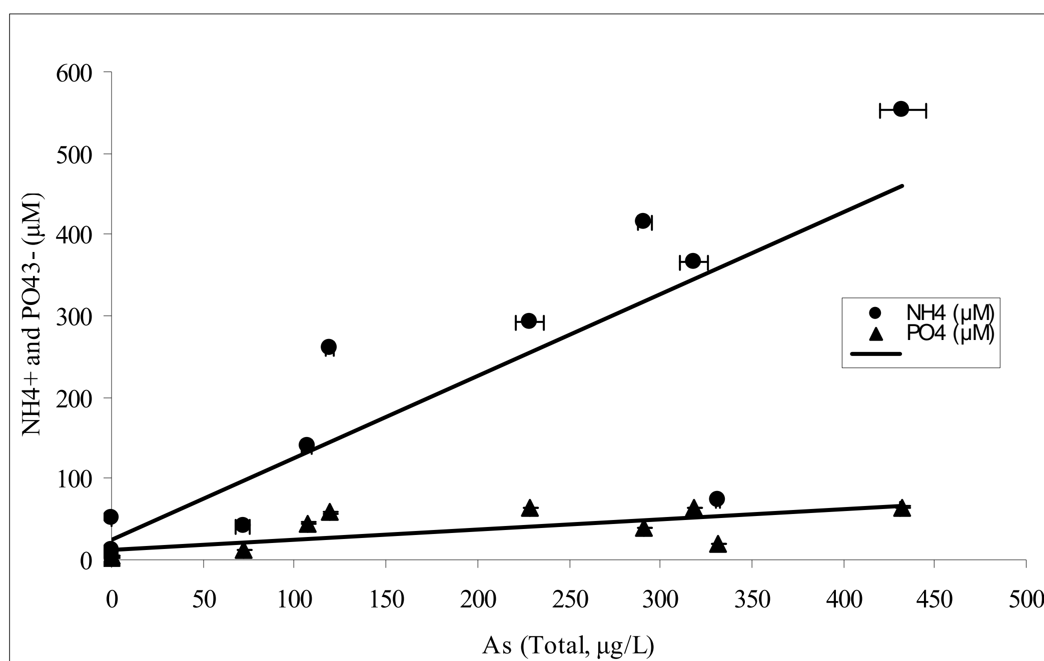

Figure 3: *NH_4^+ (●) and PO_4^{3-} (▲) versus As concentration. Error bars are one standard deviation. R^2=0.706 for NH_4^+ and R^2=0.558 for PO_4^{3-}.*

Microbial population analysis

To assess the bacterial diversity present in deep and shallow tube wells, 16S rDNA, concentrated and purified from water samples, was used for DGGE and in the creation of clone libraries. DGGE yielded 24 sequences (from 30 bands), of which 16 came from shallow tube wells and 8 deep tube wells (Fig. 4). Of these sequences, 84% are Betaproteobacteria (Table 1). The remaining 16% are made up of Bacteroidetes (1 sequence), Alphaproteobacteria (1 sequence), and Gammaproteobacteria (2 sequences). Similar distributions were observed in clone libraries of DTW4, STW5, and STW10 (Table 1). Overall 75% of the 209 clones were Betaproteobacteria. Shallow tube wells had higher diversity than deep tube wells, as observed both in the number of DGGE bands and in the clone library diversity (Fig. 4, Table 1). Of the 79 clones for DTW4, 92% were Betaproteobacteria*,* in contrast to STW5 and STW10, in which around two-thirds of clones were of this class. An exception is observed in STW7, where high As concentrations (430 µg/L) were found. Deep tube wells show a limited microbial population (DTW1, DTW6, DTW8, DTW11), with DTW4 as a notable exception.

In addition to differences in diversity, the composition of clone libraries for shallow tube wells is statistically different than that of the deep tube well. Separate comparison of sequences from DTW4 with those from STW5 and STW10 produces p values of 0.001 for both XY and YX comparison, indicating that the composition is significantly different within a 95% confidence

interval (webLIBSHUFF). In contrast, the shallow tube well clone libraries are more similar, producing p values of 0.001 for YX comparison, but 0.043 for XY comparison.

The phylogenetic tree of sequences found in shallow tube wells in DGGE and clone library analysis shows similarity to species identified in previous incubation studies (Gault et al. 2005); (Lear et al. 2007), found to sustain growth in As contaminated environments, and in some cases arsenite and Fe(II) oxidation (Fig. 5 and 6). DGGE bands are indicated by the well name (i.e. STW5) and the band letter (i.e. a) (Fig. 4). Species of the family Comamonadaceae, found in STW5_a (FJ196255) and STW9_a (FJ196237), were prominent in the microbial populations both in the *in situ* and As(V) amended incubation experiments with As containing sediments from Cambodia (Lear et al. 2007)); and arsenate resistance has been noted (Ma et al. 2007). STW5_c (FJ232946) and STW10 clone library contained sequences showing similarity to *Acidovorax*, was also found by Lear et al. (2007) to be prominent both before and after As(V) amendment and is known to be highly arsenite resistant. The two adjacent sequences found in STW12_a and STW12_b (FJ196245, FJ196246) show >99% sequence identity to *Acinetobacter junii* and *A. baumannii*, which were also observed in the STW10 clone library, and which have been identified in West Bengal sediment incubations (Gault et al. 2005). Arsenite oxidase activity has been confirmed for this genus (Fan et al. 2008). Similarly, a sequence found in STW2_b (FJ196249) showed >97% similarity to *Hydrogenophaga*, which has been identified in arsenic oxidizing biofilms (Salmassi et al. 2006) and can oxidize arsenite, however only under oxic conditions (van den Hoven & Santini, 2004). Whereas no known Fe(III) reducers were identified, sequences showing >97% similarity to denitrifying Fe(II) oxidizing bacteria were identified in STW2_d (FJ196251), STW5_b (FJ196256), and STW9_b and STW9_f (FJ196238, FJ196241) (Straub et al. 2004). These NO_3^- reducing bacteria allow anoxic Fe cycling in fresh water sediments. However, in this study, ferric iron reducers were not found in As contaminated sediments.

DGGE and clone library analysis from deep tube well bacterial DNA was dominated by sequences showing similarity to *Aquabacterium* (Table 1). Specifically *Aquabacterium hongkongensis*, identified in DTW4_a (FJ196253), DTW8_a (FJ196259), and DTW11_a (FJ196243), was especially prevalent (Fig. 6). Additionally, 39 of the 79 clones in the DTW4 clone library were of this genus. *Aquabacterium*, also associated with denitrifying Fe(II) oxidizing sediments, are facultative aerobes (Straub et al. 2004). Sequences showing similarity to *Limnobacter* (DTW1_a, FJ196247) and *Roseomonas* (DTW6_a, FJ196257), were also identified in fresh water lake sediments (Spring, Kampfer & Schleifer (2001), Jiang et al. 2006) and are obligate aerobes (Gallego et al. 2006).

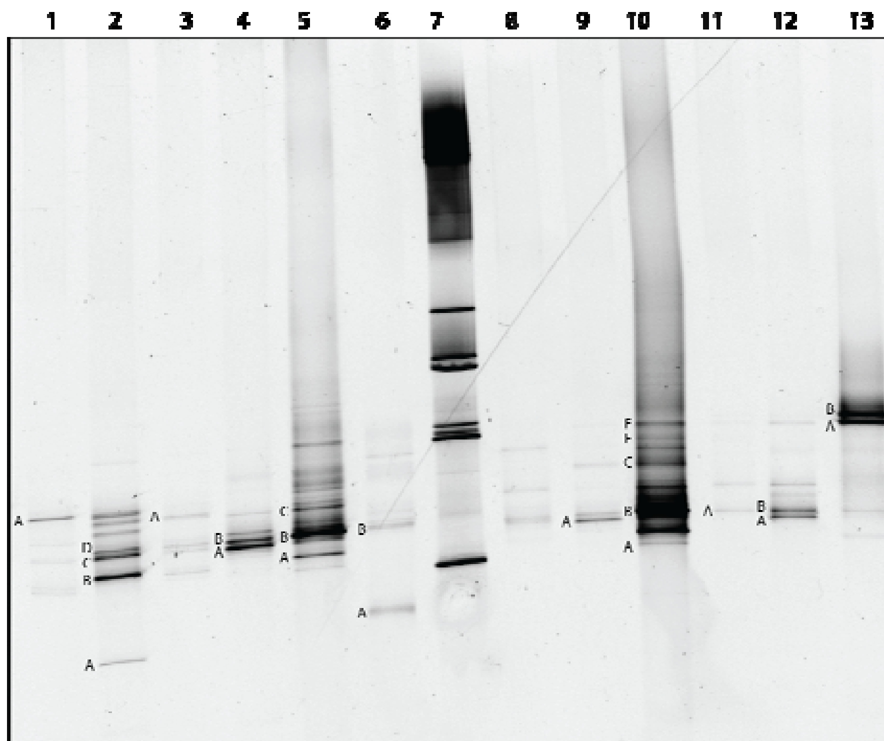

Figure 4: *DGGE of bacterial 16S rRNA gene fragments from deep and shallow tube wells, labelled DTW and STW, respectively. Bands are labelled with a letter, which is used for reference in the text and Figures 5 and 6. (+) denotes wells marked as being above the BDWS, (-) were marked as uncontaminated. Lane 1, DTW1; Lane 2, STW2 (+); Lane 3, STW3 (-); Lane 4, DTW4; Lane 5, STW5 (+); Lane 6, DTW6; Lane 7, Ladder; Lane 8, STW7 (+); Lane 9, DTW8; Lane 10, STW9 (+); Lane 11, STW10 (-); Lane 12, DTW11; Lane 13, STW12 (+).*

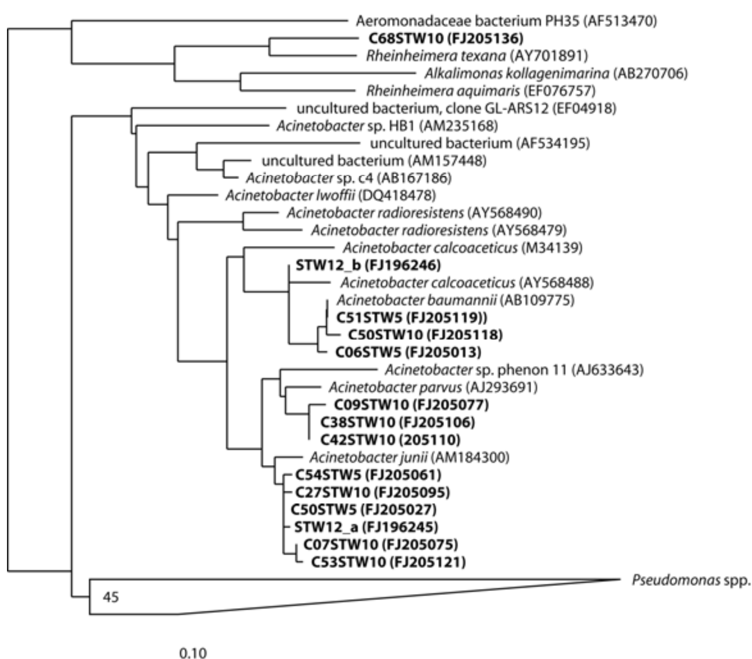

Figure 5: *Phylogenetic tree based on 16S rRNA sequences of the gammaproteobacteria. Sequences determined in this study are in boldface. Sequence accession numbers are shown in parentheses. White triangles indicate compressed sections of the tree, with the ratio of sequences from this study to total sequences indicated. DGGE sequences are identified by the well name (STW12) and band letter (a). Clone library sequences are identified by the clone number (C68) and location (STW10).*

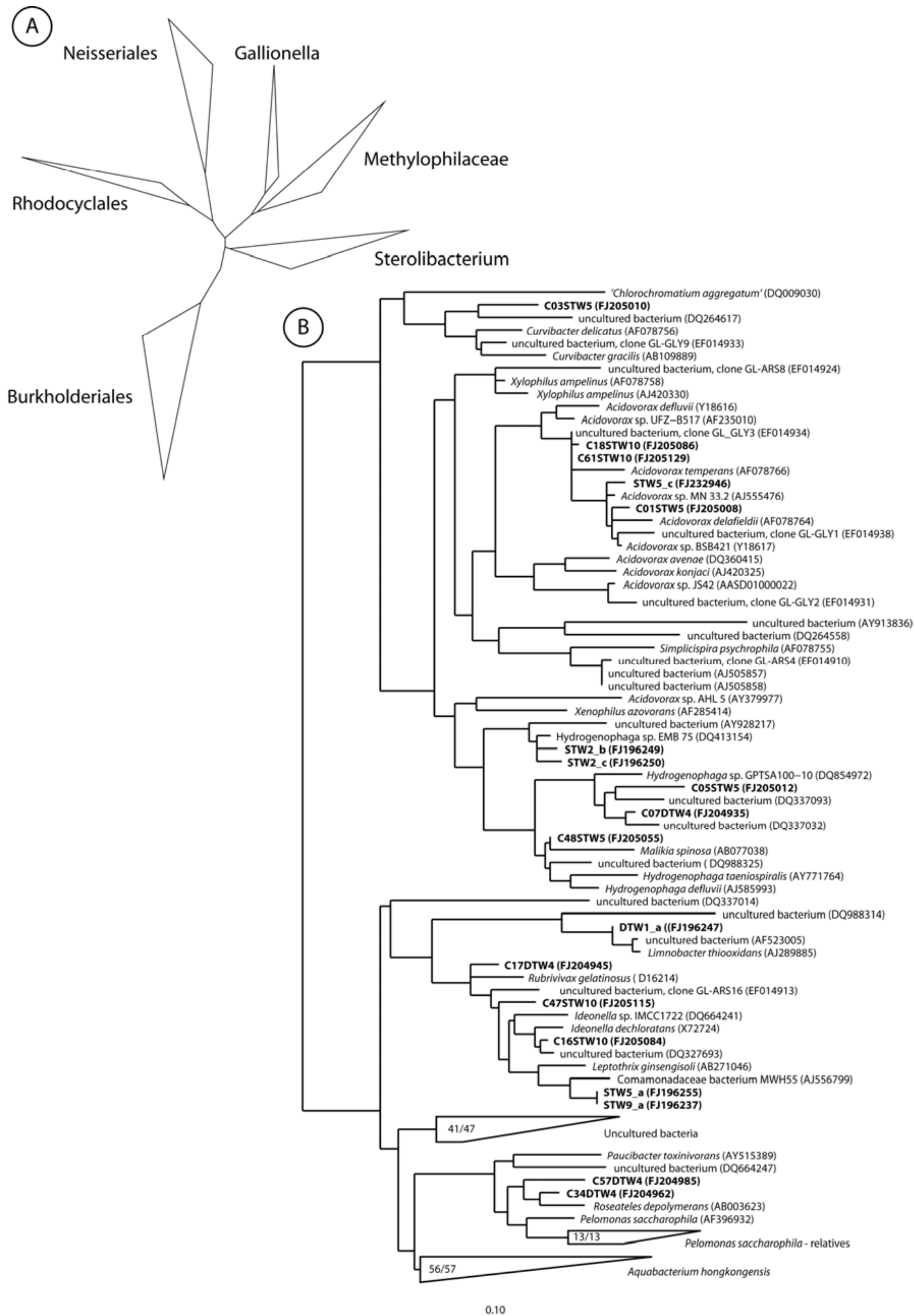

Figure 6: *(A) Phylogenetic grouping based on 16S rRNA sequences of the Betaproteobacteria. (B) Expanded phylogenetic tree of sequences from the order Burkholderiales. Sequences determined in this study are in boldface. Sequence accession numbers are shown in parentheses. Triangles indicate compressed sections of the tree, with the ratio of sequences from this study to total sequences indicated. DGGE sequences are identified by the well name (STW12) and band letter (a). Clone library sequences by their clone number (C68) and location (STW10).*

Discussion

Indications of reducing conditions due to microbial activity, as seen in higher PO_4^{3-}, NH_4^+, and Fe concentrations and the absence of NO_3^- , were markedly more prominent in shallow tube wells as compared to deep tube wells. The higher DOC levels observed in the Holocene aquifer most likely support the increased bacterial diversity observed and associated higher As concentrations. STW7 shows limited diversity, which may be due to the exceptionally high As concentrations found here (431 μg/g). In contrast, low DOC concentrations are found in deep tube wells, which in contrast to previous investigations of wells accessing the Pleistocene aquifer, did not contain As (Swartz et al. 2004) and showed a reduced assortment of species. More bands are observed in DTW4; however, as this well was installed a week before sampling, contamination during the drilling process may have instigated additional bacterial growth. These results support the role of prokaryotes in the creation of reducing conditions associated with As mobilization in the shallow subsurface.

Unfortunately, the shallow tube wells marked as uncontaminated that were sampled in this study contained As concentrations above the BDWS. Although this does reinforce the importance of continued and accurate test campaigns, we are unable to compare microbial populations in shallow tube wells with and without As under similar geological and hydrological conditions.

Shallow tube well bacterial populations indicative for As tolerance, not mobilization

Analysis of microbial diversity from DGGE and clone libraries did not produce any sequences associated with Fe or As reduction, which questions the role of dissimilatory iron or arsenate reduction in As mobilization. Additional analysis targeting the functional genes for As and Fe reduction in both the water and sediment bound bacterial communities is required to make this statement more robust. However, as previous work did not successfully produce PCR amplification of the *arrA* gene for As reduction in native sediment samples (Lear et al. 2007), our study focused specifically on the *in situ* microbial diversity.

The bacterial community identified in the shallow tube wells studied share similarity to those identified previously in As-contaminated sediments (Gault et al. 2005); (Lear et al. 2007); (Anderson & Cook, 2004); however their prominence is more likely due to As resistance than function. Comamonadaceae, identified here in STW5 and STW9 with As concentrations of 318 and 107 μg/L, respectively, and *Acidovorax*, seen in STW5 and STW10 (119 μg L^{-1}), were also documented to dominate the microbial population in contaminated Cambodian sediments (Lear et al. 2007). Upon amendment with acetate and 10 mM arsenate (740 mg L^{-1} As), the proportion of these species relative to the total population increased, indicative of either As resistance or

utilization. Considering that the *arrA* gene for As reduction was not found for this genus in work by Lear et al. (2007), the role of Comamonadaceae in As mobilization is unclear. Additionally, although *Acidovorax* does possess the *arsC* arsenate reductase gene, this functions as a form of As resistance rather than for dissimilatory growth. Whereas activation of this resistance pathway could cause some As mobilization, it is expected that the relative yield of reduced As is small compared to that produced by dissimilatory growth. Similarly, *Acinetobacter* identified here in STW5, STW10 and STW12, the latter containing 229 μg L^{-1} As, was found in previous work with Bengal delta sediments (Gault et al. 2005) and at an As contaminated site in New Zealand (Anderson & Cook, 2004)). This genus shows exceptional As tolerance, able to sustain growth in the presence of 320 mM As(V) and 14 mM As(III), several orders of magnitude higher than the concentrations observed in this study (Achour, Bauda & Billard, 2007). Although the species *A. calcoaceticus* is able to mobilize As on copper arsenate-treated timber, this has only been observed under aerobic conditions (Clausen, 2000).

Sequences showing similarity to bacteria able to oxidize arsenite were also observed. Microbial As oxidation has been suggested as a mechanism in household filters for removal of As (Berg et al. 2006). *Acinetobacter* has been shown capable of As oxidation (Fan et al. 2008); however, the As resistance genes *ars*R and *ars*H are indicative of a survival strategy rather than a means of chemotrophic growth (Fournier et al. 2006). Species within the genus *Hydrogenophaga*, identified in STW2 with an As concentration of 332 μg L^{-1}, have been found in association with As oxidizing biofilms (Salmassi et al. 2006).

In contrast to the commonly held theory that dissimilatory Fe reduction is responsible for As mobilization, only sequences similar to Fe(II) oxidizing bacteria (STW2, STW5, STW9) were found in this study (Straub et al. 2004). Such organisms could explain As mobilization in conjunction with anoxic Fe cycling. Under this scenario, As associated with Fe(II) biominerals produced through microbial activity (Islam et al. 2005) would be mobilized upon the structural changes associated with ferrous oxidation. Inconsistent correlations between Fe and As concentrations could be explained by the limited accessibility of Fe(II) atoms on the surface of the biominerals to microbial oxidation. However, in view of the fact that no Fe(III) reducers were found in this study, in contrast to the experiments with *Geobacter* and *Geothrix* where As affinity for Fe(II) biominerals was observed, this explanation is speculative.

Deep tube well microbial diversity indicative of oxic conditions and low DOC

Bacteria identified with DGGE in wells accessing the Pleistocene aquifer were indicative of oxic or suboxic conditions. Sequences showed >97% similarity to *Limnobacter* and *Roseomonas*, both obligate aerobes, and *Aquabacterium*, a facultative aerobe (Straub et al. 2004); (Spring, Kampfer & Schleifer, 2001); (Gallego et al. 2006). This finding supports previous geochemical work indicating that the Pleistocene is oxic. Zheng et al. found dissolved oxygen concentration up to 7 mg L^{-1}; and at 274 m, the depth analyzed here, 0.8 mg L^{-1} was detected (Zheng et al. 2004). As their investigation was performed on the uplifted Pleistocene Madhupur terrace, which would have been more exposed during the last glaciation, it is not surprising that in this study less oxic conditions were observed. Rather, the presence of *Aquabacterium*, which is a facultative aerobe able to use oxygen or nitrate as electron acceptors (Kalmbach et al. 1999), is consistent with by the suboxic conditions found in Munshiganj Pleistocene sediments (Swartz et al. 2004).

The low concentration of organic matter found in this study prevents microbial respiration and the onset of reducing conditions. Although unable to grow autotrophically, *Limnobacter* (DTW1) is able to grow on very low organic carbon concentrations (Spring, Kampfer & Schleifer, 2001). Notably, this aerobe was only found in the deep tube well in Jessore with the lowest DOC concentration of this study (0.91 mg C/L). Pleistocene aquifer organic matter abundance in Jessore, an area which also may have undergone additional weathering during the low sea levels of the last glaciation, agrees with the Zheng et al. estimate of 1% for a similarly oxidized area (Zheng et al. 2004).

Theories of As mobilization

The absence of previously characterized Fe(III) or As(V) reducers in DGGE analysis does not preclude their presence in small numbers or role in As mobilization. However, this observation, in conjunction with the inconsistent community distributions seen across contaminated wells, leads us to conclude that As mobilization may not occur within the wells. Rather, as suggested by Polizzotto et al., reducing conditions caused by microbial activity in conjunction with redox cycling at the surface mobilizes As via chemical or biological processes, which is subsequently drawn down to well depth by pumping (Polizzotto et al. 2006). This conclusion is supported by the observed inverse relationship between well depth and As concentration (R^2=0.520, only shallow tube wells).

Well contamination due to recharge with water containing dissolved As explains a number of inconsistencies in this study. If mobilization occurs at the surface, the concentration of As in the well is not dependent upon the availability of DOC at that depth, as observed here (R^2=0.026 for

DOC vs. As correlation). Preferential usage and thus increased recharge at wells previously identified as being within BDWS limits would explain why this study found such wells to be contaminated. Additionally, the recent or sudden influx of As would initially reduce microbial diversity in these wells, as observed here, until a new community of As resistant bacteria forms.

Deep tube well sustainability

The lack of investigations to similar depths as the deep tube wells examined here makes predictions of As contamination in wells accessing the Pleistocene aquifer challenging. Core analysis in the Munshiganj district is only to a depth of 165 m, whereas this study investigates 240 m deep wells (Swartz et al. 2004). Although this study indicates that current conditions are not favorable to As mobilization (oxic or suboxic with limited DOC), water extraction could change this. As theorized for the Holocene aquifer (Polizzotto et al. 2006), and observed in the Hanoi, Vietnam Pleistocene aquifer exploited for municipal drinking water (Berg et al. 2008), downward recharge could cause contamination in the deep tube wells studied here.

Whether drawdown could transport As or promote microbial activity through the introduction of DOC is a question of lithology and geology. Although a 30 m clay layer has been identified in the Munshiganj district (Swartz et al. 2004), similar confining layers were not identified in Jessore (Akai et al. 2004) or Faridpur (BGS and DPHE, 2001). The absence of dissolved As in the Pleistocene has been attributed to the absence of electron donors and the abundance of Fe(III) oxides available to adsorb As (Polizzotto et al. 2006); (Stollenwerk et al. 2007). However, adsorption and modeling experiments utilizing sediments collected near Dhaka indicate that the solid phase buffer capacity is highly dependent upon the depth of the well screen relative to the confining layer, local lithology, and extraction practices (Stollenwerk et al. 2007). Considering that tens of thousands of deep tube wells have already been sunk without scientific investigation into the geological and hydrological constraints of the Pleistocene aquifer, future research must focus on illuminating the processes responsible for As contamination and determining the sustainability of deep tube wells in order to circumvent tragedies similar to those seen with Southeast Asian shallow tube wells.

Conclusions

In an attempt to illuminate the geochemical and biological conditions that lead to arsenic mobilization, water was analyzed from deep tube wells and shallow tube wells labelled as contaminated (red) and uncontaminated (green) during previous testing campaigns. Major chemical constituents were quantified and the bacterial community was analyzed using DGGE and clone libraries. The following conclusions were made:

Although labelled otherwise, all shallow tube wells had arsenic concentrations above the BDWS of 50 μg L^{-1}, indicating the need for continuous testing.

No arsenic was found in any of the 5 deep tube wells tested. The bacteria species identified were indicative of aerobic conditions and included members of the genera *Aquabacterium*, *Limnobacter*, and *Roseomonas*.

The microbial populations of shallow tube wells were dominated by species associated with arsenic tolerance and observed in previous investigations of arsenic contaminated environments, including Comamonadaceae, *Acidovorax*, *Acinetobacter*, and *Hydrogenophaga*. No known dissimilatory Fe(III) or As(V) reducers were identified.

Results including that (1) no bacteria responsible for arsenic mobilization were identified, (2) the observed inverse correlation between well depth and arsenic concentration, and (3) no relationship between DOC and arsenic was seen, speaks in favour of the theory of Polizzotto et al., that contamination of shallow tube wells is due to drawdown of As enriched surface water (Polizzotto et al. 2006). Therefore, further research should investigate the sustainability of deep tube wells for extraction of drinking and irrigation water.

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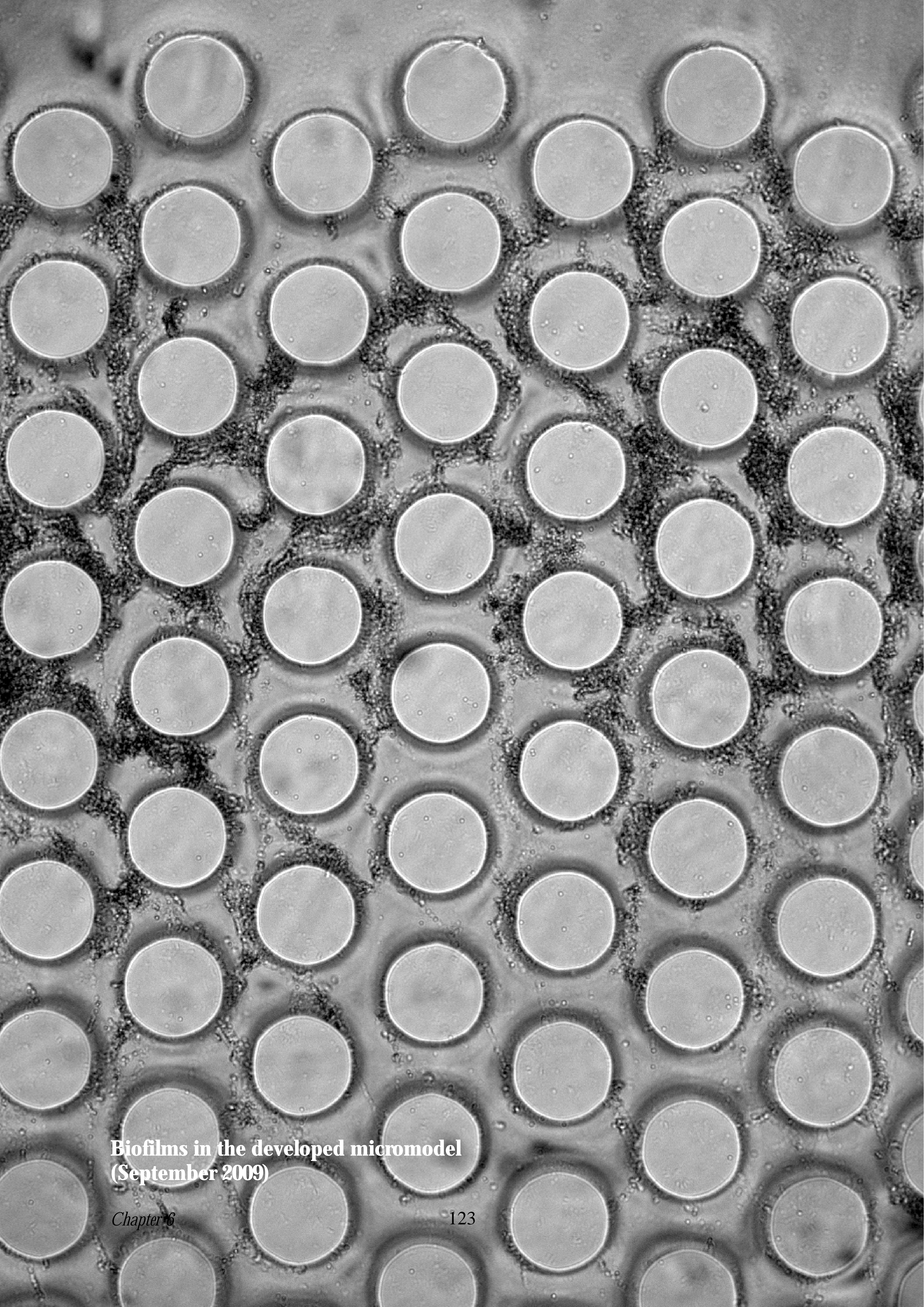
Supplemental Data

Table S1. Summary of chemical analysis for all wells tested. At each location a deep tube well (DTW) and the nearest shallow tube well marked as contaminated (STW (+)) was sampled. When available, water from shallow tube wells marked as having As concentrations below the BDWS (STW (-)) was also collected.

Sample		DTW 1	STW2 (+)	STW3 (-)		DTW4	STW5 (+)	DTW6	STW7 (+)	DTW8	STW9 (+)	STW10 (-)	DTW11	STW12 (+)	STW13 (-)
Location: District Upzilla (Area) Village	**Jessore:**	Jhikorgacha (North Mothbari) Godkhali			**Munshiganj:**	Lohajang Masadgau		Lohajang Kumarbhong		Srinagar (Balashur) Tetamara			Lohajong (Kanakshar) Singerati		
Well Depth (m)		240	37.5	22.5		210	21	210	37.5	210	75	60	240	30	27
Well age (yr)		0.5	16	6		0	10	2	10	0.5	2	2	1	1	4
Total As (µg/L)		0	332	72		0	318	0	432	0	107	119	0	229	291
As(III) (µg/L)		0	270	70		0	280	0	320	0	80	90	0	210	240
As(V) (µg/L)		0	62	2		0	38	0	112	0	27	29	0	19	51
pH		7.79	7.02	7.03		7.18	7.10	7.05	6.96	7.14	6.70	6.86	7.23	7.11	7.06
EC (uS)		694	723	721		512	844	514	727	503	541	571	515	551	597
ORP (mV)		-93	-41	-46		-51	-45	-46	-42	-53	-26	-36	-62	-29	-48
Temp. (°C)		26.5	27.5	27.5		28.5	28.5	32	28.5	30	28.5	29	29.5	28.5	28.5
Alkalinity (meq/L)		3.12	3.52	3.28		1.56	3.46	1.82	2.78	2.08	2.12	2.44	2.02	1.90	2.34
DOC (mgC/L)		0.91	1.56	6.97		2.193	5.896	2.277	9.399	2.209	24.030	8.675	1.173	4.963	3.076

Table S1. (continued)

Sample		DTW 1	STW2 (+)	STW3 (-)		DTW4	STW5 (+)		DTW6	STW7 (+)		DTW8	STW9 (+)	STW10 (-)		DTW11	STW12 (+)	STW13 (-)
Location: District Upzilla (Area) Village	**Jessore:**	Jhikorgacha (North Mothbari) Godkhali			**Munshiganj:**	Lohajang Masadgau			Lohajang Kumarbhong			Srinagar (Balashur) Tetamara				Lohajong (Kanakshar) Singerati		
Ca (mM)		0.88	2.50	2.88		0.75	1.64		0.85	2.10		0.77	1.19	1.23		0.68	0.93	1.45
Cl (mM)		0.62	0.40	0.26		0.61	0.41		1.01	0.47		0.31	0.13	0.13		0.64	0.27	0.33
Fe (μM)		1	71	77		14	72		16	154		5	196	92		6	39	117
K (mM)		0.11	0.06	0.16		0.08	0.19		0.09	0.14		0.08	0.09	0.13		0.41	0.15	0.14
Mg (mM)		0.93	0.91	0.74		0.68	1.44		0.74	0.77		0.63	1.00	1.24		0.55	1.08	0.97
Mn (μM)		0.50	2.28	2.84		0.62	0.83		0.75	12.70		3.93	3.33	2.99		3.03	0.47	3.73
Na (mM)		3.49	0.52	0.33		2.01	2.54		2.02	0.73		2.19	0.64	0.67		2.24	1.02	0.53
Total P (μM)		1.35	18.16	13.14		5.12	62.98		4.05	64.67		4.65	42.97	58.06		5.00	63.07	38.89
PO_4^{3-} (μM)		1.54	19.00	12.35		5.32	63.90		4.43	65.05		4.92	45.26	57.93		5.10	64.02	39.27
SO_4^{2-} (μM)		0	0	0		18.32	0		11.35	0		8.02	0	2.29		19.26	16.97	60.17
Si (mM)		0.53	0.66	0.62		1.07	0.86		1.08	0.79		0.88	1.30	1.04		0.75	0.81	0.73
NO_3^- (μM)		0	0	0		0	0		0	0		0	0	0		1.77	0	0
NO_2^- (μM)		0	0	0		0	0		0	0		0	0	0		0	0	0
NH_4^+ (μM)		52	73	42		10	366		11	552		13	141	262		6	292	415



Biofilms in the developed micromodel (September 2009)

6

Development and usage of an etched transparent flowcell for visualisation of biofilm induced heterogeneities in porous media using Particle Image Velocimetry.

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Abstract

Flow cells (or 'micromodels') are widely used to observe complex processes in two dimensional (2D) porous media. The objective of this study is to design, develop, and apply a wet-etched glass micromodel suitable for the observation of biofilm formation, transport of microorganisms and flow measurements using Particle Image Velocimetry (PIV). PIV in this case can be used for the visualisation and quantification of flow diversion. The wet-etching technique applied here is relatively straightforward. The chosen etching depth of around 25 μm proved to be well suited for transport experiments with microorganisms. We show that the micromodel containing a wet-etched cell (wafer) provides a high quality image of transport and growth of microorganisms in porous media. The microorganism *Pseudomonas chlororaphis* was used as a model strain to perform the experiments on biofilm formation and transport. In the transparent cell, we could clearly observe biofilm formation. Moreover, we showed that PIV techniques can indeed visualize the change in flow pattern caused by biofilm presence.

Introduction

Biofilm formation and transport of microorganisms play an important role in numerous biological-mediated processes in the subsurface. Much of the research regarding transport of microorganisms is done for safe drinking water and bioremediation (Murphy, 2000). There is renewed interest in biological aspects in the petroleum industry; not only for Microbial Enhanced Oil Recovery (MEOR) applications (Bryant, 2002), but also to reduce H_2S emissions or for the analysis of microorganisms found in wells as possible additional information source for reservoir performance (Pronk, 2009), (van der Kraan et al. 2010). As opposed to more conventional environments, oil reservoirs have more extreme conditions like high temperatures and salinities, but still hold diverse microbial communities. Transport of microorganisms and biofilm formation in the subsurface are important aspects if microorganisms are to be used in MEOR applications or as information carriers from oil reservoirs (Foppen & Schijven, 2005), (Zandvliet, 2008), a process commonly known as biomonitoring (Röling, 2002). In these environments microorganisms can occur in many forms; examples are: suspended cells, aggregates, or biofilms. Biofilms offer a protective environment for the bacteria to e.g. oxygen stress or biocides (Tolker-Nielsen & Molin, 2000). Important for all of the mentioned applications are the attachment and detachment processes of micro-organisms and the formation of biofilm structures in porous media. Examples of processes that trigger biofilm formation are, biobridging (formation of chains of microorganisms that stretch from sand grain to sand grain or in our case from pillar to pillar), clogging of pores by released pieces of biofilm and adsorption due to interaction of bacteria with the pore-wall, (Rijnaarts, 1999), (van Loosdrecht et al. 1990). It is asserted that these mechanisms can be visualized in “two dimensional” transparent flow cells (also known as micromodels). Visualization is an indispensable tool for a better understanding of the phenomena associated with the presence of biofilm and its effect on flow properties and vice versa (see references and text below).

Chatenever and Calhoun (1952) were the first to use a visual approach for the study of fluid flow in porous media. They used micromodels in which a single layer of glass beads was placed between two flat glass plates. Mattax and Kyte (1961), were the first to use glass etching in order to create networks. They used the then innovative etching techniques for the construction of a micromodel. In their study, a wax covered glass plate in which a pattern was drawn was etched chemically with hydrofluoric acid. Their purpose was to study multiphase flow in porous media. Davis and Jones (1968) superseded the wax with photo resist, thus introducing photo etching techniques, which greatly improved the versatility of this approach.

Micromodels differ from an ideal 2D shape, which is not always explicitly stated in micromodel studies. Chemical (wet) etching, as described in this manuscript expands spherically from the points exposed to the etching agent. Consequently the pillars in between, which constitute the porous skeleton, obtain a bell shape. The spherical expansion in combination with sufficiently deep pores to prevent spurious attachment to bottom and top plate excludes the creation of pores with a high aspect ratio. Therefore, the depth of the micromodel must exceed the diameter of the microorganism several times (in our case in the order of 10 μm). Consequently the width of the pores in the micromodel equals roughly twice the depth of the pores. Wet-etched micromodel pores are therefore larger than pores in a real porous medium. The features described above may play an important role in the interpretation of the microbial trapping mechanisms and are therefore mentioned.

A number of studies has been devoted to the study of microbes in transparent micromodels. Paulsen and Oppen used glass micromodels to visualize oil degradation and mobilisation with pore throats ranging from 120-600 microns and a depth of 200 micron (Paulsen, 1999). The microorganisms used were obtained from a marine oil-waste biotreatment plant. Steward and Fogler (2001) studied the formation of pore plugs in porous media by applying exopolymer producing Bacteria. They used the technique developed by Wan et al. to create a micromodel with a triangular pore structure arranged in a hexagonal pattern (Wan, 1996). The pore throats had a width between 30 and 300 micron. The applied bacterial strain was *Leuconostoc mesenteroides*, a facultative anaerobe that grows under mesophilic conditions. They continued their investigation by focussing on pore scale level clogging development in porous media. A short paper on biofilm accumulation and transport of microorganisms was published by Dunsmore and Lappin-Scott (Dunsmore et al. 2004). They created a micromodel in which an image of a thin slice of sandstone rock was etched chemically in a glass plate. They used *Desulfovibrio spp.* as model organism. Research towards microbial improved oil recovery was performed by Soudmand-asli et al. (2007). A micromodel has been applied to study enhanced oil sweep induced by bacterial activity. They also address the situation found in fractured reservoirs. Their study uses 2 bacterial strains, i.e., *Bacillus subtilis* and *Leuconostoc mesenteroides.* The latter is able to produce large amounts of dextran under anaerobic conditions. The *Bacillus subtilis* strain is known for its interfacial tension reduction capabilities (Abtahi, 2003). Only a few papers can be found on studies where micromodel observations of microbes were combined with flow quantification, an example is (Yarwood, 2006).

Objective

The objective of the research reported in this chapter is to give a full description of the design, construction and operation of a conventional micromodel that allows observation of transport of microorganisms and biofilm growth in porous media, combined with flow field visualization/quantification. Visualization of flow diversion is performed using PIV. For the achievement of this objective this paper describes a combination of two methods.

1) The construction of a glass transparent micromodel that allows transport of microbes.

2) A method in which Particle Image Velocimetry is used to track particles passing through a micromodel elucidating heterogeneities created by biofilms.

Included is the procedure to grow the strain *Pseudomonas chlororaphis,* which is known for its biofilm formation capabilities. This strain was used as a model strain to grow the biofilm; subsequently flow experiments were performed in the presence of this biofilm.

Outline

First the construction of the etched glass cell (wafer) will be explained, giving a description of the techniques applied in the etching of the wafers. This also involved pattern and mask plate development and the procedures that are applied to transfer the developed patterns to the wafers. This is followed by a detailed description of the construction of the holder for the wet-etched glass wafers constituting the 2D micromodel. Then a description of the usage of the complete micromodel is given including the microscope and camera. The bacterial strain *Pseudomonas chlororaphis,* used for the transport and biofim experiments, is shortly described. Methods to grow the strain, including nutrient media, and induce biofilm formation in the micromodel are given. The usage of the PIV technique is elucidated subsequently. We end with providing results, demonstrating a proof of principle, and conclusions.

Development and description of the micromodel

Materials

Glass "Borofloat© 33" wafers were purchased from Plan Optik (Elsoff, Germany), which contain 81.3 % SiO_2, 12.75% B_2O_3, 2.4% Al_2O_3 and 3.55% Na_2O. The rings for the micromodel holder were constructed from a Perspex plate (Polymethyl methacrylate or acrylic glass, $(C_5O_2H_8)_n$). Perspex is naturally transparent, which allows optical visualization of the fluid flow through the micromodel holder. Standard equipment, tools and chemicals were obtained from standard local resources.

Micromodel mask design – Litography (mask plate development)

A 2 mm thick mask plate was created in order to transfer the created micromodel pattern on the wafers. The designed pattern was created in the mask design program L-Edit. (L-Edit version 12.61, Tanner EDA, California, USA). The mask plate is made of glass on which a thin chromium (Cr) layer is deposited. This chromium layer has been coated with AZ 1518 photo-resist by the manufacturer. The pattern of the micromodel was then written to the photo-resist by using a UV Laser Beam Pattern generator, (LBPG, Heidelberg instruments, Germany). The laser causes changes in the photo-resist so that it can be dissolved later with the development liquid MF322 (Micro-posit, Shipley, MA, USA). After this step, the mask plate is exposed to an acidic bath etching away the Chromium, which is exposed to the acid. Subsequently the photo-resist is removed by dissolving it in an organic liquid, leaving the desired pattern on the mask plate. In our case, the mask contained a grid of circles (400 × 253) with a diameter of 150 μm and at a minimum distance of 3 μm between the edge of the circles. Of the total area of 6 × 6 cm^2, the area containing the circles has a size of 6 × 3.8 cm^2, which is the medium of interest. The circles are placed in an equidistant staggered grid. The remainder consists of two rectangles (1.1 × 6 cm^2) on both sides of the medium of interest, designed to create highly permeable zones for uniform inflow, with 2 inflow channels, which are 3 mm wide. The pattern data was then transferred to a Laser beam pattern generator. The pattern includes all the areas that are created by the etching process, viz., the inlet channels, the high permeable areas and the area that holds the pillars constituting the porous skeleton. The inlet channels are the connections from the porous medium to the micromodel holder. This mask plate can now be used as a template to be copied (transferred) into the wafers used in the micromodel.

Micromodel mask design – Protection layer deposition

All Borofloat 33 glass wafers were first coated with an 800 nm poly-silicon (poly-Si) layer by means of Low Pressure Chemical Vapour Deposition (LPCVD). The PolySi was deposited during a 9 hour procedure using SiH_4 as a gas. The deposition was performed at a temperature of 570 ºC, and simultaneously takes place on both sides of the wafers. The second coating consists of silicon carbide (SiC). A 500 nm thick layer was deposited through Plasma Enhanced Chemical Vapour Deposition (PECVD). As a basis, a mixture of SiH_4 and CH_4 was used. The procedure was performed in a Novellus concept one deposition system (Novellus, Ca, USA), and takes 8 minutes for one wafer and an additional 1.15 minutes for each extra wafer. The deposition was performed at a temperature of 400 ºC, and only takes place at the front of the wafer. To deposit the lower side, the wafers need to be flipped over and the process has to be repeated.

Micromodel mask design – Transfer of the pattern

The pattern transfer is done by the same lithographic method as used for the patterning of the mask plate. The wafers are first coated with SPR3017M photo-resist (3 µm thick) obtained from Shipley Company (Marlboro, Massachusetts, U.S.A.). Photo-resist deposition on the wafers was performed on an EVG 120 system (EV group, Austria) by spin coating. Subsequently the wafers are illuminated with UV light, which is guided through the mask plate thereby copying the pattern onto the wafers. Illumination is performed on an EV420 contact aligner (EV group, Austria). The wafers are then developed on the same EVG 120 system with Microposit MF-322 developer, also obtained from Shipley Company.

Micromodel mask design – Protection layer plasma etching

Prior to wet etching of the glass, the photo-resist pattern is transferred into the glass protection layers by plasma etching in an Alcatel Gir300 machine (Alcatel, Annecy, France) with a mixture of CF_4, SF_6 and O_2 gasses. This procedure takes 12 minutes. After the glass etching the machine is also used for removing the residue of the protection layers on both sides of the wafers. In this case the procedure takes 12.5 minutes on average for the front and 15 minutes for the back of the wafer.

Micromodel mask design – Chemical etching of the glass wafers

Wet-etching of the Borofloat 33 glass wafers is done in a heated mixture of HF and H_3PO_4. During this process the acid mixture will etch the glass on the unprotected areas, thus transferring the pattern into the glass. Deposition of the poly-Si and SiC masking layers on the

flipside of the wafers was necessary to prevent this side from also being attacked. The wet-etching procedure took about 45 minutes to reach a depth of 25 μm.

Micromodel mask design – Cutting of the etched wafers (post etching modifications)

The etched wafer was positioned on a Wafer/frame tape applicator, were a double-layered Nitto foil layer was applied. This layer was required to keep the wafer in place and to protect it during the cutting procedure. The layered wafer was then positioned in a Disco DAD321 Automatic Dicing Saw (Disco Corporation, Tokyo, Japan) and aligned accordingly. A strip holding a width of 2.5 mm was cut out of the glass wafer. Subsequently, the cut wafer was cleaned in a GS Ultratech Model 2066 High pressure cleaning station (GS Ultratech equipment, USA) where DI-water was used at a pressure of 30 bars. CO_2 is dissolved in the water to reduce its interfacial tension. This open end in the wafer was required to allow liquid to exit the model avoiding an increase in pressure in the constructed cell itself.

Construction and assembling of the holder including an etched wafer.

For the construction of the complete micromodel set-up the etched wafers were mounted correctly in the developed (wafer) holder. This holder consists of two Perspex rings with a thickness of 5 mm. Both rings hold an inner diameter of 90 mm and outer diameter of 130 mm. In the inside of the bottom ring, a secondary ring with a width of 10.4 mm and a depth of 1.2 mm was carved out allowing the correct placement (alignment) of both wafers (both have an outer diameter of 10 cm). These wafers are adhered to the Perspex using a Teflon elastomer (CAF 4 Silicone elastomer (Bluestar Silicones, Lyon, France)), an additional 0.4 mm deep area was carved to compensate for the thickness of this material. In order to create a leak free, waterproof micromodel the wafers were clamped and sealed between the two Perspex rings. To this purpose, plugholes were drilled through both rings (holders) where screws could be fitted in. (Fig. 1. locations of these holes)

To allow liquid flow from the Perspex holder to the created cell (consisting of 2 wafers), a reservoir ($l \times w \times h = 30 \times 4 \times 4$ mm^3) and two identical connecting channels ($l \times w \times h = 40$ mm × 2mm × 1.2 mm) were made in the bottom ring, to serve as the liquid inlet and bubble trap. Liquid is transferred from this reservoir via these channels to the wafers. As a liquid inlet from the infusion pump hose to the reservoir, a 3 mm steel tube was inserted along the long axis of the Perspex ring penetrating the reservoir from the side. The total volume of the reservoir including both channels is 0.672 ml. The created top ring has the same dimensions as the bottom ring,

although it has no reservoir or channels, nor does it have a carved ring to position the wafers. In both rings, indicator marks were applied to be able to align them correctly later on.

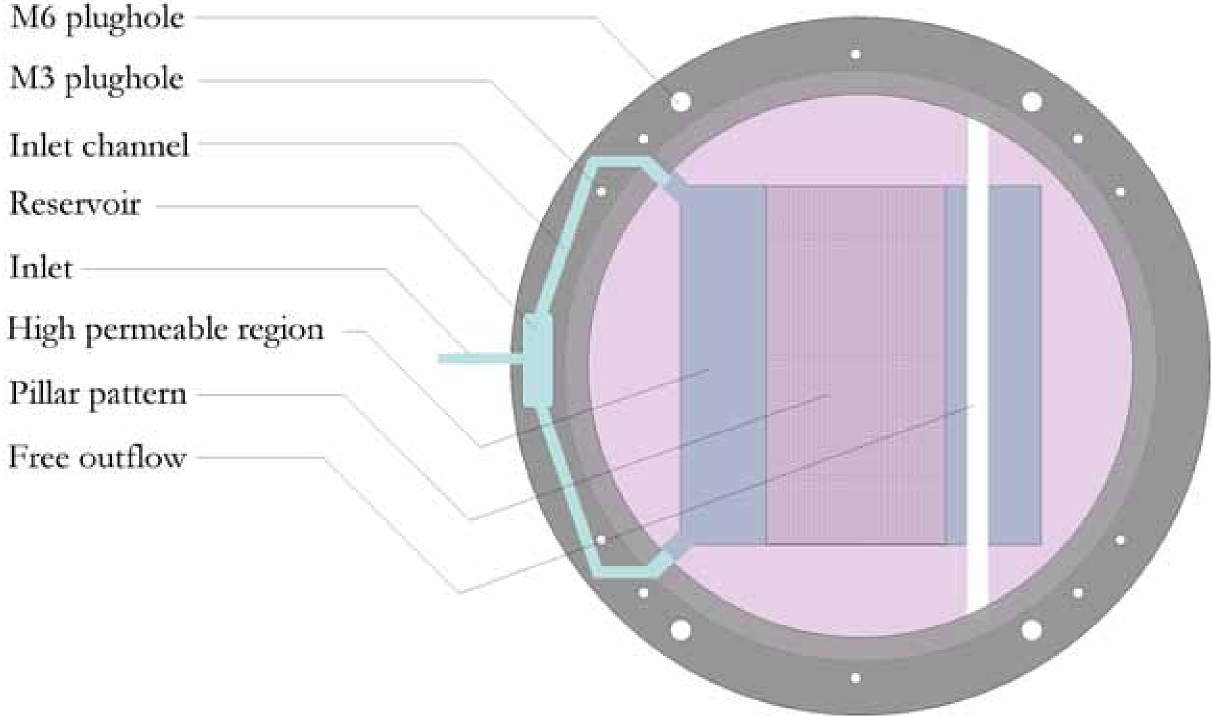

Figure 1: *Schematic overview of the micromodel. Four M6 and ten M3 plugholes were created in the holder.*

Application of the micromodel and the observation of biofilm development.

Description of experimental set-up and utilization of the micromodel

The fully assembled micromodel was connected to a Cole-Parmer Single-Syringe Infusion Pump (Series EW-74900-00, Cole-Palmer, Illinois USA) with a silicon hose. The inflow velocity of the pump during the experiments was 0.100 ml h^{-1}. The fluid traversed the micromodel in approximately 34 minutes, corresponding to a horizontal fluid velocity of $4.52*10^{-5}$ m/s or 3.9 m/day (interstitial velocity) corresponding to a Darcy velocity of 1.6 m/day. This value is somewhat larger than an average Darcy velocity in oil reservoirs (1 m/day).

Bacteria were grown on growth medium overnight. They were introduced in the micromodel by three hours of suspension pumping as an inoculum at about the same rate as used in the experiments. Flow was then stopped for one hour to give the bacteria time to adhere to the solid surface areas of the porous medium. Subsequently, fresh sterile medium was flushed through the micromodel. Biofilm formation was observed with the microscope at enlargements of 100× and 400×. Images were taken at different time frames, commonly every day.

Description of the used microbial strain applied for the testing of the set-up

The biofilm growth experiments were performed using the bacterial strain *Pseudomonas chlororaphis* (ATCC 55729). This strain was obtained from CBS (Centraal Bank Schimmelculturen, Utrecht, the Netherlands) on behalf of the Delft University of Technology. *P. chlororaphis* is a rod-shaped, motile, and facultative aerobe that is Gram negative. Strains of *P. chlororaphis* typically contain 4-8 polar flagella. It is able to grow at temperatures between 5 and 37 ºC, with an optimum at 30 ºC (Haynes & Rhodes, 1962). *P. chlororaphis* is a level 1 terrestrial microorganism that is known not to be hazardous to any extent. It is, however, well known for its biofilm forming capabilities. The medium used to cultivate this microorganism is adapted from Stoodley et al. (2005) and consists of the following compounds (mg L^{-1}): KH_2PO_4 70, K_2HPO_4 30, $(NH_4)_2SO_4$ 110, Glucose 1000, $CaCl_2$ 40, NaCl 585, trace elements/$MgSO_4$ solution consisting of the following components (mg L^{-1}): EDTA (Trilon B) 5, $FeSO_4 \cdot 7H_2O$ 2, $ZnSO_4 \cdot 7H_2O$ 0.1, $MnCl_2$ 0.03, H_3BO_3 0.3, $CoCl_2 \cdot 6H_2O$ 0.2, $CuCl_2$ 0.01, $NiCl_2 \cdot 2H_2O$ 0.02, Na_2MoO_4 0.02, $MgSO_4 \cdot$ 7H2O 0.2. Buffer containing only KH_2PO_4 and K_2HPO_4 was autoclaved at 120 ºC. A 20% w/v $(NH_4)_2SO_4$ was prepared separately and autoclaved at 120 °C. A 20% w/v glucose stock solution was prepared and autoclaved at 110 °C. Trace metals and $MgSO_4$ were autoclaved at 120 °C separately. All compounds were added together under sterile conditions. This was tested by incubating sterile medium bottles at 30 °C overnight followed by a contamination check. Biofilm formation was

induced by using a medium with a C:N ratio of 20. This ratio is believed to be favourable for biofilm formation and extracellular polysaccharide (EPS), production.

Microscopy study

Images were made on a Zeiss Axioplan 2 microscope (Carl Zeiss Imaging Solutions GmBH, München, Germany). The used camera is an AxioCam MRm (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). The software package used to analyze the images is a Leica Qwin pro software package version 3.2.1 also from Leica microsystems. The SEM images of the etched wafers were made on a Philips XL electron microscope (FEI company/Philips, Eindhoven, the Netherlands).

Particle Image Velocimetry

During PIV experiments, the micromodel was placed under a combined stereo/mono epifluorescent microscope (Leica MZ 16 FA). The stereo mode is used for preparation of the experiments only (e.g., for tracer injection). The motion of these tracer particles is recorded on a digital camera (PCO Sensicam QE, 1376 × 1040 pixels using 2×2 binning; acquisition rate 5 Hz). Local cross-correlation is used to determine the local displacement and thus velocity (by dividing the displacement by the temporal separation between consecutive images). The set-up is controlled using a PC running DaVis 7 software (LaVision GmbH). This software is also used for data acquisition and storage. Polystyrene spheres with a diameter of 1.28 micron were used as tracer particles, containing a fluorescent dye Rhodamine 6G (Microparticles GmbH). The particles are bio-inert or 'stealth', because of a poly-ethylene glycol (PEG) coating. The particles are naturally buoyant and have a very small (Stokes) response time.

Typically 1500 images are recorded for each measurement. This corresponds with a 300 second time interval, at a frequency of 5 Hz. Background image subtraction was performed to circumvent dominant reflection originating from the biofilm itself. Hereby the first image is used as a reference image, and all subsequent images are mapped onto this image. To determine the required image transformation, the disparity between the images is determined by local cross-correlation using 96 × 96 pixels interrogation windows with 50% overlap, covering the entire raw image. A second-order polynomial fit is performed using the disparity data. Although in general the corrections needed were small, this step is crucial in the PIV process. A more detailed description of this technique can be found in the article by Poelma (2008).

Results

Micromodel development and the wet-etching of the glass wafers

The wet-etching procedure used here, posed little difficulties with respect to the etching process. The obtained depth was around 25 μm. This depth is well suited for transport experiments with microorganisms and did not cause operational problems while using the micromodel. It was observed that the pillars assumed a characteristic bell shape, as mentioned. It was also observed that the pillars do not obtain a perfect circular shape when observed perpendicularly from the top. The reached upper diameter of the pillars was 110 μm. The average pore throats were 40 μm holding a final aspect ratio throat width/depth of 1.6. As can be seen in Figure 2, the pattern was transferred correctly. In the mask plate the spherical etching (undercutting) was taken into account. The porosity of the pattern can be described as the pore volume divided by the total volume and is 41% in this case.

a b

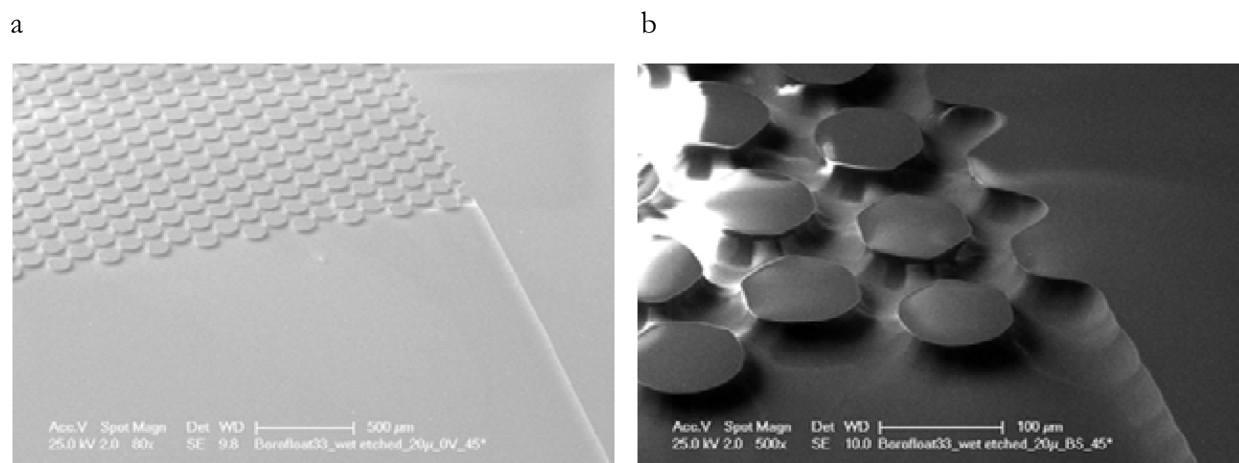

Figure 2: *SEM image showing a: wet etched pattern of the wafer (80xs magnification); b: effects of spherical etching (500xs magnification). Note the irregularities (lines) originating from the pillars in figure 2b, caused by the wet etching procedure. Note in addition the bell-shaped channels.*

Biofilm development and the formation of specific structures in 2D porous media: a proof of principle.

After inoculation of the micromodel with the microorganism *P. chlororaphis*, biofilm growth was observed in the micromodel already after one day. The formation of biobridging was clearly observed throughout the model. These structures are described as a chain of attached microorganisms that form a bridge between soil particles. In the case of the micromodel, a biobridge is equivalent to the formation of a bridge between two adjacent pillars. This phenomenon is observed at numerous locations in the micromodel. Biobridge structures in progressing stages are visualized (Fig. 3). When chains of microorganisms stretch from pillar to pillar, the bridge is complete (Fig. 3a). Often, crossing bio-bridges merge together to form a web

of biofilm (Fig. 3b). Over time, these webs increase in size and become clusters, which can block a significant fraction of the pores (Fig. 3c). It could be seen that the thick biofilm grew longer every day, following the direction of flow (Fig. 3d). Individual microorganisms could be distinguished in these structures, surrounded by layers of EPS. During the initial microbial inoculation, the cell density was relatively low. With subsequent flushing with fresh medium, the cell density increased over time. Furthermore, transport of both single microorganisms and released pieces of biofilm was visible during pulses with fresh media. These released pieces showed similar transport behaviour as single organisms.

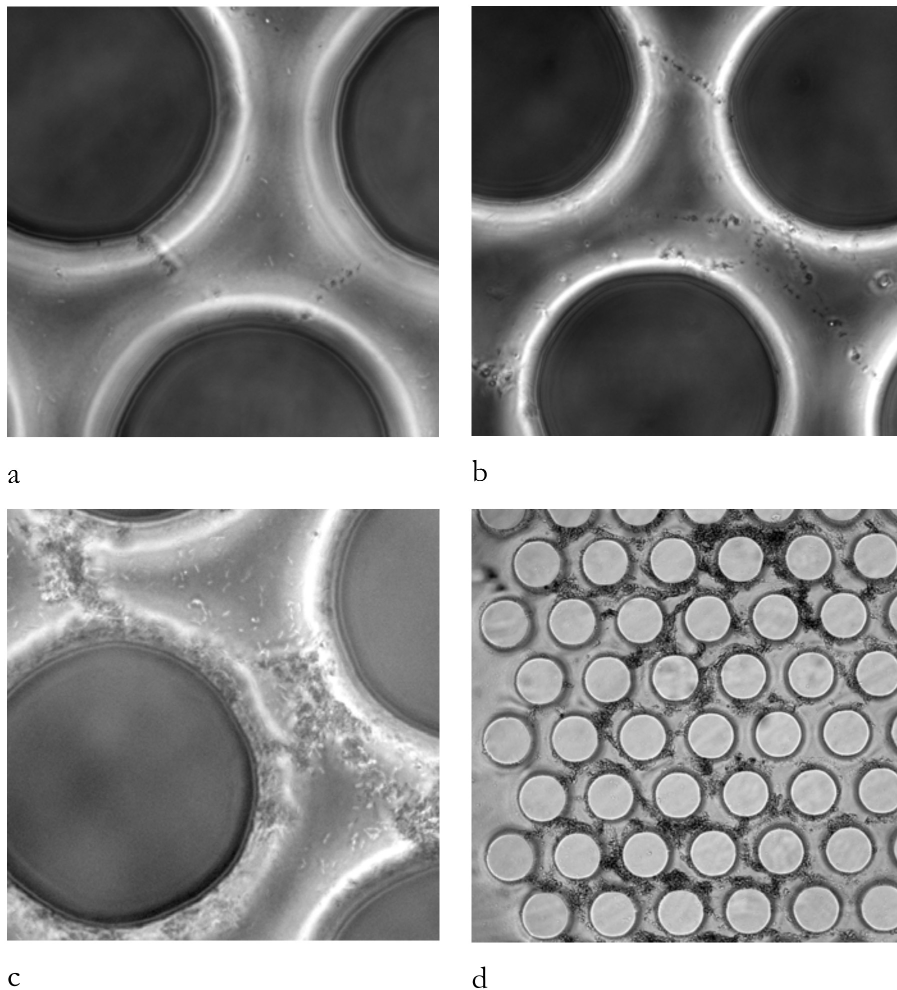

Figure 3: *Close up of micromodel showing different stages of biofilm development. Figure 3a,b&c, the pillars show up as dark disks, where porespace is the lighter region in between. Magnification in (a,b,c) 400× and in (d) 100×. Refraction and reflection cause the white and black circles surrounding the pillars, which are 110 μm in diameter. a: Initial attachment observed as string of bacteria connecting one pillar to the other. Also we observe individual bacteria, as black or white dots and initial elongation of the string. b: Formation of biobridging and subsequent cluster forming. c: Formation of the mature biofilm. d: Overview of bioweb (The pillars now show up as white disks).*

Particle Image Velocimetry

The flow patterns in the micromodel are visualized by means of microscopic Particle Image Velocimetry (Santiago, 1998). A recent review can be found in (Lindken et al. 2009). The micromodel is perfused at a flow rate of 0.100 ml h^{-1} with a medium containing 1.28 µm fluorescent particles, which act as tracer particles for fluid motion. First the flow pattern in the micromodel without microorganisms or any form of biofilm is documented (data not shown). Subsequently, PIV measurements in micromodels with biofilm are performed (Fig. 4). The flow pattern is represented by a vector field, in which both the vector length and colors indicate the velocity magnitude. To obtain a better overview, the length is only an indication of the magnitude of the velocity but not proportional to it. The velocities typically range from 0.5e-5 ms^{-1} (dark blue) to 6e-5 ms^{-1} (red). The vector field has been superposed on a dark field image of the model, which shows the location of biofilm clusters (white patches).

In the figure, it can clearly be observed that the presence of biofilm alters the flow pattern, as can be seen in the red rectangle (Fig. 4). Particles follow preferential pathways, on occasion also against the direction of the overall main flow direction. On a small scale, movement through narrow pathways led to an increase in velocity. On a larger scale, it could be seen (due to its size it could not be included in this paper) that in certain regions (wakes), lying behind thick biofilm formations, the flow rate as a whole was lower than in adjacent areas. To quantify the permeability reduction the parts of the flow pattern must be interpreted, using a flow simulation. This is, however, outside the scope of the research presented in this paper. However, with such an interpretation models of biofilm growth in porous media can be improved by incorporating a relation between permeability and biofilm concentration. Furthermore, the reproducibility of the PIV-measurements was determined by measuring the same region four times, (Fig. 5). This figure shows that even if the direction of flow is similar; the tracer particles show fluctuations in the flow rate.

Figure 4: *Example of a PIV measurement in the micromodel. The colour of the arrow represents the particle velocity, ranging from blue (slow) to red (fast). The white structures are a web of biofilm. The white dot on the left hand side of each pillar is a reflection artefact. In this picture, two types of regions can be distinguished: one with biofilm formation and subsequent alteration of initial flow patterns (left) and one with little or no biofilm and a more regular flow pattern (right).*

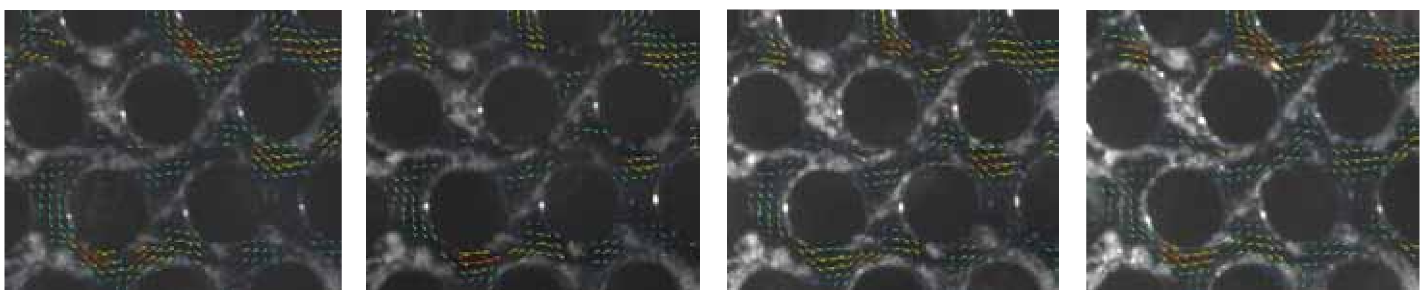

Figure 5: *Recordings of the same area in the flowcell during four separate measurements using a time interval of 10 minutes. The direction of flow remains similar; however we see small variations in the flow rates (color of arrows) and intensity. The intensity changes are caused by small movements of the light source. Reasons for the changing color pattern are discussed in the text.*

Discussion

The performed experiments are relevant for many applications in petroleum engineering and hydrology (Cunningham, 1991). Much attention is devoted to improving oil recovery using metabolic activity of micro-organisms. The most important envisioned application, however, is in bio-diversion or bio-sealing. Therefore, the interest here is on biofilm formation and pore clogging. Bio-diversion in this context implies that high permeable regions bounded by impermeable shale layers that are watered out are clogged such that for example the oil from lower permeable regions can be produced (Vermolen, 2004). A well is usually connected to a number of layers with different permeabilities. These layers may be separated by impermeable shale layers. In bull-headed injection in the production well, the placement of nutrients will be more effective in high permeable layers. Hence microbial growth will be more effective in the high permeable layers, in which a reduction of permeability and hence flow occurs. Consequently flow will be redirected via the low permeable layers from which oil will now be produced.

Indeed, microbes including EPS occupy the pore-space thus considerably reducing the permeability as observed in the experiments discussed in this paper. Furthermore observation of microbes in the wells can be used as a marker of processes occurring in the reservoir. All these applications require understanding of mechanisms of transport of microbes in the subsurface. In this, formation and destruction of biofilms play an important role. In biofilm growth nutrient supply, inhibitors and hydrodynamics influence its formation.

Proof of principle: The combination of a micromodel and the use of PIV techniques.

The combination of a 2D micromodel set-up and PIV techniques as demonstrated and applied in this study is a powerful method to study transport and attachment of microbes in porous media, e.g., in water management and oil recovery processes. Biofilm growth starts with attachment of single microbes, from where clusters of microbes develop. Sometimes clusters of microbes move in the porous medium (Vadas, 1973). Pore bridges originate and expand presumably by collector effects of bacteria and cell division. The origin of the observed biobridges can also be the effect of the wet-etching procedure in which a bacterium at a certain time is retained by the irregularities (lines caused by the wet etching procedure, (Fig. 2b.). This implies that bacterial attachment is enhanced by irregularities, which are naturally present at the porous medium surface (Mitik-Dineva et al. 2008). When the clusters develop biofilms are formed. It was observed that the biofilm formation affects both direction and velocity of the fluid. Large biofilm clusters cause the flow to divert into regions with less biofilm. Hereby, preferential flow pathways originate, as has been shown in this research. An important aspect of visual observations is the

interpretation of the results. A clear observation is that flow avoids regions of high microbial concentrations. However, the flow rates flowing towards a node point and away from the node point should balance. From the observed rates, i.e. the color of the arrows, one would conclude that this balance is not satisfied at some node points. Below we list a number of artefacts that can lead to misinterpretation of the results.

Artefacts of the PIV measurement in the micromodel.

As can bee seen in Figure 4, at some places flow paths seem to start and end abruptly. This, however, is likely to be an artefact caused by the autofluorescence of the biofilm itself. If the biofilm is thick enough, the fluorescence of the biofilm can at some places overrule the signal of the tracer particles. This would lead to a distortion of the vector pattern. Another plausible possibility is that the particle moves out of the field of vision, due to 3D effects, e.g., a fluorescent particle passing below a piece of biofilm.

In Figure 5, it was illustrated that the flow direction in the different pictures was comparable in the region that was measured four times. It can be expected that the number of tracer particles fluctuates in the region under consideration. Therefore, the flow rate shows small fluctuations. This artefact will decrease when time interval of the measurement is increased, due to an averaging effect with the increased amount of captured tracer particles.

Advantages of the micromodel.

The application of wet etching in the construction of the etched cells is a relatively easy technique to create patterns in glass or SiO_2. A suitable depth (20-40 μm) to perform flow experiments, unlike, e.g., with plasma etching (Metwalli, 2003), is easily reached. We used a depth between 20-25 μm and a width of 40-50 μm. however, if a micromodel with small pores is required different etching techniques are needed.

The choice to use ordinary glass wafers for the construction of a micromodel has been proven fruitful. Glass allows a better quality of observation than an intransparent silicon (Si) wafer covered with a glass plate. Additionally, glass is hydrophilic resembling most subsurface environments; this in contrast to many other transparent materials like SU8, which are commonly hydrophobic. Since a mask plate has been designed, multiple wafers can be constructed allowing multiple flow experiments in cells that have the same etched pattern.

Limitations of the applied etching technique and the constructed micromodel.

In the current design we used two inlet channels followed by a high permeable area, before the area of interest is reached. However, it does not create a completely uniform waterfront as intended. All the same it did improve the uniform shape of the waterfront compared to earlier versions of the model in which the high permeable areas were not created. Furthermore, the use of conventional wafers with a thickness of 0.5 mm as commonly used in the electronic industry has its limitations. The use of chemical etching as mentioned earlier is rather straightforward and therefore frequently used in micromodel studies. It, however, puts a limit on the size of the pores. The fact that the pores used in our micromodel are relatively large excludes certain mechanisms in which microorganisms can clog pores, like size exclusion (filtration effects) and the observation of effects due to inaccessible and excluded pore volumes. The fact that this type of etching causes small irregularities in the pore structure bottom plate requires a critical interpretation of the observed bacterial attachment processes.

Conclusions

Chemical etching techniques can be used to construct transparent glass or silica micromodels, which have the advantage, as opposed to silicon models, that they are transparent and hence can provide clearer images of processes on the micro-scale. The wet etching technique limits the resolution of the pore sizes due to the undercutting artefact, i.e., minimal pore size is twice the depth. This limitation effectively rules out the construction of small pores, and hence the observation of size exclusion, inaccessible and excluded pore volume or filtration effects.

It is possible to successfully introduce *Pseudomonas chlororaphis* into the model. Biofilm formation was successfully induced. The designed holder functions properly with a wet-etched micromodel into place and it is shown that biofilm growth can be observed. Preliminary observations show that micromodels are a versatile method for the observations of microbial processes in porous media. They provide detailed insights in processes on the pore level. Experiments have shown that some of the mentioned processes, e.g., bio-bridging, that are related to biofilm formation can be observed. The biobridging, however, can also be a consequence of the created imperfections by the wet etching procedure. Therefore it is advised to describe the structure of the pore network in detail and to verify the experimental observations regarding bacterial attachment with the pore network structure.

The combination of 2D micromodels with PIV techniques allows the observation of flow irregularities caused by biofilm development at specific locations. On these locations, preferential flow pathways and wake zones were observed. The constructed micromodel has proven to be well suited for observations of these kinds of phenomena.

In principle the PIV measurments can interpreted in terms of permeablity modification. Such an interpretation would allow incorporation of a permeability-biofilm relation in the modelling.

This set-up provides an experimental tool for elucidating some of the transport mechanisms that determine the movement of microbes in oil reservoirs and aquifers.

Results regarding transport of microbes and biofilm formation in porous media from computer models can now be compared and verified to real experiments. This is a welcome contribution towards a better understanding of these processes, also on a larger scale.

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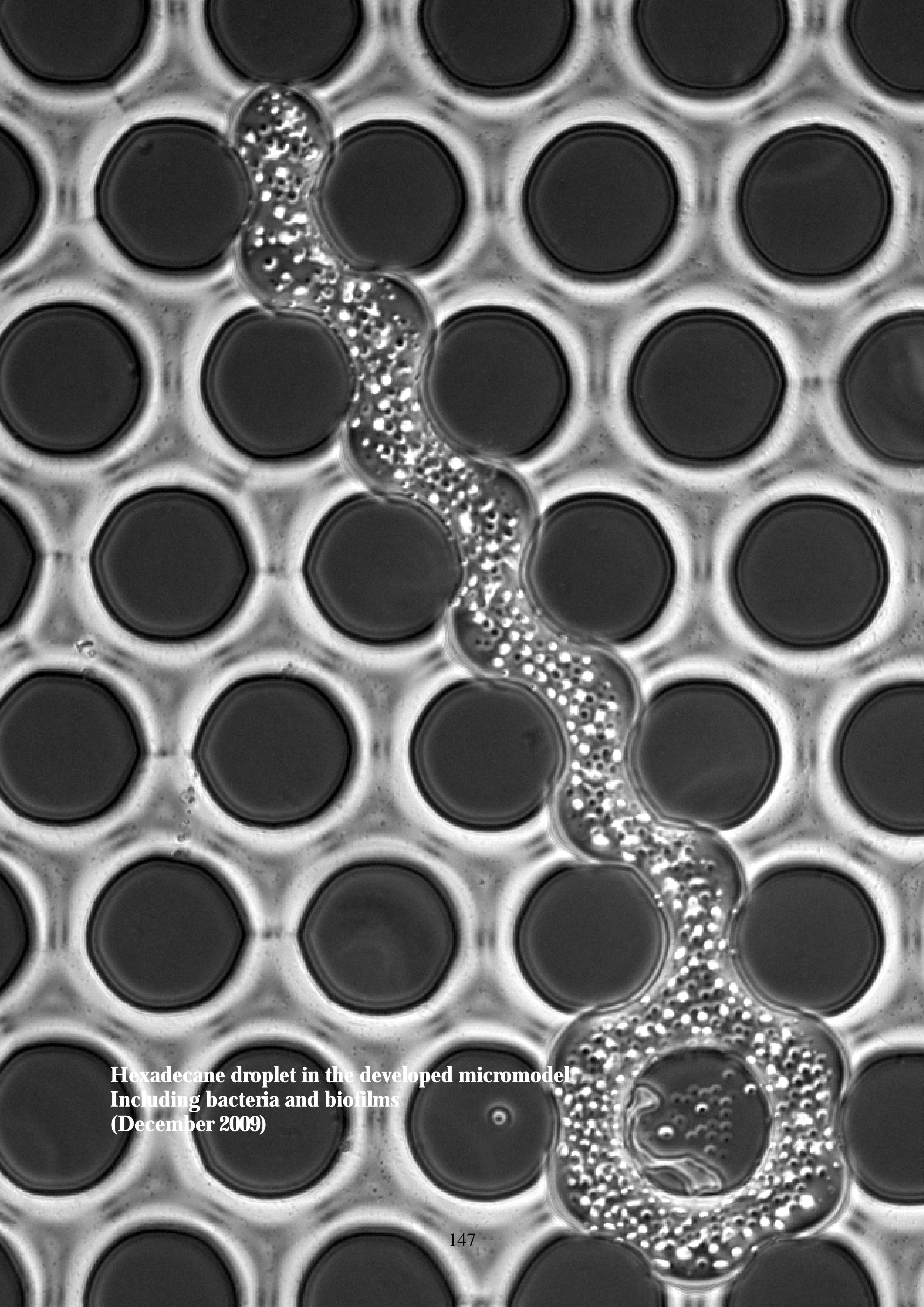


Hexadecane droplet in the developed micromodel
Including bacteria and biofilms
(December 2009)

6 extended

Applications in the developed micromodel: Quantification of biofilm surface and a final 2-phase follow-up experiment.

&

An attempt to perform plasma etching in the creation of an improved pillar pattern

Geert M. van der Kraan, Maarten de Ridder, Mark C. M. van Loosdrecht & Johannes Bruining

Introduction

A proof of concept for the development and application of the micromodel has been given in Chapter 6. In this extension on chapter 6, (a) preliminary results of additional methods for image analysis will be presented. (b) The results of a single experiment involving oil are given and (c) attempts to use plasma etching for more versatile construction of micromodels are described. In theory, plasma etching is a technique that can create more detailed etched structures allowing 'closer to ideal' 2D structures without the artefacts associated with chemical etching.
These preliminary experiments are presented to facilitate follow-up research for biofilm development on the pore level in oil associated environments.

Aim of the preliminary experiments

To explore the possibilities of the micromodel and to relate this work, via a final experiment using hexadecane as a petroleum equivalent, to aspects of interest to the oil industry.

Additional methods for the image analysis and the 2-phase system

'Flow Porosity' Analysis

A biofilm surface coverage determination was performed on dark field microscope images of the micromodel holding biofilm structures, using Imaging Analysis software (QWinPro 3.2.1, Leica Microsystems, Rijswijk). A threshold grey-value was chosen dependent on the image intensity, creating a binary image selective for the biofilm coverage. This biofilm surface coverage is a measure for a decrease in 'flow porosity' in the system.

Two-phase system with hexadecane

In a subsequent biofilm monitoring experiment, the same experimental set-up (chapter 6) was used to create a two-phase system in the micromodel. Hexadecane was first introduced in the micromodel, before *P. chlororaphis* was flushed with the same flow rate (0.1 ml h^{-1}) and medium as described in chapter 6. After two hours of suspension perfusion, the flow was stopped for two hours; subsequently sterile medium was introduced into the micromodel. Cell density increased slower than in previous biofilm monitoring experiments, hence the flow was at some points stopped for several hours. This was done to prevent washing out of the bacteria. After four days the micromodel was perfused for three days with a fresh bacterial suspension. After eleven days, to increase cell density further and stimulate biofilm growth, the standard medium was enriched with YPD (1:50 ml/ml). YPD stands for, Yeast (10 g L^{-1}) Peptone (bacto) (20 g L^{-1}), Dextrose (22 g L^{-1}) medium.

Biofilm surface coverage determination using image analysis

A biofilm surface coverage determination of the pore space in the micromodel was performed on dark field microscope images displaying a mature biofilm (Fig. 1a). This image is a combined (stitched) image of all the dark field microscope images used in the successful PIV measurements that have been performed (chapter 6). The area was divided into fifteen equally large regions and was analyzed using imaging analysis software regarding the biofilm surface coverage of the same fragments (Fig. 4). It shows that the biofilm surface coverage was highest at the entrance of the pore complex. The maximum biofilm surface coverage calculated in a fragment was 40.1%

Additional methods for the plasma (dry) etching

Plasma etching of the quartz wafers

Double Side Polished pure fused silica (SiO_2) wafers with a diameter of 100 mm and a thickness of 0.5 mm were obtained from the University Wafer, (Boston, USA). These wafers had no flat, like normal silicon wafers but do have smoothed etches on the side to prevent easy damage by soft impacts to the wafer. In the electronic industry, the word flat is used to indicate that the circular wafer is cut-off yielding a straight edge. These SiO_2 wafers were specifically ordered for dry-etching since they contain no impurities.

For the plasma etching a mixture of CHF_3 and C_2F_6 was applied in a ratio of 4:1. The actual etching was done in a Drytek Triode 384T plasma etching machine (company taken over by LAM research, California, USA) and was performed at 180 millitorr pressure for 30 minutes. The energy supplied to the plasma was 300 Watt. The used frequency in this case was 13.56 MHz.

Results from the surface coverage determination

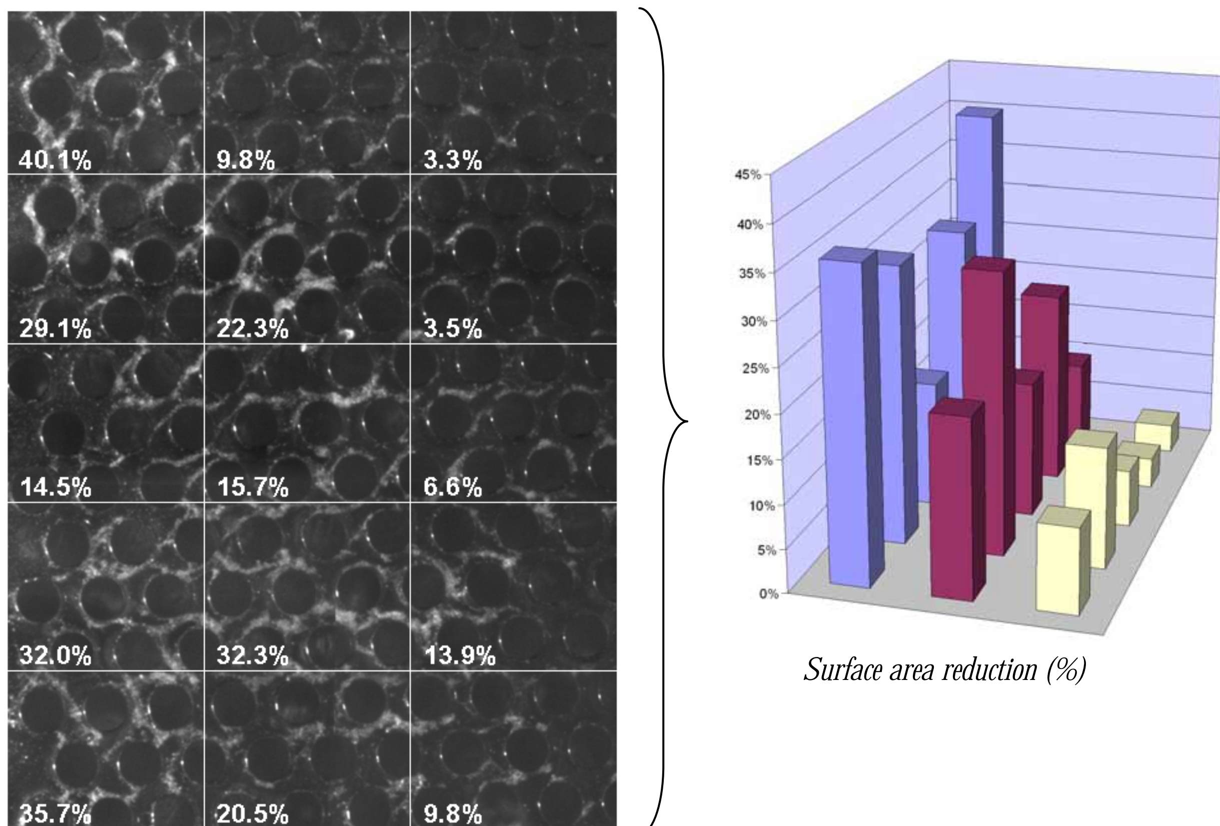


Figure 1a: *Result of the biofilm surface coverage determination at the entrance of the pore complex. The direction of flow is left to right. The given numbers in each area is the calculated surface coverage. The porosity of the empty pore complex is 41%. The indicated area is 2500 μm in height.* ***b:*** *3D visualization of biofilm surface coverage in the same region. In this figure 1b, the direction of flow is from left to right*

Biofilm development in a two-phase system

In a follow-up experiment on biofilm monitoring, a second phase was introduced in the micromodel using hexadecane, as equivalent of oil. In this two-phase environment, the cell density increased slower than during the oil-free experiment. From day eleven, the micromodel was perfused with standard medium, enriched with YPD (1:50 ml/ml). At day fourteen, biofilm formation was observed. In some cases, biofilms attached to a hexadecane film (Fig. 2). The biofilms were more flexible than the biofilms observed in preceding experiments (Fig. 3). Furthermore, the biofilms were less thick and contained less EPS. Whereas in the oil-free experiment the biofilms were clustered in a web like structure close to the entrance of the pillar pattern, biofilms in this experiment occurred more as small individual clusters. Some of them were located deeper in the micromodel (Fig. 4.) Furthermore, hexadecane fronts of various sizes were observed. Over time, it could be seen that bubbles were captured within areas filled occupied by hexadecane. Furthermore, spreading the micromodel, bubbles were observed on top of and connecting to various pillars (Fig. 4).

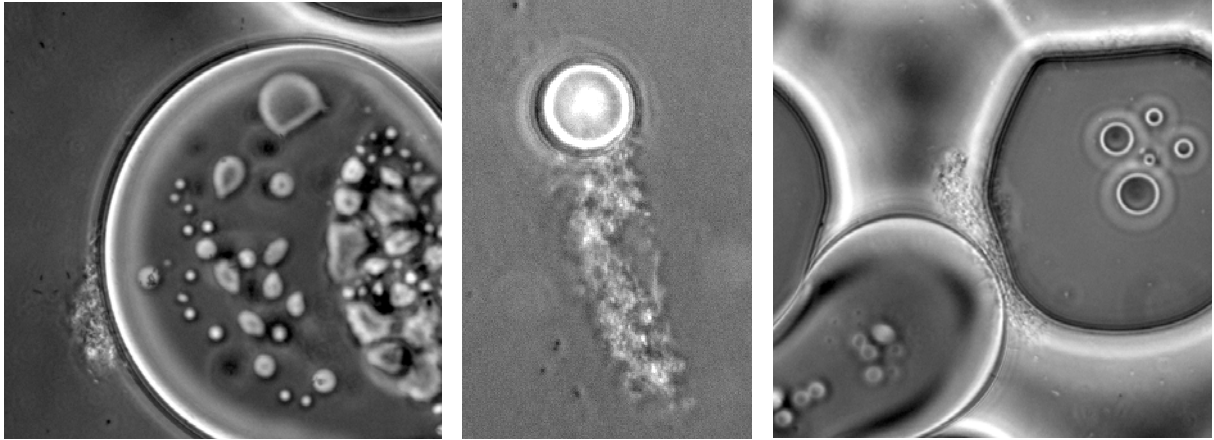
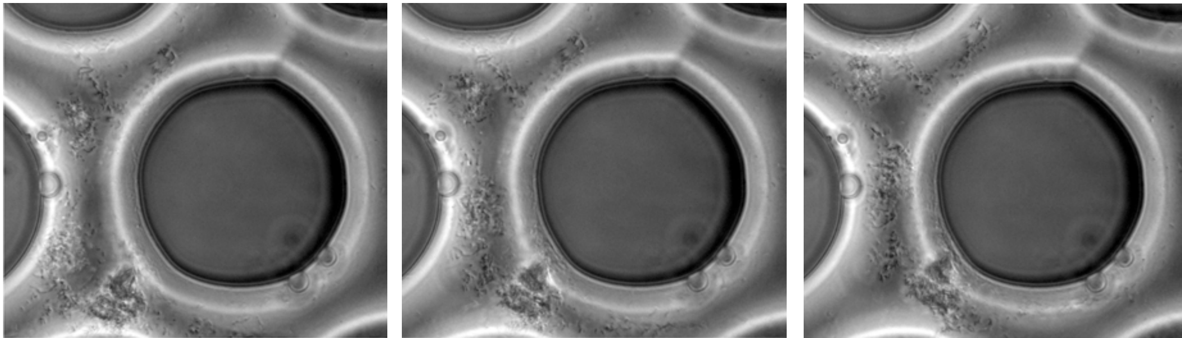


Figure 2: *Microscope images of the micromodel (400x magnification). Regions occupied with hexadecane are within the black/white/grey perimeter. Due to a reflection artifact, the perimeters are white at their border. The biofilms attachment on a hexadecane front can be seen in all three images outside the hexadecane region. Trapped emulsion droplets in hexadecane formations can be distinguished as light structures inside the hexadecane regions. In the right picture we observe the irregular shaped pillar, which has a white / grey / black perimeter.*



t = 0 sec **t = 2 sec** **t = 4 sec**

Figure 3: *Visualization of biofilm flexibility in the two-phase micromodel (400×s magnification). Attached to the left pillar, a droplet of hexadecane is observed. The dotted structure is the biofilm. It is observed that the biofilm moves in an upward direction.*

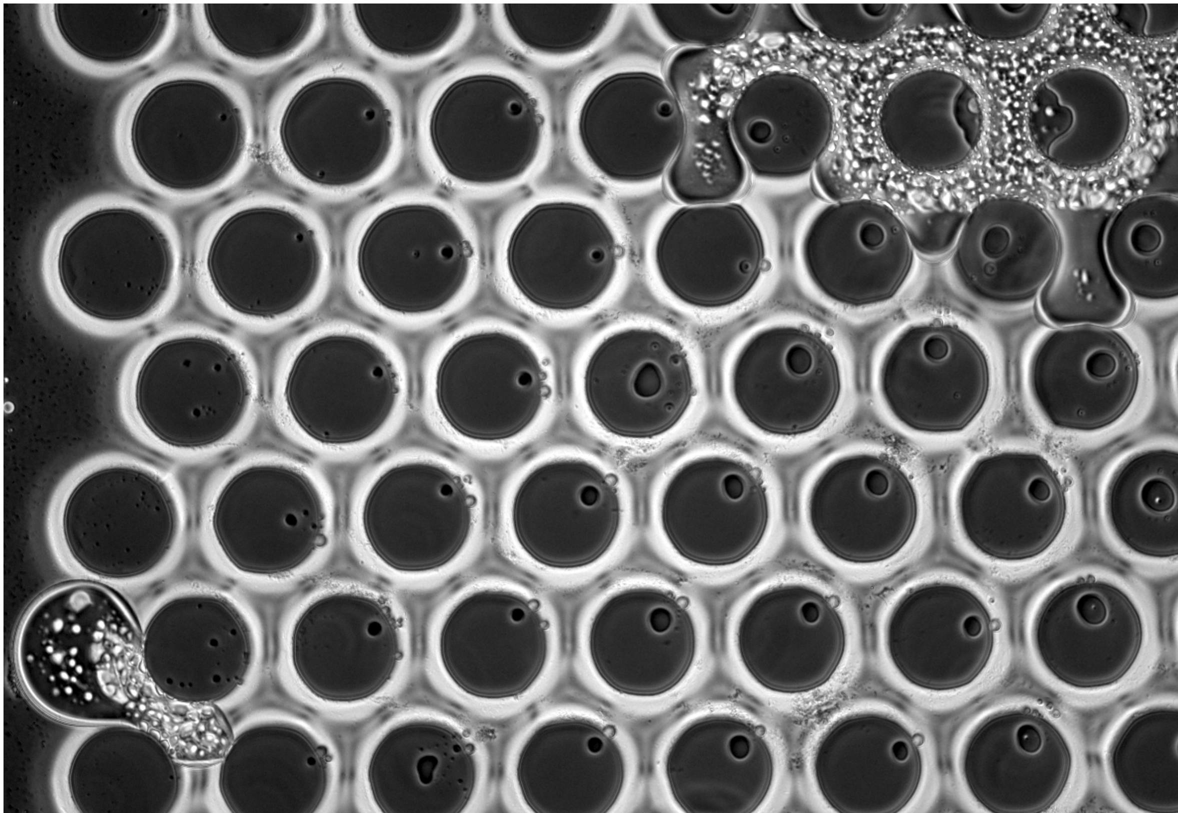


Figure 4: *At the bottom left and top right droplet aggregates (bright spots) in the bubbles of hexadecane fills up the pores between the pillars. The black disks between the pillars is an artefact. Also, a separation of phases was observed on top of most pillars 'crater-like strcuture' and connecting to the right-hand side of most pillars, as round shapes. Furthermore, in pore spaces throughout the micromodel, individual clusters of biofilm (dotted structure) are observed. The image is a 100× magnification. The direction of flow is left to right.*

Localization of biofilm formations

After the micromodel was perfused with hexadecane, it was observed that the inoculum was not homogeneously distributed throughout the micromodel. The same applies for medium distribution. It was observed that an increase of cell density and biofilm formation occurred mainly in regions that were well perfused.

PIV in the two-phase system

An attempt was done to perform PIV experiments in the micromodel holding the two-phase system. However, the tracer particles were captured all over the micromodel, even with a ten-fold increase of flow, which made reliable PIV measurements impossible. Hence conventional PIV methods cannot be applied in the presence of oil or a oil equivalent.

Results of plasma etching

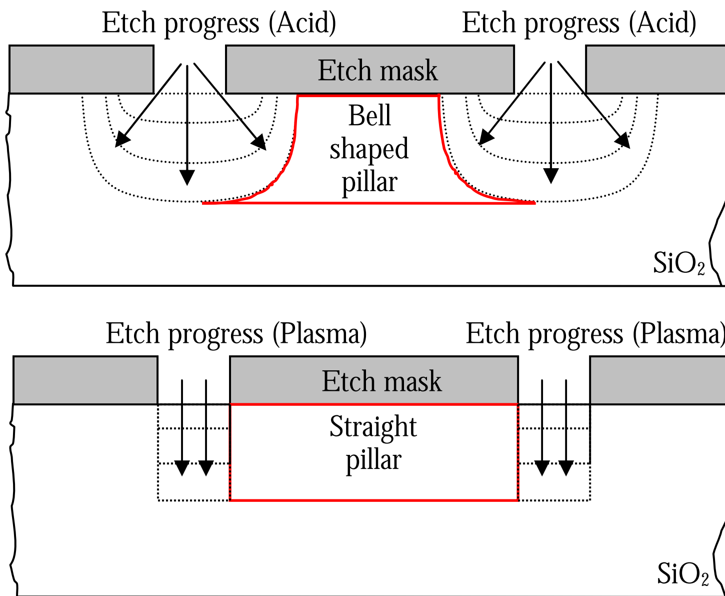


Figure 5: *Schematic overview of dry (plasma) and wet (chemical) etching*

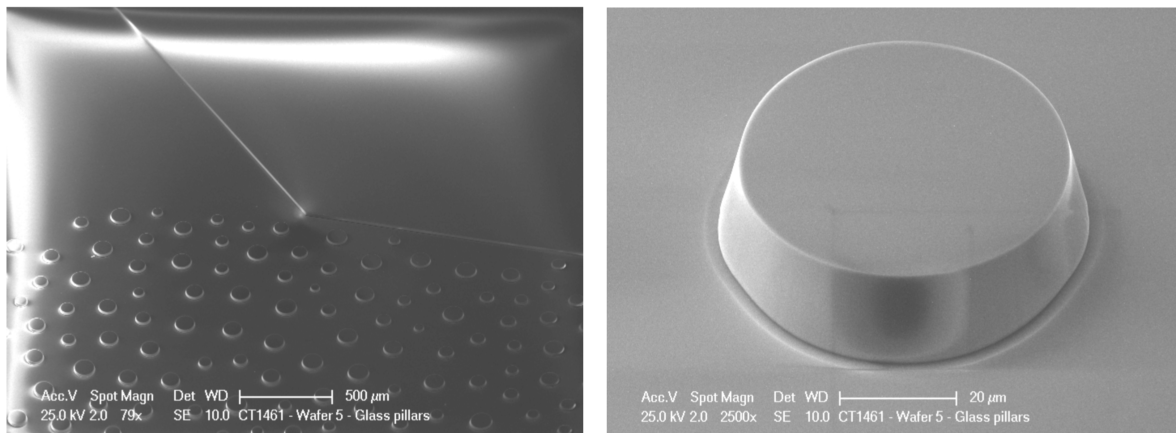


Figure 6: *SEM images of dry etched pattern and a close up of a pillar. It is clear that while using plasma etching, an improved pattern can be created. Pillars still assume a conical shape.*

Although plasma etching gave a promising result with respect to the transfer of the pattern, it is hard to reach an appropriate depth for micromodel experiments. This is due to the plasma etching itself, which is achieved by 3 processes; ion sputtering, etching by radical formation, and impact by photons. Unlike chemical etching, in plasma etching everything is etched. The maximal depth that can be obtained is therefore limited by the resistance of the masking layer. If this protection layer is etched away, the wafer will be etched uniformly and there will be no net increase in pattern depth. With the method described in this paper, a depth of 6 µm is eventually reached with dry-etching, which is still not deep enough for transport experiments with microorganisms. The developed dry-etching method needs improvement in order to obtain a depth that exceeds 10 µm, but we must leave this for future research. It was shown that with the

developed technique, the designed pattern was transferred without major artefacts to the wafer (Fig. 6a&b). It was demonstrated that also with dry-etching the shape of the pillars is not completely cylindrical. Pillars obtained a conical shape; the conical shaped pillars showed a much steeper inclination angle than obtained with chemical etching and approach a true cylindrical shape.

Discussion

Permeability decrease during biofilm growth

Significant biofilm surface coverage was calculated using image analysis software (Fig. 1a). Although a biofilm may not block an entire pore, the polymers 'stick out' in the water, hereby drastically hampering the flow of water in this pore, i.e. dropping the permeability. This interpretation is corroborated by live observation of the movement of the tracer particles (data recorded, but not shown) during the PIV experiments. However, it is expected that the pore would be more permeable for apolar petroleum, since this would force the polymers flat to the surface. In this position, the polymers would have less effect on flow of oil. A biofilm can therefore act as a relative permeability modifier for water, leaving the oil permeability more or less unaffected. Hence, the biofilm surface coverage is roughly a measure for the decrease in (water) permeability.

It was observed that the biofilm formations were located near the entrance of the etched pore structure. This can be due to oxygen limitation: although *P. chlororaphis* can both grow under aerobic and anaerobic conditions, growth rates are higher during aerobic growth. The introduced growth medium is O_2-saturated. Yet, due to the increasing concentration of cells, less oxygen might be available for bacteria that are located further away from the entrance of the micromodel. At these locations, there was also an increase in cell density and biofilm formation; however, the rate in which this occurred was far lower than at the entrance of the micromodel.

Observations and wetting effects in the micromodel holding the two-phase system

During the experiment, where apolar hexadecane was introduced as a second phase, separations of phases could clearly be observed. Since the micromodel in this experiment was initially perfused with hexadecane after which (polar, watery) medium was introduced, at many different locations throughout the micromodel 'captured' areas with hexadecane were observed. There were microscope field wide regions in the micromodel that were covered with hexadecane, i.e., regions of several pillars surrounded by hexadecane; however, also small captured bubbles of hexadecane were positioned lying connected to or in some cases even on top of a pillar.

Captured hexadecane formations can be explained by wettability effects. In the micromodel it can be expected that the surface energy between water and glass is less than between oil and glass. Water has a larger contact area with the glass surface than oil (Fig. 7). A solid surface for which the surface energy of water with the solid is less than the surface energy of oil is called water-wet. Water will be enveloping regions filled with hexadecane. For this reason, it can be assumed that most fluid bubbles, e.g. the 'craters' on top of pillars (Fig. 4), were small hexadecane formations, surrounded by water.

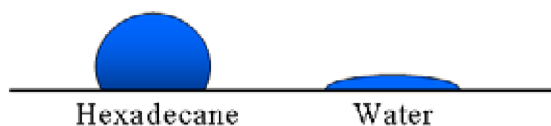


Figure 7: *Wetting effect of hexadecane and water (schematically). Hexadecane has a lower surface energy than water, hence it maintains less contact with the solid (glass) surface.*

Surfactants

In the micromodel, it was observed that bubbles were captured in hexadecane formations when time progressed. From the observation it cannot be determined whether the bubbles are gas bubbles or micro-emulsion droplets. The appearance of these bubbles indicates the presence of surfactants. In the growth medium, no surfactants were present. They were introduced by *P. chlororaphis*, since bacteria contain and excrete molecules that behave like surfactants.

Biomonitoring in the two-phase system

It was observed that the inoculum and medium were not homogeneously distributed throughout the micromodel due to hexadecane formations. Cell density increase and biofilm formation occurred mainly in regions that were well perfused. This can be expected, since these regions contained the highest nutrient supply.

In comparison with the biofilm monitoring experiments in the one phase system, the increase of cell density and formation of biofilm took significantly more time. For enhanced growth, the standard medium was enriched with (glucose containing) YPD. This unbalanced the ideal C:N ratio of 20:1, for optimal biofilm formation. This could make the biofilm formations less rigid than in the oil-free system. Indeed, microbial growth rates were lower and biofilm formation was observed in a less mature state, meaning less thick biofilm and less EPS secretion. Biofilm clusters were not part of a large web, it were individual clusters. In the early stage of biofilm monitoring during the oil-free experiment, individual biofilms were observed too. This

emphasizes that biofilm growth in the two-phase system was not developed into a full web of biofilms. Also possible toxicity of hexadecane should be taken into account.
In some cases, it was observed that biofilm formations were attached to a hexadecane droplet. This seems to indicate that hexadecane resembles a solid phase, hereby allowing the bacteria to bond.

PIV measurements

During PIV measurements in the two-phase system, tracer particle motion was disturbed and accurate flow measurements were not possible. This is likely to be a result of the different interaction of oil with the injected particles than the glass, which repels the particles.

Preliminary conclusions

In a two-phase system with hexadecane, accurate PIV measurements could not be performed yet. However the formation of biofilms in the 2-phase system were clearly observed. These biofilms remained more isolated and smaller. Small bubble formation indicates the formation of surfactants.

Image analysis provides a sophisticated tool to obtain experimental data that are quantifiable. This, in combination with pressure drop measurements, can provide correlations with biofilm formation and plugging of the micromodel in future experiments. This information can in its turn be used as basis for obtaining upscaled models on the core scale, which can be used in simulators for reservoir management.

The developed plasma etching technique is not yet suitable for micromodel construction, as state of the art techniques suffer from a limited depth reach regarding the phenomena we want to observe. This plasma etching technique however still looks promising considering that there is much room for improvement of the technique. However, also the pillars obtained with dry-etching are not completely cylindrical and have a conical shape. The pore throat diameters that can be obtained with dry-etching are smaller than those obtained with the wet-etching technique leading to a micromodel that is more suitable for interpretation.

7

Concluding Remarks

Summary and outlook

Inzichten voor de toekomst

Dankwoord (Acknowledgements)

Over de auteur

Publicatielijst (List of publications)

Concluding Remarks

Oil associated ecosystems like subsurface oil reservoirs, hydrocarbon seeps, and artificially created environments like oil separation tanks and pipelines host very specific microbial communities. The metabolic activities of these communities commonly can have detrimental effects like H_2S formation and M(icrobially) I(nduced) C(orrosion), but also can be potentially used to our advantage e.g. as information source of these environments and in several envisioned Microbial Enhanced Oil Recovery techniques like thief zone plugging and methane formation. The research as performed in this thesis was set out to make several steps towards answering two questions from which the overall two research fields, smart well technology and MEOR, could benefit. 1) Can community analysis be used as an indicator for environmental change, 2) What are the processes that lead to pore clogging.

Chapter two sets out to investigate the possibility of 'community analysis' as an information source of oil associated ecosystems. As a case study environment, the Berkel oil field (The Netherlands) and its associated oil-water separation surface facility units, was chosen. This choice was based on the fact that each unit holds water and oil from the same oil field, but the different treatments to separate the oil from the water in each unit posed a unique ecosystem to investigate the community and see if these differences caused specific shifts in the communities. Water samples were taken and their microbial communities were analyzed. It was shown that indeed the communities found, reflected their environment, regarding temperature, pH, presence of various electron acceptors and salt concentration. Our results additionally showed indications that the detection of specific single species might be used as indicator rather than the community as a whole. Examples of these species were *Anaerobaculum thermoterrenum*, *Halanerobium congolense* and *Methanocalculus halotolerans*, detected in the produced brine water (all isolated from oil field brine water samples), various members of the *Marinobacterium* genus in the oil-water separator tanks and *Thermodesulfovibrio yellowstonii* in the wash tank in which SO_3^{2-} is added for removing traces of oxygen before the water phase is reinjected. The research as described in this chapter points in favor of both community analysis and the use of specific species as indicators of subsurface environments but more research in this field needs to be performed even if many open questions remain.

In chapter 5 this research was extended towards a non-hydrocarbon subsurface environment. The work in this chapter showed that community analysis can indeed provide information about the environment itself which is obscured to our direct observations (the same holds for oil reservoir environments). In rural areas of Bangladesh, the communities of deep and shallow aquifers were investigated and compared, this with the purpose to investigate the role of

microorganisms in arsenic release into drinking water. Water samples were taken from various deep and shallow tube wells. Also geochemical characteristics were combined with the molecular biological results. In this chapter we showed that no arsenic was found in the deep aquifer and that the bacteria species identified were indicative of aerobic conditions and included members of the genera *Aquabacterium*, *Limnobacter*, and *Roseomonas*. In addition we found that the microbial populations of the shallow aquifer was dominated by species associated with arsenic tolerance and observed in previous investigations of arsenic contaminated environments, including *Comamonadaceae*, *Acidovorax*, *Acinetobacter*, and *Hydrogenophaga*. No known dissimilatory Fe(III) or As(V) reducers were identified. This led us to believe that bacteria were not responsible for arsenic mobilization in the shallow aquifer environment.

In chapter 3 the impact of a biocide on an oil-water separation plant was assessed. It was found that biocide indeed has an effect on the overall population; this effect however was short lasting. It was therefore concluded that multiple treatments are required to get a good effect to diminish microbial growth.

In chapter 4 a different type of sample was investigated, in this case the community of a core sample retrieved from an oil field was examined. Core sample studies are rare, since retrieving a core is difficult and costly. Still these studies are required to give definite answers to the main questions addressed in chapter 2. Core samples give a more complete overview of a downhole community since it contains also those species that are attached to surfaces and therefore will not be present in brine water wellhead samples. In this particular case an African core sample from the Rabi oil field (Gabon) was analysed using PCR-DGGE. We showed that this hypersaline core mainly contained microorganisms related to hypersaline environments. Sequences showed most identity to the Halobacteria, Clostridia, *Alphaproteobacteria* and *Gammaproteobacteria* Examples are *Orenia salinaria* and *Halanaerobium kushneri*. These nearest neighbours of all organisms should be able to sustain growth at *in situ* temperatures of the reservoir and were all known to display anaerobic fermentation. Interestingly, the analyzed spatial positions in the core displayed only minor differences on the DGGE gel, which indicates an equal distribution of microorganisms in the core.

In this thesis we already showed that community analysis can potentially be used as change indicators in oil associated environments, subsurface aquifers and cores. However if microorganisms are to be used as a change indicator from subsurface environments as a full application in smart well applications, efforts should be made to understand and investigate their

behavior and transport through subsurface reservoir rock. This is required since it is expected that it will be an essential part in the interpretations of the results of community analyses.

Next to the petroleum microbiology and their potential applications, also the formation of biofilms in porous media has been studied as the second topic in this PhD thesis.

In chapter 6 an experimental model system for the observation of biofilm formation at a pore level scale has been developed. The micromodel allowed us to monitor biofilm formation and flow diversion retardation due to the presence of this biofilm in the model system by combining the developed micromodel with a Particle Image Velocimetry set-up. We were able to demonstrate biofilm formation of the species *Pseudomonas chlororaphis* in an idealized "2D" reservoir rock system. These experiments provide detailed insights in processes on the pore level. Observed were bio-bridiging and straining as biofim initiators. With PIV techniques we showed that the biofilm induced flow irregularities at specific locations. On these locations, preferential flow pathways and wake zones were observed.

In the extension of chapter 6 the influence of a second liquid phase (hydrocarbon) on the biofilm formation was investigated during a preliminary experiment, which will be the basis of further research in this area.

Summary and outlook

Microbiology within the oil industry is relatively unknown and is often viewed with some suspicion. Yet all people that are employed in the oil industry cannot ignore the fact that the activities of microbes in oil reservoirs may have serious consequences. Consider the example of reservoir souring in which H_2S production poses a serious issue. Throughout the oil industry historically the philosophy has been that everything that is alive in the oil reservoir: 1) is irrelevant or 2) should be killed as soon as possible. Fortunately this is changing rapidly. Petroleum microbiology currently is an emerging discipline.

The past four years I have been working in the field of the microbiology of oil reservoirs and oil associated ecosystems. During my research I found that oil associated microbial life also provides opportunities for the petroleum industry. However these possibilities have, in most cases not been validated towards real applications and therefore remain for the time being fictive applications that linger only in the academic world. The proposed validations are still in their infancy, although in some areas much has been done and studied already.

A good example is the injection of nitrate to counterbalance sulphide accumulation. This nitrate injection is based on the natural substrate competition between sulphate reducers and nitrate

reducers, which will be won by the last group and thus will prevent unwanted H_2S production. This is one of the few biological techniques that on a small scale have been applied so far.
One reason for the near absence of well-studied (direct) applications is that reservoir engineering and biotechnology are two very different fields of expertise that are a world apart. Understanding of both disciplines and moreover understanding between the two disciplines is needed to come to good research and eventually a usable application. The fact that both fields are a world apart is logical, since there are 4 to 6 orders of magnitude between the scale on which the research is performed. Reservoir engineering is the complex field of flow modelling and uses to this purpose complete reservoir models. Often the model grid blocks in the reservoir model have dimensions of e.g. 100 × 100 × 20 m. Through the use of complex computer models the bulk flows are predicted in the reservoir. The research is predominantly focussed on making predictions regarding the production of oil and gas in the future. To serve this purpose, history matching is an important part; tuning the models and making simulations fit to match the production amounts as they occurred in the past to subsequently extrapolate the models towards the production amounts in the future. The reservoir is often regarded as a 'black box' environment based on a geological model and often research is aimed at calculating phase flows in the reservoir itself. Upscaling (exporting experiments to the field scale) is an important part of this research. To this purpose also practical research is being performed. Mechanisms are usually understood at the level of the drill core. The then applied equations and assumptions however are only valid at this scale. On a large scale different equations apply. A good example is Darcy's law. On the scale of the core permeability is isotropic (similar in x, y and z direction). This is however not true at reservoir scale. In this case the permeability perpendicular to the sediment is by approximation a factor 10 -100 lower than in the direction of the sediment. In these situations a permeability tensor is required holding 3 separate components.
It is important to know that within the oil industry only a few standard methods such as secondary recovery techniques, gas and water injection, components that are naturally present in the reservoir, are used. Tertiary recovery is, considering the entire oil industry, used very little. An example is the most popular tertiary extraction method 'steam soak' which only produces 3 to 5 % of the global oil production. The steam soak application can be summarised as follows: Steam is injected, which transfers its heat into the reservoir, subsequently the local oil in place becomes less viscous. This oil is then much easier back produced. Eventually it boils down to the following questions: What was produced yesterday, what are we producing today, and how can we apply this knowledge to predict what we are producing tomorrow?

Biotechnology deals with processes that occur at micro scale. From this first sentence it is already clear that this area of expertise does not fit within the just described framework of the reservoir engineering. The average size of the prokaryotic cell is around 1 μm^3. Freely translated, the term biotechnology comprises the application of microorganisms in different fields like the fine chemicals industry. Within the biotechnology area research is done towards processes that microorganisms are carrying out and mostly on how these are performed and if we are able to influence these processes to our advantage. This can be done at multiple levels. Examples are: genetics, metabolism and ecology. More than within the reservoir engineering, laboratory research is carried out. This is due to the fact that within the field of reservoir engineering, experimental research at field scale is difficult. Also interaction of microbes and (bio)chemical reactions are not scale dependent and can therefore be easily done in a laboratory. In an oil reservoir however many processes are scale dependent, this provides a practical problem when performing research at lab scale.

The differences just mentioned are reflected in the literature. From the title of the article it can often already be conducted if the article was written from the perspective of the biotechnology or the petroleum engineering. Both fields often ignore basic principles and important aspects which are basic in the other field. Microbiologists often claim to have discovered a Microbial Enhanced Oil Recovery technique, but they regularly ignore the many aspects of the reservoir engineering field like flow, diffusion of chemicals, fluid retention times and specific characteristics of the oil reservoir, which may eliminate desired activities of microbes. What reservoir engineers ignore to point as a priority is that small-scale processes can have significant effects on a large scale. Events that occur on the pore scale affect the macro-scale. For example the plugging of many pores on the micro scale with EPS or biomass, which on the macro-scale has the same effect as a permeability modifier. These molecules are polymers which are soluble and sorbed to the reservoir rock; due to these properties they retard water flow. When oil is present, these molecules (or their biological equivalents) precipitate on the pore wall to let the oil through. It is however difficult to take processes that happen on the small scale, into account within macro scale models. To do this requires an understanding of the processes on the micro-scale to eventually incorporate these phenomena in these models. Here lies the real challenge to which this thesis contributes. There are, within the large simulation programs, options for microbial aspects. Enough possibilities to start.

In the previous sections, I have briefly described the essence of both disciplines and the difficulties that have to be faced when the two are to be combined. On this basis, in combination

with information found in the literature and my own findings, I would like to address priority areas in the field of petroleum microbiology that according to me may lead to new insights and applications, and likewise, areas that I would not recommend to study further.

The overall described and desired MEOR mechanism in which is proposed that microbes perform wanted activities throughout a complete reservoir, will probably (for the large part) remain a hypothetical application. This conclusion can be drawn when one considers the often present large heterogeneities in oil reservoirs and the slow fluid flows in a reservoir. This combination makes it almost impossible to grow microbes at reservoir scale in a way that *we* intended. Main issues are, how does one get enough nutrients in the right place, and how does one create enough surface area between the oil and water phase to make the oil molecules as carbon source accessible for the micro organisms. It will be difficult to grow microorganisms within the complete reservoir and have them produce chemicals (like surfactants, or biomass) within an economically realistic time frame to eventually make an improved oil recovery possible.

A better option is perhaps to grow micro-organisms locally in a water producing layer of a producer (bioclogging). This process is local and more controllable. Due to the reduced watercut caused by the present biomass, this potentially can be an MEOR technique. The proposed application may also lead to more oil production through the effect of pore plugging within a high permeable layer. This forces the injected water from the water injector into low permeable layers which still contain oil.

In our research we already demonstrated that microorganisms are indeed capable of pore plugging and thus can modify the permeability of parts of the reservoir. It was also demonstrated that micro-organisms can be used for soil reinforcement purposes (van Paassen et al. 2009). This opens the road towards a microbiological well stimulation or a 'reduced water cut' mechanism.

Research should be expanded with core experiments and 3D imaging techniques to reach a better understanding of biofilm formation in porous media. Furthermore it is clear from the performed research, that the much discussed biomonitoring process is a realistic option which can be introduced into the petroleum industry. A change in a certain ecosystem affects the complete population, other species become dominant as a consequence of the induced change. This change can be picked up by the used Molecular Biological Methods; see previous chapters in this thesis. Little conversion of substrate is needed to support a substantial number of specific cells that later on can be picked up by the used methods. This indicates their sensitivity. The application of biological indicators to monitor changes in oil reservoirs is feasible and could be construed into a so-called 'smart-well' application as it was originally proposed within the ISAPP framework (see chapter 1, scope and outline). All knowledge that is obtained should be integrated

into one single database. Additional *in situ* research is however required to expand this database or to complete it. Found species have to be linked to changing reservoir conditions eventually to find species that match a specific combination of conditions and become dominant during this combination. This should lead to a technique in which changing conditions (wanted or unwanted) can be detected in an early stage. This would also require a standardisation of the used molecular techniques to allow a quick screening of the wells. More research is however required to see if species can indeed be linked to the conditions downhole. As the temperature of the reservoir is higher, this will become more difficult. At higher (reservoir) temperatures, the concentration and species richness of microbes will be different and possibly less divers. This allows additional growth contained higher up in the well pipe at lower temperatures, which disrupts the measurement. This argument is often heard from the oil industry. The studied production water may not be representative for what is actually happening downhole. Research is however performed towards stratification in high temperature reservoirs during flooding. At low production rates, a clear temperature change will occur in the pipe.

From the oil industry requests are made to develop a so called 'dipstick' method (a standardised high throughput method). This to me however seems far away since the used techniques are specific, require a time investment and a good overall knowledge of the molecular methods. Also the possible target parameters for the, to be developed, dipstick method are not yet known. A good example is the research towards the genes and proteins that are involved in the anaerobic alkane activation through fumarate addition (chapter 1), which is still in its early stages. In this thesis we therefore chose to look into the total microbial diversity. The upcoming metagenomics techniques can contribute to the knowledge on oil field ecosystems and the found species. This is becoming more attractive as the price of sequencing is decreasing fast.

The overall conclusion that I want to draw from all that is said above is that the petroleum microbiology should be more studied and developed towards tools intended for reservoir information collection. If microbes are to be used as acting agents in the reservoir, it should be noted that I would recommend to first study and develop methods at the well level. These methods have better chances of success since the flows at the well level are higher and the well level is smaller; this is what makes the whole process better controllable.

Success to you all,

Geert M. van der Kraan

May 31, 2010

Samenvatting en inzichten voor de toekomst.

Microbiologie binnen de oliewereld is relatief onbekend en wordt vaak met enige argwaan bekeken. Toch kunnen alle mensen die binnen de oliewereld werken niet om het feit heen dat de activiteiten van deze kleine beestjes in oliereservoirs grote gevolgen kunnen hebben. Denk als voorbeeld aan 'reservoir souring' waarin de productie van H_2S een enorm probleem is. Binnen de oliewereld is vanuit het verleden de filosofie dat, alles wat er aan microorganismen in het reservoir leeft 1) niet relevant is of 2) zo snel mogelijk moet worden afgemaakt. Gelukkig is dit in hoog tempo aan het veranderen. De petroleum microbiologie is momenteel een opkomend vakgebied.

De afgelopen 4 jaar heb ik mij bezig gehouden met de microbiologie van oliereservoirs en olie geassocieerde ecosystemen. Tijdens mijn onderzoek ben ik er achter gekomen dat het microbiële leven dat met petroleum is geassocieerd ook mogelijkheden biedt voor de petroleum winning. Echter zijn deze mogelijkheden vaak nog weinig tot helemaal niet gevalideerd ten behoeve van een reële toepassing, en blijven deze alleen (fictief) bestaan binnen de academische wereld. Validatie staat nog in de kinderschoenen, ondanks dat er op enkele gebieden toch al veel is gedaan. Een goed voorbeeld is het injecteren van nitraat om sulfide accumulatie tegen te gaan. Deze nitraatinjectie is gebaseerd op de natuurlijke substraatcompetitie tussen sulfaatreduceerders en denitrificeerders die door de laatste groep zal worden gewonnen, wat ongewenste H_2S productie tegengaat. Dit is een van de weinige biologische technieken die op kleine schaal wel al wordt toegepast.

Een van de oorzaken voor het grotendeels ontbreken van goed onderzochte (directe) toepassingen is dat de vakgebieden biotechnologie en reservoir engineering ver uit elkaar liggen. Begrip van beide vakgebieden en tussen beide vakgebieden is noodzakelijk om tot een goed onderzoek en een uiteindelijke applicatie te komen. Het feit dat deze twee vakgebieden een wereld van verschil zijn is logisch, aangezien er tussen de 4 en 6 ordegroottes zitten tussen de schaal waarop onderzoek wordt gedaan. Reservoir engineering is het complexe vakgebied van de modellering van stromingen en maakt gebruik van complete oliereservoir modellen. Vaak wordt er gewerkt met model gridblokken van bijvoorbeeld 100 × 100 × 20 m waarin via complexe computermodellen naar bulkstromingen wordt gekeken. Het onderzoek is vooral toegespitst op het doen van voorspellingen van de olie en gas productie in de toekomst. Hiervoor is 'history matching' een belangrijk onderdeel, modellen afstellen en het doen van simulaties om in het verleden behaalde productie resultaten te matchen om deze vervolgens te extrapoleren naar de toekomstige productie. Het reservoir wordt vaak gezien als een black box gebaseerd op een geologisch model en veelal zijn onderzoeken erop gericht informatie over fasestromingen in het

reservoir te berekenen. Opschaling (experimenten brengen naar verldschaal) is een belangrijk aspect en hier wordt ook een stuk praktisch onderzoek aan verricht. Mechanismen in poreuze media worden meestal begrepen op boorkern niveau; echter zijn de dan toegepaste vergelijkingen en aannames alleen geldig op deze schaal. Op grote schaal echter gelden andere vergelijkingen. Een goed voorbeeld hiervan is de wet van Darcy. Op de schaal van de boorkern is de permeabiliteit isotroop (hetzelfde in de x,y en z richting). Dit geldt echter niet op grote schaal waar de permeabiliteit loodrecht op de laagrichting door de bank genomen vaak een factor 10 tot 100 lager is dan in de laagrichting.

Over het algemeen is er dan een permeabiliteits tensor nodig met drie onafhankelijke componenten. Belangrijk is om te weten dat er binnen het overgrote deel van de oliewinning slechts enkele standaardmethoden zoals secundaire winningstechnieken; injecteren van gas en water. i.e., vloeistoffen die van nature reeds in het reservoir aanwezig zijn, worden toegepast. Tertiaire olie winning wordt, over de gehele industrie bekeken, slechts mondjesmaat toegepast. Bijvoorbeeld zorgt de meest populaire tertiaire winningmethode, steam soak, slechts voor 3 tot 5 % van de globale olieproductie. De 'steam soak' techniek komt in het kort op het volgende neer. Stoom wordt geïnjecteerd, die zijn hitte afgeeft in het reservoir, met als gevolg dat de viscositeit van de olie afneemt en hierdoor makkelijker kan worden geproduceerd. Uiteindelijk draait het om de volgende vragen, wat produceerden we gisteren aan olie gas en water, wat produceren we vandaag, en hoe kunnen we deze informatie gebruiken om te voorspellen wat we mogelijk morgen produceren.

De biotechnologie houdt zich bezig met processen die zich op microschaal voordoen. Uit deze eerste zin blijkt al dat dit vakgebied niet binnen het net beschreven algemene kader van de reservoir engineering valt. De gemiddelde prokaryote cel grootte ligt rond de 1 μm^3. Vrij vertaald staat de biotechnologie voor het toepassen van levende micro-organismen binnen allerlei applicaties waaronder de fijnchemicaliën-industrie. Binnen het vakgebied van de biotechnologie wordt gekeken naar processen die levende (micro)organismen uitvoeren en vooral hoe ze dat doen en of wij daar mogelijk invloed op kunnen uitoefenen. Dit kan op verschillende niveaus; voorbeelden zijn genetica, metabolisme, ecologie. Meer dan binnen de reservoir engineering, wordt er laboratorium onderzoek gedaan. Dit komt voornamelijk omdat binnen de reservoir engineering, onderzoek op veldschaal moeilijk is. Ook is het zo dat interacties tussen microben en (bio)chemische reacties niet schaalafhankelijk zijn. In een reservoir zijn veel processen schaalafhankelijk, dit levert een praktisch probleem op als men onderzoek op laboratoriumschaal doet.

De net genoemde verschillen zijn terug te vinden in de literatuur. Uit de titel is vaak al duidelijk of het artikel geschreven is vanuit de biotechnologie oogpunt of uit het oogpunt van de petroleum winning. Beide kanten gaan vaak voorbij aan belangrijke aspecten en basis-limitaties uit de beide vakgebieden. Zo claimen veel microbiologen dat ze een microbial enhanced oil recovery methode hebben gevonden maar gaan ze vaak voorbij aan de vele aspecten die met reservoir engineering hebben te maken zoals, stromingen, diffusie van stoffen, vloeistof retentietijden in oliereservoirs en specifieke karakteristieken van oliereservoirs, die de effecten van microben teniet doen. Wat reservoir-engineers niet direct als prioriteit stellen, is dat processen op kleine schaal belangrijke gevolgen kunnen hebben op grote schaal. Ook dingen die op porieniveau gebeuren hebben invloed op macroschaal processen. Een voorbeeld is het op grote schaal pluggen van poriën die op macroschaal hetzelfde effect hebben als een "permeability modifier". Dit zijn polymeermoleculen die oplosbaar zijn en geadsorbeerd zijn aan het reservoir gesteente; daardoor reduceren ze de waterstroming, als er echter olie voorbij komt slaan deze moleculen (of hun biologisch geproduceerde equivalenten) neer op de poriewand en laten de olie beter door. Het is echter erg moeilijk om de processen op microschaal mee te nemen binnen macroschaal modellen. Om dit te doen is er een begrip nodig van de processen op microschaal opdat deze in een later stadium begrepen kunnen worden en wel meegenomen kunnen worden in de modellen. Hier ligt de echte uitdaging waar wij (mijn team en ik) aan hebben bijgedragen. Er zitten binnen de grote simulatieprogramma's al opties voor microbiologische aspecten, mogelijkheden genoeg.

In de vorige paragrafen heb ik kort de essentie van beide vakgebieden beschreven evenals de moeilijkheden die er zijn in het combineren van beide vakgebieden. Aan de hand hiervan wil ik in combinatie met de in de literatuur beschreven onderzoeken en mijn eigen bevindingen binnen het vakgebied van de petroleum microbiologie de volgende speerpunten voor vervolgonderzoek aangeven die mijns inziens tot nieuwe inzichten en applicaties kunnen leiden evenals richtingen die ik zou afraden:
Het alom beschreven en gewenste reservoir scale MEOR mechanisme waarin een voorstelling gemaakt wordt dat microorganismen door het gehele reservoir activiteiten ontplooien, zal waarschijnlijk een grotendeels hypothetische toepassing blijven. Tot deze conclusie kom ik door de vaak aanwezige grote heterogeniteit in reservoirs en lage vloeistof stroomsnelheden in een oliereservoir. Deze combinatie maakt het nagenoeg onmogelijk om microben op reservoir scale te laten groeien op de manier waarop *wij* dat willen. Hoofdproblemen zijn, hoe krijg je voldoende nutriënten op de plaats waar je ze hebben wilt en hoe creëer je voldoende oppervlak om de olie

als voedselbron toegankelijk te maken. Het zal moeilijk zijn om micro-organismen te laten groeien in het gehele reservoir en binnen een economisch realistische tijdschaal gewilde chemicaliën (zoals surfactants) of biomassa te laten produceren om zo een verbeterde oliewinning mogelijk te maken (micro-organismen kunnen tot 25% van de olie omzetten voordat het economische verlies significant wordt)

Een betere optie is het lokaal laten groeien van bacteriën in een waterproducerende laag van een producer (bioclogging). Dit is een proces dat beter controleerbaar en lokaler is. Vanwege de verminderde waterproductie kan dit worden gezien als een potentiële methode om microbiologie te gebruiken als toepassing om de olieproductie te verbeteren. Dit proces kan mogelijk ook leiden tot meer olieproductie door het mogelijke effect van pluggen van poriën binnen een hoog permeabele laag. Dit dwingt het geïnjecteerde water in een nog olie bevattende laag.

In dit onderzoek is aangetoond dat microorganismen inderdaad in staat zijn tot het pluggen van poriën en dus de permeabiliteit van een reservoir kunnen aanpassen. Ook is al aangetoond dat microorganismen in grondversteviging kunnen worden toegepast (van Paassen et al.). Dit maakt de toepassing van een biologische well stimulation c.q. een “reduced watercut mechanisme” mogelijk. Het onderzoek zou moeten worden uitgebreid met core experimenten en 3D beeld technieken om tot een beter begrip te komen van biofilm formatie in poreuze media.

Verder is duidelijk uit het gedane overzicht dat het veelbesproken biomonitoring een reële optie is om in te voeren binnen de petroleum industrie. Een ecosysteem verandering heeft zijn weerslag op de soorten die dominant worden en dit kan dus worden opgepikt met moleculair biologische methoden; zie eerdere hoofdstukken in dit proefschrift. Aangezien er weinig conversie van substraten nodig is om meer cellen van een specifieke soort te ondersteunen en later op te pikken met deze methoden, geeft een hoge gevoeligheid aan. De toepassing van biologische indicatoren om reservoir veranderingen te volgen is dus haalbaar en zou kunnen gaan gelden als een zogenoemde ‘smart-well application’ zoals voorgesteld binnen het ISAPP raamwerk, (zie hoofdstuk 1, scope and outline). Alle kennis die al is opgedaan zou moeten worden geïntegreerd in één database. Echter extra *in situ* onderzoek is nodig om deze database mogelijk uit te breiden of te completeren. Gevonden soorten moeten worden gekoppeld aan veranderende reservoir karakteristieken opdat er echt specifiek passende soorten worden gevonden die onder bepaalde omstandigheden dominant worden en dus bepaalde gewilde of ongewilde verandering al in een vroeg stadium kunnen aangeven. Ook zou hiervoor een standaardisatie van de Moleculair-Biologische methoden nodig zijn opdat putten snel kunnen worden gescreend. Meer onderzoek is echter nodig naar de vraag of de gevonden soorten ook daadwerkelijk zijn terug te koppelen aan de down-hole condities. Naarmate de temperatuur van

het reservoir hoger ligt, zal dit moeilijker worden. Bij hogere (reservoir) temperaturen zal de concentratie en soortenrijkheid van microben anders in samenstelling en mogelijk minder divers zijn zodat eventuele additionele groei in de put bij lagere temperaturen het beeld kan verstoren.
Dit is een bezwaar dat vaak gehoord wordt vanuit de hoek van de oliemaatschappijen.
Het onderzochte productiewater is mogelijk niet meer representatief voor wat er op de eigenlijke diepte gebeurt. Er wordt echter al onderzoek gedaan naar de stratificatie binnen hoge temperatuur olievelden tijdens reservoir flooding. Bij lage productiesnelheden zal er in de pijp een duidelijke temperatuursverandering binnen putten van hete oliereservoirs optreden. Er wordt vanuit de olie-industrie veel gevraagd om een zogenoemde dipstick methode (een gestandaardiseerde high throughput screenings methode). Echter lijkt mij dit nog ver weg, aangezien Moleculair Microbiologische Methoden erg specifiek zijn en veel kennis vergen. Ook is de zogenoemde doelparameter van een mogelijke dipstickmethode nog niet bekend. Een voorbeeld hiervan is dat het onderzoek naar de genen en eiwitten betrokken bij de anaerobe alkaan activatie door fumaraat additie (zie hoofdstuk 1) nog in de kinderschoenen staat. In dit proefschrift hebben we daarom gekeken naar de microbiele diversiteit als geheel. De steeds populairder wordende metagenomics projecten kunnen wel een belangrijke bijdrage leveren aan de kennis betreffende olieveld ecosystemen en soorten. Dit wordt aantrekkelijker aangezien het "sequencen" van genetisch materiaal steeds minder kosten met zich meebrengt
De conclusie die ik uit het bovenstaande wil trekken is dat mijns inziens de biotechnologie meer richting de reservoir informatiewinning zou moeten worden ontwikkeld. Als men dan toch microben wil inzetten als actoren in het reservoir doet men er zeker goed aan eerst toepassingen te onderzoeken/ontwikkelen op put niveau, deze hebben meer kans van slagen aangezien de stroomsnelheden bij de put hoger zijn en het putniveau kleinschaliger is; dit maakt het hele proces beter controleerbaar.

Succes,

Geert M. van der Kraan
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Ik kan natuurlijk niet eindigen zonder een woord van dank richting mijn naaste familie, die mij support hebben gegeven gedurende mijn 4 jarige onderzoeksproject. Mijn ouders Chris en Gonnie en mijn (kleine) zusje Mathilde (en haar Niels), bedankt voor jullie luisterend oor, interesse en support. Als allerlaatste kom ik aan bij mijn allerliefste schat Daniëlla, met wie ik al meer dan 8 jaar een gelukkig en vrolijk leven deel en die al die tijd vertrouwen heeft gehad in mij en vaak juist die dingen zei die nodig waren om mij weer te stimuleren om door te gaan.

Als laatste een woord aan de toekomst:
Je bent nu nog zo klein en zit nog in de buik van mama, ik hoop dat ik je binnenkort mag begroeten als een persoon die ons leven verrijkt, ik ben nu al zo benieuwd naar wat je gaat worden en hoe je leven eruit gaat zien. Op een goed gesprek over een jaar of 20. Maar eerst zal ik vooral mijn best doen een goede vader te zijn.

Over de auteur

Op 5 november 1979 tijdens een koude herfstmorgen, werd Geert geboren in het Zeeuwse Goes. Vervolgens groeide hij op in Kats, een klein Noord-Bevelands dorpje, waar hij ook naar de basisschool ging. Deze school, de prinses Margrietschool legde de basis voor een nieuwsgierige inborst, hier haalde Geert ook zijn eerste officiële diploma's (zwem, schaak, judo). Na de basisschool vervolgde hij in 1993 zijn opleiding op de Stedelijke scholengemeenschap "Het Goese Lyceum," waar hij in 1998 zijn HAVO diploma haalde. Tijdens deze tijd op de middelbare school kwam hij erachter dat hij een interesse had in de vakgebieden Biologie, Scheikunde en Natuurkunde. Na een aantal prettige gesprekken met zijn ouders heeft hij toen besloten na het HAVO nog 2 jaar VWO te doen, wat een overstap naar de Universiteit mogelijk maakte. In 2000 haalde Geert zijn VWO diploma. Na een middag op de Utrechtse studiebeurs datzelfde jaar was de keuze snel gemaakt, Geert verhuisde naar Delft en begon met de opleiding Life Science & Technology, een toen nieuwe opleiding van de Universiteit Leiden en de Technische Universiteit Delft. Het was duidelijk dat hij zijn interessegebieden in deze opleiding gecombineerd waren en het een goede keuze was. In de periode 2001-2005 was Geert betrokken bij het LS&T informatieteam, en gaf hij voorlichting over de opleiding op middelbare scholen en studiebeurzen.
In 2003, deed hij zijn B.Sc onderzoek bij de vakgroep Biocatalysis and Organic Chemistry van de TU Delft onder begeleiding van dr. ir. Luuk van Langen waar hij onderzoek deed naar het

immobiliseren van het enzym S-Oxynitrilase, waarmee moleculen met een aldehyde groep bij milde condities voorzien konden worden van een reactieve CN groep. Het geïmmobiliseerde enzym is getest op de substraten kaneelaldehyde, benzaldehyde en acroleïne. Uiteindelijk is er een manier gevonden om het enzym zonder verlies van activiteit te immobiliseren en te hergebruiken, wat de weg opende richting industrieel gebruik. Dit onderzoek is in 2006 gepubliceerd in Advanced Synthesis & Catalysis.
Vervolgens liep hij in 2004 bedrijfsstage bij het bedrijfje CLEA Technologies waar hij zich bezighield de hydrolysereacties van aminozure esters met behulp van alcalase enzymen en het opsluiten van enzymen in alginaatbolletjes. Belangrijk was het onderzoek naar de stabiliteit van de bolletjes waar een optimum voor is gevonden. Begin 2005 is Geert actief geweest als studentassistent aan de Universiteit Leiden waar hij de practica Biochemie I en II begeleide. In september 2005 begon hij met zijn M.Sc afstudeerwerk bij het bedrijf PURAC Biochem onder begeleiding van dr. ir. Diana Visser en dr. ir. Mickel Jansen, waar hij bezig is geweest met het opzetten van een proces waarin Tapioca zetmeel direct omgezet werd naar natuurlijk melkzuur. Aan het einde van zijn stage realiseerde hij een "proof of principle" van dit proces op laboratoriumschaal. In dit proces werd zetmeel eerst "koud" vervloeid en versuikerd met behulp van een combinatie van een α-amylase en een glucoamylase. De gevormde carbohydraat moleculen werd vervolgens omgezet met behulp van een microorganisme tot melkzuur. De uitdaging zat in het combineren van de optimale eigenschappen van de 'enzym combinatie' en het microorganisme. Nadat hij in 2006 zijn ingenieurstitel haalde is hij voor korte tijd nog in dienst geweest bij PURAC als onderzoeker. Het was echter duidelijk dat hij nog een promotie onderzoek wilde doen. In september 2006 is Geert begonnen bij de vakgroepen Milieubiotechnologie en Petroleum engineering (Beide TU Delft) onder begeleiding van prof. dr. ir. Mark van Loosdrecht, prof. dr. Hans Bruining, dr. Gerard Muyzer, dr. Bart Lomans (Shell), en Cor Kuijvenhoven (Shell) op het gebied van de petroleum Microbiologie. Dit heeft geleid tot een exploratief onderzoek binnen het brede kader van dit onderzoeksveld waarvan het resultaat thans beschreven is in dit proefschrift. In de zomer van 2007 vertrok Geert voor korte tijd naar de Verenigde Staten waar hij aan het Marine Biological Laboratory (Woods Hole, MA) een kort onderzoek heeft gedaan op het gebied van ecosysteem successiestaten op microbieel niveau. Op 8 augustus 2008 trouwde Geert met Daniëlla van Leeuwen, wie hij in 2002 had leren kennen Thans wonen zij samen happy in Zeeland en verwachten zij hun eerste kindje eind oktober 2010.
Vanaf november 2010 zal Geert als Microbioloog aan de slag gaan bij 'The DOW Chemical Company' binnen de 'Microbial Control Group.'

Daniëlla en Geert op de Oost Zeedijk, Noord-Beveland, 08-08-2008

List of Publications

Journal papers

Geert M. van der Kraan, Floris Buijzen, Maarten de Ridder, Barbara Thuss, Mario Laros, Gerard Muyzer, Mark C.M. van Loosdrecht & Johannes Bruining. *Development of an etched transparent micromodel for observation of microbes in porous media.*
Accepted for presentation at the 2010 SPE Annual Technical Conference and Exhibition (ATCE) to be held in September 2010. Florence, Italy. With its associated SPE paper (requested to be refereed).

Geert M. van der Kraan, Johannes Bruining, Bart P. Lomans, Mark C. M. van Loosdrecht & Gerard Muyzer. *Microbial diversity of an oil-water processing site and its associated oil field: The possible role of microorganisms as information carriers from oil-associated environments.* FEMS Microbiology Ecology, Vol 71 (3), pages 428-443, (2010)

Nora B. Sutton, **Geert M. van der Kraan**, Mark C.M. van Loosdrecht, Gerard Muyzer,
Johannes Bruining & Ruud J. Schotting. *Characterization of geochemical constituents and bacterial populations associated with As mobilization in deep and shallow tube wells in Bangladesh,* Water Research, Vol 43, pages 1720-1730, (2009)

Gerard Muyzer & **Geert M. van der Kraan.**
Bacteria from hydrocarbon seep areas growing on short-chain alkanes.
Trends in Microbiology, Vol. 16, No 4, (2008)

Book chapters

Geert M. van der Kraan, Maarten de Ridder, Cor A. T. Kuijvenhoven & Gerard Muyzer, *Sampling and nucleic extraction procedures from oil reservoir samples*, Applied Microbiology and Molecular Biology in Oil Field Systems ISBN: 978-90-481-9251-9 (2010), (Including a method chapter)
1st Edition, Whitby, Corinne, Lund Skovhus, Torben (Eds.)

Geert M. van der Kraan, Floris Buijzen, Bart P. Lomans & Gerard Muyzer, *PCR-DGGE case study on an oil field core sample,* Applied Microbiology and Molecular Biology in Oil Field Systems ISBN: 978-90-481-9251-9 (2010), (Including a method chapter)
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Salimi, H, **Kraan, G.M. van der**, Laros, M, & Bruining, J, *Construction of glass micromodels with pore-size distributions in the 10-100 μm range using wet- and dry-etching techniques for the observation of counter-current imbibition.* In: 10th International Symposium on Reservoir Wettability and its Effect on Oil Recovery, Abu Dhabi, AE, 26-28 October 2008 (pp. 1-13). Abu Dhabi. (2008).

Other journal publications

Andrzej Chmura, **Geert M. van der Kraan**, Filip Kielar, Luuk M. van Langen, Fred van Randwijk & Roger A. Sheldon. *Cross-Linked Aggregates of the Hydroxynitrile Lyase from Manihot esculenta: Highly active and Robust Biocatalysts.* Advanced Synthesis and Catalysis, Volume 348, Issue 12-13, (2006)

Popular scientific publications

Geert M. van der Kraan, Floris Buijzen, Maarten de Ridder, "Biogeo-engineering," *Unraveling subsurface processes using model systems,* DCMaterials, Newsletter, December 2009
Geert M. van der Kraan, *"De Heilige graal," Microben in de olie,* Chemisch 2 Weekblad (C2W), Jrg 105, #11 (2009)
Geert M. van der Kraan, "Tussenstand," In de Olie, Delta, weekblad van de Technische Universiteit Delft (2009)

Relevant talks

Development of a glass-etched micromodel for the observation of biofilm formation in 2D-porous media (The effects of biofilm presence on flow diversion), International Conference on Non-Linearities and Upscaling in Porous Media, (NUPUS) at the Universität Stuttgart, Stuttgart, Germany, October 5-7, 2009

Microbial Diversity of an Oil-water Processing Site and its associated Oil Field Production Water , International Symposium on Applied Microbiology and Molecular Biology in Oil Systems (ISMOS-2) at the Danish Institute of Technology (DTI) Aarhus, Denmark, June 17-19, 2009.

Microorganisms as information carriers from subsurface environments and industrial sites, ISAPP-Symposium, Delft University of Technology, October 29, 2008

What about microbial enhanced oil recovery? Enhanced Oil Recovery Workshop, Delft University of Technology, September 10, 2008

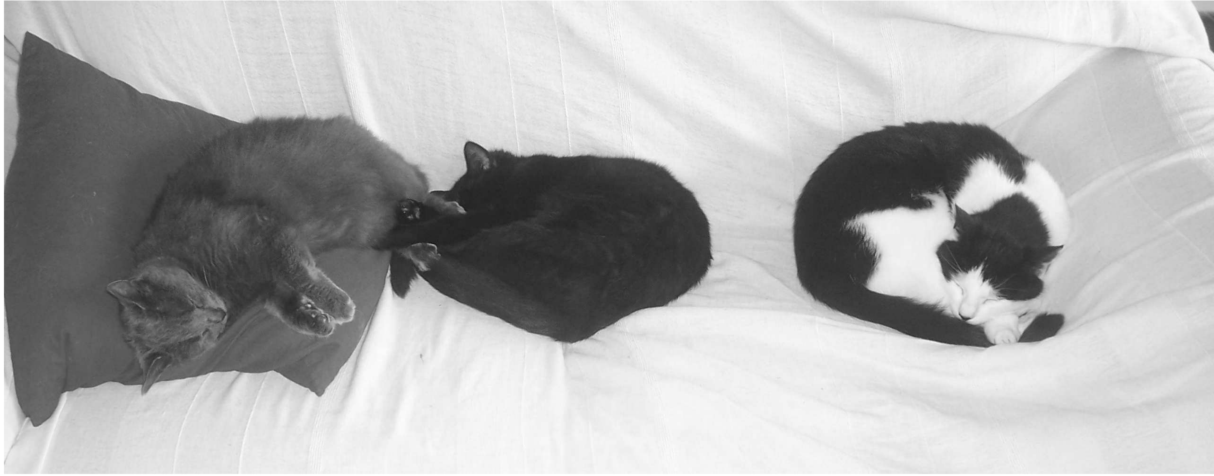
Microbial detection in oil water systems, Research day, department of Geosciences, Delft University of Technology, February 21, 2008

Measurement of biological activity during oil recovery, Meeting Shell Exploration&Production/Statoil, Shell Exploration&Production, 20 September 2007.

Microbial Detection in oil wells, Biofilm day, Technical University of Eindhoven, 6 September 2007

Measurement of biological activity during Microbial Enhanced Oil Recovery (MEOR), ISAPP-Symposium, Delft University of Technology, 7 June 2007.

Microbial detection in the subsurface, Workshop Microbial Enhanced Oil Recovery, Shell Exploration and Production, March 20, 2007



Doenja *Smokey* ……………*en* *Taz*

Op de bank, in het nieuwe huis (augustus 2010)