Chapter 9
Subsurface Petroleum Microbiology

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9.1 Introduction

Biogeochemical processes carried out by the microorganisms in near or deep subsurface sediments have been the subject of much interest during the last two decades (Magot et al. 2000; Van Hamme et al. 2003; Jones et al. 2008; Voordouw 2011). The possibility for living organisms to survive or thrive in extreme oilfield environments depends on the ecosystem physicochemical characteristics. Plate tectonics, basin, and petroleum system formation are believed to be the vital processes that affect deep subsurface biological systems and are integral to interactions between the biosphere and geosphere in petroleum reservoirs (Head et al. 2003). They alter the physical environment, control the formation and distribution of organic carbon, and influence delivery of nutrients and oxidants that can be utilized by resident subsurface microbes.

Petroleum reservoirs, generally found in porous and permeable sediments such as sandstones and limestones, exhibit a wide range of temperatures (30–180 °C), salinities (up to 300 g/L NaCl), and pressures (up to several hundred bars). Essentially anaerobic, these extreme subsurface ecosystems were long considered

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too hostile for microbial life. However, with the exception of those exhibiting very high temperature and salinity, there is now evidence that many petroleum reservoirs contain active and diverse populations of chemolithotrophic and heterotrophic microorganisms with a wide range of metabolic abilities (Magot et al. 2000; Head et al. 2003; Galperin and Kaplan 2011). Besides potentially being indigenous, some of these microbial communities may have been introduced as contaminants during reservoir flooding with seawater, during drilling processes, or after migration through aquifers from terrestrial and sub-terrestrial sites (Jones et al. 2008). Currently, the World's petroleum inventory is dominated by the biodegraded petroleum reservoirs, but determining the microbial processes that have led to the biodegradation of hydrocarbons remains a challenge due to the inherent difficulties in collecting representative samples from deep subsurface petroleum reservoirs. An overview of our current knowledge on subsurface petroleum microbiology including the microbial diversity and metabolic processes in petroleum oilfields and reservoirs are provided in this chapter together with a shorter section providing examples of microbial processes in deep aqueous environments.

9.2 Metabolic Processes

Although the role of microorganisms in subsurface petroleum transformation and the likelihood that they are almost exclusively anaerobic are now well accepted, important details such as the identities and specific metabolic activities of indigenous microbes are still obscure (Röling et al. 2003). It has been hypothesized that the majority of microbial activity occurs at the interface between oil-saturated and water-saturated regions of reservoirs, which is the zone of recharge with water-soluble nutrients and oxidants (Larter et al. 2003).

Geothermally heated oil reservoirs contain an excess of reduced electron donors including a range of hydrocarbons, H_2, and organic acids. The most commonly found fatty acids in many formation waters are acetate, butyrate, formate, propionate, and benzoate. These substrates may be oxidized by anaerobic microbial communities, which include sulfate-reducing bacteria (SRB) and methanogenic *Archaea*. More complex organic acids such as naphthenic acids are present in crude oil and amino acids may also accumulate during the kerogen maturation process (Foght 2010). Hydrogen, possibly resulting from abiotic reactions (mineral hydrolysis) at high temperature in the deep subsurface, may also act as a significant source of energy for the hydrogenotrophic microorganisms retrieved from oil reservoirs. The use of H_2 may favor the anaerobic oxidation of hydrocarbons in the presence of sulfate and CO_2 as terminal electron acceptors or may cause a shift in electron flow during fermentative processes, resulting in the formation of less-reduced end products of metabolism such as acetate, propionate, and isobutyrate. Hydrogenotrophic sulfate reducers and methanogenic *Archaea* have been frequently isolated from formation water and there are reports on homoacetogens having the ability to oxidize hydrogen and reduce CO_2 into acetate (Jones

et al. 2008; Ollivier and Alazard 2010). In the deep biosphere, H_2 generated from abiotic (mineral hydrolysis) and biotic (hydrocarbon oxidation, fermentative processes) reactions seems to be a primary source of energy for methanogenic *Archaea* and SRB.

The type of metabolic processes occurring in oil reservoirs depends to a great extent on the availability of electron acceptors. Since many reservoirs have limited dissolved oxygen, it is unlikely to be available in significant amounts to enable efficient aerobic biodegradation (Skaare et al. 2011). Similarly, nitrate is relatively rare in oil reservoirs, except when specifically added to injection water to prevent souring. Dissimilatory iron-reducing microorganisms and methanogenic *Archaea* have been isolated from many production water samples and other extreme environments within the deep subsurface, linking subsurface hydrocarbon degradation to iron reduction and methanogenesis/CO_2 reduction (Head et al. 2003). The availability of fixed nitrogen likely does not limit microbial activity in reservoirs because ammonium ions buffered by reservoir minerals, N_2 gas, and N heterocycles could provide adequate N in situ. However, the availability of water-soluble nutrients like phosphorus and/or oxidants (terminal electron acceptors such as Fe^{3+}, SO_4^{2-}, or CO_2) is more likely to limit microbial activity.

Since stratal waters often contain sulfate and carbonate, sulfate reduction, methanogenesis, fermentation, and homoacetogenesis may be expected as the major metabolic processes within oil reservoirs (Ollivier and Alazard 2010):

Sulfate reduction: $SO_4^{2-} + 4H_2 + 2H^+ \rightarrow H_2S + 4H_2O$
Hydrogenotrophic methanogenesis: $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$
Acetoclastic methanogenesis: $CH_3COOH \rightarrow CH_4 + CO_2$
Homoacetogenesis: $2HCO_3^- + H^+ + 4H_2 \rightarrow CH_3COO^- + 4H_2O$

9.3 Biotransformation of Petroleum Hydrocarbons

Microbial degradation of oil in petroleum reservoirs is a globally significant biogeochemical process. The physical and chemical changes that result from reservoir biodegradation of crude oil have important operational and economic consequences. Removal of the lighter saturated hydrocarbon fraction increases the polar fraction of resins and asphaltenes in the oil resulting in increased viscosity and production costs and reduced American Petroleum Institute (API) gravity and oil value. In addition, biodegradation results in increased acidity and sulfur content leading to corrosion problems during oil transport, refining, and processing. Out of Earth's estimated ten trillion barrel oil inventory, heavy oils and tars constitute around six trillion barrels (Head et al. 2003).

Hydrocarbon-degrading bacteria able to exploit petroleum hydrocarbons for producing energy and biomass were first isolated almost a century ago (Head et al. 2003). The two principal modes by which microorganisms can harvest energy for building the new cell material are respiration and fermentation. Biodegradation

of hydrocarbons by aerobic bacteria is supported by the presence of oxygen in petroleum reservoirs, whereas anaerobic heterotrophic microorganisms requiring nitrate, sulfate, iron, manganese, or carbon dioxide as electron acceptors are responsible for biodegradation of hydrocarbons in the absence of oxygen. Water controls both physical and physiological processes of microbial activity and hence plays an important role in subsurface microbial habitats. In order for a microbial cell to metabolize a compound, the enzymes of the cell must gain direct access to the compound and that must either be transported across the cell membrane into the bacterial cell or at least directly contact membrane-bound enzymes at the cell surface in the case of hydrophobic substrates such as saturated hydrocarbons (Wentzel et al. 2007). Substrate uptake takes place through either passive or facilitated diffusion at the point of contact (Van Hamme et al. 2003).

The effect of microbial activity on the total petroleum hydrocarbon composition in oils is well documented and reflects the removal of different compound classes of petroleum oils in anaerobic subsurface reservoirs (Magot et al. 2000; Head et al. 2003; Jones et al. 2008; Galperin and Kaplan 2011). The most degradable compounds are straight chain *n*-alkanes followed by the branched acyclic and monocyclic hydrocarbons, polycyclic steroidal and triterpenoidal hydrocarbons, and some aromatic hydrocarbons. Within the *n*-alkanes, multiple observations of anaerobic petroleum biodegradation in oil reservoirs, near-surface petroleum-contaminated soils, and controlled laboratory experiments have demonstrated a systematic relationship between decreasing relative degradation rates and increasing chain length for *n*-alkanes from *n*-C10 to *n*-C25 and higher (Aitken et al. 2004; Galperin and Kaplan 2011). In biodegraded reservoirs, alkylated naphthalenes and other two- and three-ringed aromatic hydrocarbons are only degraded after significant removal of *n*-alkanes and modification of acyclic isoprenoids such as pristane and phytane. This contrasts what occurs under aerobic conditions, where removal of aromatic compounds such as alkylated naphthalenes can occur before the alteration of *n*-alkanes can be observed.

A major fundamental difference between aerobic and anaerobic metabolism that affects subsurface biodegradation processes is the cellular energy yield resulting from the use of terminal electron acceptors with different free energies of formation. Anaerobic microbes gain considerably less energy from substrate biotransformation processes and, as a result, form much smaller amounts of biomass compared to aerobic microbes (Widdel and Rabus 2001). Aerobic degradation usually proceeds more rapidly and efficiently; consequently aerobic reactions require less free energy for initiation and yield more energy per reaction (Schink 2006; Wentzel et al. 2007). Laboratory experiments reveal that the cultivation of microorganisms with hydrocarbons as growth substrates under anaerobic conditions is more demanding with slower growth rates than cultivation of conventional anaerobes that grow along with facultative aerobic microorganisms (Widdel 2010). Doubling times of aerobic hydrocarbon degraders were in the range of several hours with high cell densities, whereas doubling times of anaerobic hydrocarbon degraders were in the order of days to even weeks (Widdel and Grundman 2010). These observations confirm earlier reports where comparison of hydrocarbon degradation under aerobic

and anaerobic conditions leads to a conclusion that rates of aerobic biodegradation of *n*-alkanes in the natural environment are high enough to be observable on a timescale measured in years to decades, whereas anaerobic degradation proceeds on a much longer timescale (Larter et al. 2003; Head et al. 2003).

Recently da Cruz et al. (2011) reported evidences from in vitro experiments that petroleum biodegradation could be jointly achieved by aerobic and anaerobic bacterial consortia in biofilms. In their study, an aerobic consortium depleted, in decreasing order, hydrocarbons > hopanes > steranes > tricyclic terpanes, while an anaerobic consortium depleted in the order of hydrocarbons > steranes > hopanes > tricyclic terpanes. Under subsurface conditions, anaerobic microorganisms could provide microquantities of oxygen to the aerobic microbiota by reducing salts like sulfate, nitrate, or perchlorate, for example, $ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^- \rightarrow Cl^- + O_2$. This oxygen might become trapped in biofilms and build microaerobic environments, stimulating aerobic bacterial growth and biodegradation. Gradual oxygen consumption would generate an anaerobic atmosphere and result in succession to anaerobic communities. Thus, the aerobic and anaerobic microorganisms might contribute to stepwise biodegradation in such a way that creates microenvironments that allow for the persistence of both microbial groups.

When in-reservoir oil biodegradation was first noticed, no anaerobic hydrocarbon-degrading microorganisms were isolated (Palmer 1993; Reuter et al. 1994). The prevalent occurrence of hydrocarbon biodegradation in shallow near-surface or deep petroleum reservoirs had been attributed to aerobic bacterial degradation stimulated by surface recharge of oxygen-bearing meteoric waters. The isolation of anaerobic bacteria and hydrocarbon degradation linked to nitrate reduction, iron reduction, sulfate reduction, and methanogenesis began to change this view in the 1990s, as all of these processes potentially play a role in oil biodegradation in anoxic petroleum reservoirs. Sulfate reduction and methanogenesis are the most likely processes responsible for in-reservoir hydrocarbon oxidation. Many SRB are known which can completely oxidize alkanes, saturated hydrocarbons that are quantitatively the most significant crude oil fraction. A multidisciplinary approach employing systematic laboratory anaerobic microcosm studies of oil degradation, microbial community analysis, petroleum geochemistry, isotopic analysis of reservoir gases, and modeling of oil biodegradation under reservoir conditions, was required to establish which anaerobic pathways are likely to be most significant for in-reservoir oil biodegradation (Jones et al. 2008; Carmona et al. 2009). Crude oil biodegradation, and the resulting patterns of compound removal observed in biodegraded petroleum reservoirs, was mimicked in oil-degrading laboratory microcosms under methanogenic and sulfate-reducing conditions. Distinct patterns of removal were observed under the two conditions that support the role of biological activity in petroleum maturation (Jones et al. 2008). A strong selection for CO_2-reducing methanogens and against acetoclastic methanogens was observed in oil-degrading microcosms inoculated with methanogenic communities. Although the reason for the predominance of syntrophic acetate oxidation over acetoclastic methanogenesis in this system is unclear, there is evidence of a detrimental effect of crude oil on acetoclastic

methanogens favoring alternative pathways of methanogenic alkane degradation via syntrophic acetate oxidation (Dolfing et al. 2008). However, there is a report on predominating acetoclastic methanogenesis in some methanogenic oil-degrading systems (Gieg et al. 2008).

Pathways for aerobic hydrocarbon metabolism are generally well known (Van Hamme et al. 2003), although new genes for alkane and aromatic hydrocarbon metabolism continue to be discovered. However, these new discoveries generally reveal that the approach to hydrocarbon metabolism is well conserved. Conversely, anaerobic hydrocarbon metabolism is not as well understood, with the majority of research being carried out only in the last 20 years. The approaches that anaerobic microorganisms use to activate hydrocarbons for oxidation and entry into central metabolism are hydroxylation with water, carboxylation, and fumarate addition. To date, anaerobic hydrocarbon metabolism has been described under a wide range of environmental conditions and for a range of alkanes and aromatics. For example, fumarate addition is now known to occur for alkyl- and trimethyl-benzenes, toluene, ethylbenzene, xylene, methylnaphthalenes, and cyclic and linear alkanes. Nitrate reducers, Fe^{3+} reducers, sulfate reducers, and anoxygenic photoheterotrophs have all been shown to use hydrocarbons as electron donors. Other terminal electron acceptors that can be used include humic acids, fumarate, and manganese. A number of recent reviews detail various aspects of anaerobic hydrocarbon metabolism (Fuchs et al. 2011; Meckenstock and Mouttaki 2011; Vogt et al. 2011).

As with aerobic metabolism, under anaerobic conditions the reduced hydrocarbon substrate is oxidized and reduced intermediates are not generated. A number of genome sequences have been reported for anaerobic hydrocarbon-degrading organisms, the most recent being *Desulfatibacillum alkenivorans* AK-01 (Callaghan et al. 2012), a sulfate-reducing bacterium able to oxidize C_{13} to C_{18} alkanes, and a range of alkenes, alcohols, organic, and fatty acids. Detailed analysis of this organism's genome has revealed some of the expected genes responsible for biochemical activities it has been shown to possess. As with other anaerobic hydrocarbon-utilizing microorganisms, the exact nature of all metabolic intermediates between parent hydrocarbon and acetyl-CoA in central metabolism is not known, although all intermediates are expected to be oxidized. As more genomes are sequenced and microbiologists learn how to grow more anaerobes in the laboratory, a clearer picture of anaerobic hydrocarbon metabolism, and the resulting impact on oil formations should emerge.

Shuqing et al. (2008) have reviewed and described in detail the geological background of the oil sands distribution in the western Canada and biodegradation in petroleum oil. The Western Canada Sedimentary Basin (WCSB) covers an area of 1,400,000 km^2 in the western part of North America. The oil sand deposits comprise of at least 85 % of the total immobile bitumen in place in the world and are an important source of economically recoverable oil. Oil sands are formed through the microbial biodegradation of light oils over millions of years, resulting in increased viscosity, sulfur, resin, asphaltenes, and metal content (Kryachko et al. 2012). There are three major oil sand deposits in the WCSB with exploitable bitumen reserves: Athabasca (~75,000 km^2; 0–500 m below ground surface), Cold

Lake (~22,000 km^2; 985–1,970 m below ground surface), and Peace River (8,000 km^2; 550–700 m below ground surface).

Oil compositions and physical properties as affected by biodegradation in the WCSB have been investigated by various groups (Brooks et al. 1988; Riediger et al. 1999; Obermajer et al. 2004; Bennett et al. 2006; Larter et al. 2006). Generally, biodegradation levels appear to intensify from west to east in the Cretaceous deposits. Current reservoir temperature at the Athabasca and Peace River are <10 °C and 16–22 °C, respectively. The Athabasca oil sands are severely affected by biodegradation (Harner et al. 2011). All *n*-alkanes, isoprenoid alkanes, and alkylated naphthalenes are completely removed. Most regular steranes are selectively removed with diasteranes being relatively concentrated or slightly attacked as well. Hopanes are in varying degrees converted to 25-norhopanes, which are often present in severely biodegraded oils and are generally considered to be the product of heavy biodegradation. The extent of biodegradation in the Peace River area is characterized by complete loss of the *n*-alkanes, acyclic isoprenoids, alkylbiphenyls, and methylated naphthalenes, but terpane and sterane biomarkers do not appear to have been affected, indicating that most of the oils suffered a moderate level of biodegradation.

9.4 Factors Affecting Occurrence of Biodegraded Reservoirs

A number of factors have been identified that may exert a major impact on the occurrence of biodegraded petroleum reservoirs. Reservoir temperature appears to be the most important factor based on the observation that biodegraded oil is rarely found in petroleum reservoirs at temperatures much in excess of 80 °C. According to the palaeopasteurization hypothesis, this is because the majority of microbial life does not persist above this temperature (Connan 1984), although many hyperthermophiles are known to exist in a variety of environments on the planet.

For example, hyperthermophilic microorganisms (*Thermococcus*, *Thermotoga*, and *Archaeoglobus* spp.) with optimal growth temperatures above 80 °C have been frequently isolated from reservoirs (Connan 1984; Grassia et al. 1996). Life at high temperatures presents some physiological challenges for the organisms, as in vitro studies at temperatures around 100 °C have demonstrated that some low molecular weight compounds such ATP and NAD have half lives of less than 30 min, and thermolabile amino acids like cysteine and glutamic acid are rapidly degraded (Orphan et al. 2000). Typically, deep subsurface petroleum reservoirs are characterized by high temperatures, with temperature increasing ~2–3 °C per 100 m depth, and biodegraded oils are found at depths of up to about ~4 km, with the most biodegraded reservoirs at up to 2.5 km below the sediment surface (Head et al. 2003). The paleopasteurization hypothesis notwithstanding, not all oil

reservoirs with a temperature less than 80 °C are biodegraded, which may be explained by other factors in their geological histories.

Salinity ranges of formation water can limit microbial activity significantly and appears to be another major factor that affects in-reservoir oil biodegradation. This is consistent with the observations that reservoirs with highly saline waters typically show limited oil biodegradation (Larter et al. 2006) and failure to culture microorganisms from reservoir waters with salinity greater than 100 g/L (Grassia et al. 1996). Similarly, the pH of formation waters can limit bacterial activity (Magot et al. 2000). Since pH is influenced by dissolution of gasses under high pressure, the pH of reservoir samples measured at atmospheric pressure does not necessarily reflect in situ pH, which is normally in the 3–7 range. Thus, for in vitro studies it is critical to consider pH when designing culture media or when making inferences about the lifestyles of indigenous microorganisms recovered from deep subsurface samples. Although pressure within oil reservoirs (up to 500 atm) can influence the physiological and metabolic properties of microbes, it is not considered to preclude their activity in situ.

As discussed above, microbial metabolic activities within an oil field or reservoir environments are governed by the availability of electron donors and acceptors. Due to deep subterranean environments being isolated from surface waters, their redox potentials tend to be very low, and some electron acceptors such as oxygen, nitrate, and ferric iron are generally absent. Stratal waters generally contain sulfate and carbonate, which have led to the assumption that the major metabolic processes occurring in such conditions are sulfate reduction, methanogenesis, acetogenesis, and fermentation (Head et al. 2010). The potential electron donors may include organic molecules and CO_2 and H_2 of geochemical or microbial origin. Availability of fixed nitrogen likely does not limit microbial activity in reservoirs because ammonium ions buffered by reservoir minerals, N_2 gas, and N-heterocycles could provide adequate N in situ, but the availability of water-soluble nutrients like phosphorus is more likely to limit microbial activity in situ (Foght 2010). Resins and asphaltenes are important fractions of crude oil that are composed of thousands of complex hydrocarbons which can also contain heteroatoms of nitrogen, sulfur, and oxygen. Although less studied, they are an abundant potential source of electron donors for anaerobic metabolism and their presence could explain the observation that diverse groups of strict anaerobes can grow with crude oil as a sole carbon and energy source without any significant modification of the alkanes or light aromatic compounds (Galperin and Kaplan 2011). Reservoir temperature, oil-charge histories with reservoir topology (relationships between oil and water volumes and interface areas), and nutrient supply from temperature-dependent mineral diagenesis appear to be the major controllers of the degree of oil biodegradation in the petroleum reservoirs (Head et al. 2003).

9.5 Microbial Diversity of Petroleum Oilfields

In anaerobic environments containing organic matter, the biogeochemical carbon, nitrogen, and sulfur cycles are intimately intertwined through diverse sulfate- and nitrate-reducing microbial communities that may use similar organic electron donors as carbon and energy sources, participate in syntrophic relationships, or share metabolic byproducts (Aitken et al. 2004; Hubert et al. 2009). For example, hydrogen sulfide produced by SRB can serve as an electron donor for nitrate reduction. The products of nitrate reduction include nitrite, which can inhibit sulfate reducers, but which also serves as an electron acceptor for the oxidation of organic or reduced sulfur-containing electron donors. Oil field microbial community composition, and their changes in response to the use of chemical addition, has been studied through culturing, by denaturing gradient gel electrophoresis, by sequencing of clone libraries, and, more recently, using metagenomic techniques (Bødtker et al. 2009; Gittel et al. 2009; van der Kraan et al. 2010; Kotlar et al. 2011; Ren et al. 2011; Stevenson et al. 2011; Hubert et al. 2012; Kryachko et al. 2012). Table 9.1 shows some of the microbial species isolated from petroleum oilfields (Magot et al. 2000; Tang et al. 2009; Ollivier and Alazard 2010).

9.5.1 Sulfate-Reducing Bacteria

SRB are widespread anaerobic microorganisms considered among the oldest microorganisms on the Earth (Wolicka and Borkowski 2007). SRB use sulfate as a terminal electron acceptor for the degradation of organic compounds, resulting in the production of sulfide which can be subsequently oxidized under oxic conditions by chemolithotrophic sulfur bacteria or under anoxic conditions by phototrophic sulfur bacteria (Fig. 9.1). In general SRBs are strict anaerobes but some of them have shown oxygen tolerance. SRB are ubiquitous and can be found in many natural and engineered environments where sulfate is present. SRB have been isolated from petroleum oilfields, hydrocarbon seeps, marine sediments, hydrothermal vents, mud volcanoes, and hypersaline microbial mats (Muyzer and Stams 2008; Tang et al. 2009). They have been detected in habitats with extreme pH values, such as acid-mine drainage sites, where the pH can be as low as 2 and in soda lakes, where the pH can be as high as 10. SRB are morphologically diverse with cell forms including cocci, curved types, rod, spiral, and disk shaped.

SRB are major contributors to biogeochemical cycling of carbon, sulfur, nitrogen, and phosphorus. In many petroleum hydrocarbon-contaminated environments, biological sulfate reduction is an important metabolic activity resulting in utilization of around 70 % of BTEX (benzene, ethylbenzene, toluene, xylene) compounds. SRB are also able to thrive in hydrocarbon seep areas and gas reservoirs where short chain hydrocarbons such as propane and butane are abundant (Kniemeyer

Table 9.1 Microbial diversity of petroleum reservoirs

Microbes isolated from oilfields	Temperature range (°C)	Salinity range (% NaCl)
Sulfate-reducing bacteria		
Archaeoglobus fulgidus	60–85	0.02–3
Desulfacinum infernum	40–65	0–5
Desulfacinum subterraneum	60	–
Desulfobacter vibrioformis	5–38	1–5
Desulfobacterium cetonicum	20–37	0–5
Desulfobulbus sp.	28–39	–
Desulfomicrobium apsheronum	4–40	0–8
Desulfotomaculum halophilum	30–40	1–14
Desulfotomaculum kuznetsovii	50–85	0–3
Desulfotomaculum nigrificans	40–70	0–4
Desulfotomaculum thermocisternum	41–75	0–5
Desulfovibrio gabonensis	15–40	1–17
Desulfovibrio longus	10–40	0–8
Desulfovibrio bastini	35–40	–
Desulfovibrio vietnamensis	12–45	0–10
Thermodesulfobacterium mobile	45–85	–
Thermodesulfobacterium thermophilum	65	–
Thermodesulforhabdus norvegicus	44–74	0–5.6
Methanogenic bacteria		
Methanobacterium bryantii	25–40	0–2
Methanobacterium ivanovii	10–55	0.09
Methanobacterium thermoaggregans	40–70	2–4
Methanobacterium thermoalcaliphilum	30–80	0–2
Methanobacterium thermoautrophicum	40–70	0–30
Methanocalculus halotolerans	25–45	5
Methanoculleus receptaculi	50–55	0–7.6
Methanothermococcus thermolithotrophicus	17–62	1.4–2.4
Methanohalophilus euhalobius	10–50	6
Methanohalobium evestigatum	30–60	15–30
Methanoplanus petrolearius	28–43	1–3
Methanosarcina mazei	10–50	0.1–2
Methanosarcina siciliae	20–50	2.4–3.6
Methermicoccus shengliensis	50–70	15–30
Fermentative bacteria		
Acetoanaerobium romashkovii	30–60	–
Anaerobaculum thermoterrenum	28–60	0–2
Dethiosulfovibrio peptidovorans	20–45	1–10
Geotoga petraea	30–55	0.5–10
Geotoga subterranea	30–60	0.5–10
Haloanaerobium acetoethylicum	15–45	6–20
Haloanaerobium congolense	20–45	4–24
Haloanaerobium salsugo	22–51	6–24
Petrotoga miotherma	35–65	0.5–10
Spirochaeta smaragdinae	20–40	1–10
Thermoanaerobacter brockii	37–75	0–4.5

(continued)

Table 9.1 (continued)

Microbes isolated from oilfields	Temperature range (°C)	Salinity range (% NaCl)
Thermotoga elfii	50–72	0–2.4
Thermotoga hypogea	56–90	0–1.5
Thermotoga subterranea	50–75	0–2.4

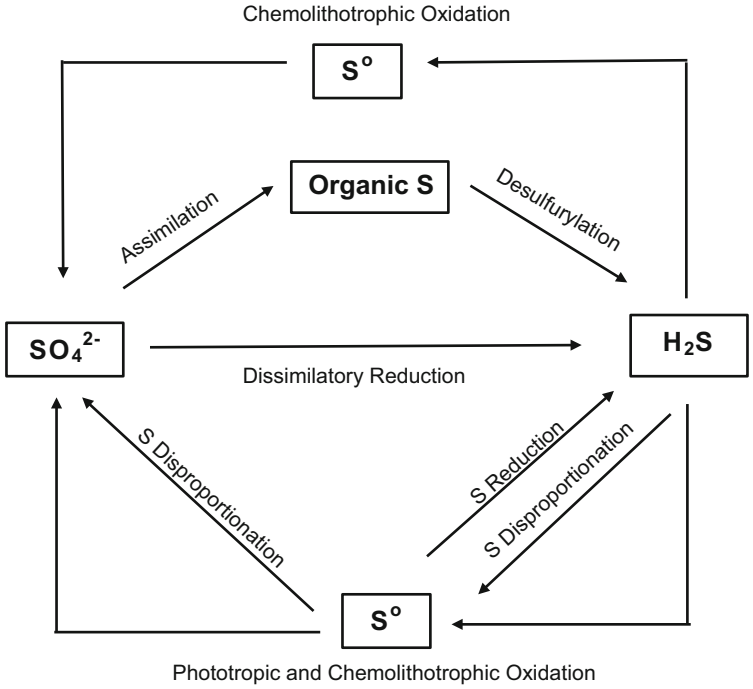

Fig. 9.1 Role of sulfate-reducing bacteria (SRB) in the biogeochemical transformation of sulfur

et al. 2007). The first extensive microbiological study describing the widespread presence of SRB in oil-producing wells was published by Bastin (1926).

Most of the information on the diversity of SRB in both natural and engineered ecosystems has been obtained by the use of marker genes such as 16S ribosomal RNA (rRNA) genes. Based on comparative analysis of 16S rRNA gene sequences, the known SRB can be grouped into seven phylogenetic lineages, five within the *Bacteria*, and two within the *Archaea* (Wolicka and Borkowski 2007; Muyzer and Stams 2008; Tang et al. 2009). Most of the sulfate reducers belong to various genera within the Deltaproteobacteria, followed by the Gram-positive SRB within the Clostridia (*Desulfotomaculum*, *Desulfosporosinus*, and *Desulfosporomusa*). *Desulfosporosinus* and *Desulfotomaculum* are able to produce endospores. Nitrospirae (*Thermodesulfovibrio*), Thermodesulfobacteria (*Thermodesulfobacterium*), and Thermodesulfobiaceae (*Thermodesulfobium*) lineages contain

only thermophilic sulfate reducers. Within the *Archaea*, SRB belong to the genus *Archaeoglobus* in the Euryarchaeota and to the genera *Thermocladium* and *Caldivirga* in the Crenarchaeota.

Metabolically, SRB are classified into two groups of complete oxidizers (acetate oxidizers), which have the ability to oxidize organic compounds to carbon dioxide, and incomplete oxidizers (non-acetate oxidizers), which carry out incomplete oxidation of organic compounds to acetate and CO_2 (Madigan and Martinko 2006). Although the growth kinetics for incomplete oxidizers is generally faster than the complete oxidizers, they are less versatile in terms of nutritional requirements. The group of complete oxidizers includes species belonging to the genera *Desulfobacter*, *Desulfobacterium*, *Desulfococcus*, *Desulfonema*, *Desulfosarcina*, *Desulfoarculus*, *Desulfoacinum*, *Desulforhabdus*, *Desulfomonile*, *Desulfotomaculum acetoxidans*, *Desulfotomaculum sapomandens*, and *Desulfovibrio baarsii*. The incomplete oxidizers include *Desulfovibrio*, *Desulfomicrobium*, *Desulfobotulus*, *Desulfofustis*, *Desulfotomaculum*, *Desulfomonile*, *Desulfobacula*, *Archaeoglobus*, *Desulfobulbus*, *Desulforhopalus*, and *Thermodesulfobacterium.*

The ability of SRB in utilizing various hydrocarbons from crude oil has severe consequences for the petroleum industry both in the underground oil reservoirs and in surface facilities (Gieg et al. 2011; Singh et al. 2012). SRB activity increases H_2S concentrations (souring) in onshore and offshore oil reservoirs subjected to water flooding and creates associated problems such as contamination of oil and gas and produced water with sulfide, plugging of oil bearing rock formations, and accelerated corrosion in production, processing, and storage facilities (Uchiyama et al. 2010; Voordouw 2011). Indeed, SRB are the main organisms involved in the microbially induced corrosion processes (MICP) that occur commonly in oil and gas production and petrochemical processes equipment and pipelines (Duncan et al. 2009). Control of biogenic sulfide production, which improves the quality of produced oil and gas and decreases production costs, could be achieved through elimination of sulfate from water prior to injection, suppression of SRB with biocides or metabolic inhibitors such as nitrite and molybdate, and addition of nitrate to the injection water (Voordouw 2011).

9.5.2 Methanogenic Microorganisms

Methanogenic microbes can degrade hydrocarbons to methane under anaerobic conditions (Jones et al. 2008; Gray et al. 2010; Milkov 2011). Crude oil hydrocarbon degradation under methanogenic conditions in the laboratory mimics the characteristic sequential removal of compound classes seen in reservoir-degraded petroleum. The initial preferential removal of *n*-alkanes generates close to stoichiometric amounts of methane, principally by hydrogenotrophic methanogenesis. This degradation process is widespread in the geosphere. In comparison with other anaerobic processes, methanogenic hydrocarbon degradation is more sustainable

over geological time scales because replenishment of an exogenous electron acceptor is not required. As a consequence, this process has been responsible for the formation of the world's vast deposits of heavy oil, which far exceed conventional oil reserves such as those found in the Middle East. Studies of the organisms, syntrophic partnerships, mechanisms, and geochemical signatures associated with methanogenic hydrocarbon degradation have identified common themes and diagnostic markers for this process in the subsurface. These studies have also identified the potential to engineer methanogenic processes to enhance the recovery of energy assets as biogenic methane from residual oils stranded in petroleum systems (Gieg et al. 2008).

Methanogenic *Archaea* have been successfully isolated from saline oil well waters at a range of temperatures from different oil reservoirs (Ollivier et al. 1998; Dolfing et al. 2008). Halophilic methylotrophic methanogens, *Methanohalophilus euhalobius* and *Methanosarcina siciliae*, and the acetoclastic methanogen *Methanosarcina mazei*, capable of utilizing acetate, methanol, and methylamine but not $H_2 + CO_2$ for growth, have been isolated under both mesophilic and thermophilic conditions (Magot et al. 2000). Hydrogenotrophic mesophilic or thermophilic methanogens such as *Methanococcus thermolithotrophicus*, *Methanoplanus petrolearius*, *Methanocalculus halotolerans*, *Methanobacterium thermoautrophicum*, *M. bryantii*, *M. ivanovii*, and *M. thermoalcaliphilum* have been commonly isolated from oil reservoirs (Ollivier and Alazard 2010). Methanogenic *Archaea* may potentially act as iron-oxidizing microorganisms in the presence of CO_2 as terminal electron acceptor and therefore may also participate together with SRB in in situ biocorrosive processes (Dinh et al. 2004; Duncan et al. 2009).

Some syntrophs such as *Syntrophus* belong to the class delta-proteobacteria where many SRBs belong (Voordouw 2011). In the absence of sulfate, these syntrophs participate in consortia along with heterotrophic nitrate-reducing bacteria. Oil sand tailing ponds can emit up to 10^7 L of methane per day from conversion of naphtha hydrocarbon diluent used in bitumen extraction (Siddique et al. 2007). Similarly oil storage tanks that contain water can also evolve significant amounts of methane. Thus methanogenic hydrocarbon degradation offers the potential to convert oil that can no longer be economically produced to methane.

9.5.3 Fermentative Bacteria

The presence of fermentative bacteria has long been believed to be the consequence of introducing water and carbohydrates during drilling or oil recovery processes (Magot et al. 2000). However, isolation of thermophilic microbes such as *Geotoga* and *Petrotoga* species of the order Thermotogales from oil reservoirs suggests that these thermophilic microorganisms may be considered indigenous to these ecosystems. Other putative indigenous microorganisms isolated from hot oil reservoirs

include the moderately halophilic *Halanaerobium* sp.; anaerobic species of *Garciella* and *Petrobacter* using nitrate; *Deferribacter* spp. using iron and Mn as terminal electron acceptors with yeast extract, peptone, and casamino acid as energy sources; and finally the homoacetogenic bacterium *Acetogenium romashkowii*.

A wide range of mesophilic and thermophilic fermentative microorganisms have been isolated from oil reservoirs, including *Bacteria* (e.g., *Halanaerobium*, *Thermotoga*) and a few hyperthermophilic *Archaea* such as *Thermococcus* and *Pyrococcus* spp. (Ollivier and Cayol 2005). Besides their ability to ferment sugars to acids, gases, and solvents, several thermophiles have been shown to oxidize hydrogen in the presence of Fe^{3+} (*Thermococcus*, *Pyrococcus*, and *Thermotoga*) or thiosulfate (*Thermotoga*) as terminal electron acceptors, thus demonstrating that hydrogen oxidation in oil reservoirs is not restricted to SRB and methanogenic *Archaea* (Ollivier and Alazard 2010). Interestingly, besides *Thermotoga* spp., a number of mesophilic (*Dethiosulfovibrio*) and thermophilic bacteria (*Thermoanaerobacter*) can also reduce thiosulfate into sulfide and may contribute to biocorrosion and possibly also reservoir souring in oil field ecosystems. Some hyperthermophilic microorganisms such as *Thermococcus*, *Pyrococcus*, and *Thermotoga* have the ability to remove sulfur from crudes and produce sulfide, thus can also contribute to the oil souring during production. *Thermoanaerobacter brockii* is capable of using hydrogen as electron donor with improved consumption of amino acids and peptides in the presence of thiosulfate besides carbohydrates (Magot et al. 2000).

9.5.4 Metagenomics of Subsurface Oil Environments

Driven by increasing interest in microbial-enhanced oil recovery (MEOR), reservoir plugging and souring, biocorrosion, biodegradation of light hydrocarbons resulting in the production of heavy oils, and methane production from heavy oil formations, metagenomic studies are beginning to appear that shed additional light on the natural microbiological processes in the deep subsurface and the impacts that oil recovery techniques have on these processes.

Ren et al. (2011) used DGGE and relatively small 16S gene clone libraries to compare the *Bacterial* and *Archaeal* communities in both injection and production waters at a water-flooded petroleum reservoir in China. Interestingly, the microbial communities in produced waters were distinct from those in injection waters, indicating that injected microorganisms did not survive in the subsurface. In this case their goal was not to introduce organisms into the reservoir in an effort to stimulate activity, but the work does highlight the need to monitor produced waters in order to gain some understanding of what impacts microorganisms are having on the recovered oil. In contrast, a study in north Texas by Struchtemeyer et al. (2011) found that drilling muds prepared in open air environments at well sites could potentially introduce microorganisms into oil formations that can be recovered in

collected drilling waters. No information was specifically collected on microbial activity in these studies, and since complete metagenomes were not sequenced, no hypotheses can be made about hydrocarbon metabolic potential.

This lack of solid data relating to metabolic capacity is common at this stage, but will undoubtedly change as metatranscriptomic methods are applied. In general, microorganisms with the potential for low molecular weight hydrocarbon biodegradation, fermentative metabolism of carbohydrates and amino acids to ethanol, acetate, hydrogen, and carbon dioxide, and methane formation are observed in these studies. Indeed, as discussed above, these observations are supported by Jones et al. (2008) who showed that methanogenic petroleum biodegradation can explain the chemical composition of heavily degraded oils in the subsurface. As is always seen, straight chain alkanes are preferentially metabolized before aromatics, branched aromatics, and alkylated naphthalenes are depleted. Their main conclusion is that "identification of the pathways inherent in subsurface bio-degradation facilitates the engineering of processes to accelerate naturally slow methanogenic biodegradation to recover energy from heavy oilfields as methane, rather than oil." This area of research is quickly evolving as new models are proposed. Historically, it was believed that oxygen permeation into oil reservoirs resulted in oil degradation. However, with the relatively recent discovery of anaerobic hydrocarbon metabolism, thermodynamic studies have shown that anaerobic alkane and aromatic hydrocarbon biodegradation can result in the formation of methane (Dolfing et al. 2008).

The first deep subsurface metagenome encompassing sequencing of total DNA rather than just 16S genes was published in 2011 (Kotlar et al. 2011). Here, DNA was extracted from water recovered from an oil reservoir 2.5 km beneath the Norwegian Sea (85 °C, 250 bars) thought to be free of impact from previous human interventions. In the study, a modest amount of metagenomic data was used to characterize the overall microbial community in the reservoir. Interestingly, the genera of *Bacteria* and *Archaea* found in the reservoir are quite similar to those found in terrestrial environments, although the reason for this is unclear. Having said that, evidence for thermophilic or thermotolerant organisms was found as would be expected. The authors focused mainly on identifying the organisms present, and methanogens and sulfate-reducing bacteria appeared to dominate. They did not describe any attempts to reconstruct metabolic potential within the reservoir through annotation of known metabolic genes, something that would be interesting for future work. Even more interesting will be to carry out metatranscriptomic experiments if sampling procedures can be developed and to focus on putative genes that may currently have no known function.

9.6 Petroleum Microbiology in Deep Aqueous Environments

Another aspect of subsurface petroleum microbiology relates to how microbes deal with petroleum hydrocarbons in deep aqueous environments. Two examples, the Deepwater Horizon marine oil spill and hydrocarbon degradation in the large fine tailings lakes resulting from oil recovery from the Canadian Oil Sands, will be considered here.

The recent BP Deepwater Horizon oil spill in the Gulf of Mexico, the largest marine oil spill in history, casts some light on how crude oil is degraded in deep aqueous marine environments. A strategy which was adopted in addressing this oil spill was to apply oil dispersants as well as physical dispersion strategies as the oil exited the well head, close to the ocean floor to prevent large oil slicks from reaching the water surface, and moving toward the coast.

Physical, chemical, and microbial studies confirmed substantial aerobic hydrocarbon biodegradation associated with the dispersed oil plumes, which resulted in a reduction in dissolved oxygen concentration, relative to oxygen concentration in water outside the oil plume. Nevertheless, the oxygen concentration in the petroleum plume or cloud was never decreased to anoxic levels (Hazen et al. 2010; Atlas and Hazen 2011). The biodegradation activity in the plume was also reflected by the greater microbial counts in the plume (~10^5) compared to ~10^3 in the surrounding waters. At an early point in the spill, propane and ethane degradation was considered the primary drivers of respiration, and later on essentially all released methane was considered to be respired, mediated by a dominant presence of methanotrophs (Valentine et al. 2010; Kessler et al. 2011). Rapid degradation of alkanes and substantial degradation of PAHs were observed in the cloud. While more than 900 subfamilies of bacteria, from 62 phyla, only 16 subfamilies of the gammaproteobacteria were found to be enriched in the cloud and three families of the class *Oceanospirillales* predominated (Hazen et al. 2010).

Nevertheless, functional gene microarray investigations confirmed that populations of microbes associated with both aerobic and anaerobic biodegradation of hydrocarbon components were present in the oil plume (Lu et al. 2012). For example, the *bbs* gene (beta-oxidation of benzylsuccinic acid), derived from *Azoarcus* and *Thauera* species, involved in anaerobic degradation of toluene, was enriched in the cloud. Anaerobic petroleum-degrading bacteria were also associated with marsh sediments, of Southeast Louisiana, which had been contaminated with crude oil from the BP spill (Boopathy et al. 2012).

The Athabasca Basin in Alberta, Canada, is estimated to contain trillions of barrels of heavy bitumen petroleum. The water used in the oil extraction process results in the generation of very large and deep tailing ponds containing fine suspended materials and residual bitumen suspended in water which densify to what are known as mature fine tailings (MFT). A small portion of naphtha, used as a diluting agent for bitumen processing, and which contains C_3–C_{14} aliphatic and

aromatic hydrocarbons, ends up in the MFTs, together bitumen-derived higher molecular weight aliphatic and ringed hydrocarbon structures.

These MFTs become anaerobic and exhibit bubbles of gas at the pond surface (Salloum et al. 2002). The microbial population in these deep ponds includes denitrifying bacteria, sulfate reducers, and methanogens. Methane biogenesis, both from naphtha and the higher molecular weight hydrocarbon substrates has been observed (Siddique et al. 2007, 2011). Given that methane is an undesirable greenhouse gas, understanding and controlling the microbial processes which generate methane in MFT ponds will be important to minimizing environmental impacts of these processes. In that regard, addition of sulfate inhibits methane generation from MFTs may represent a solution, although the associated generation of H_2S may represent a problem (Salloum et al. 2002).

9.7 Conclusion

As fossil energy resources dwindle, the need to better understand the distribution and occurrence of biodegraded petroleum deposits increases. While our knowledge of biodegraded petroleum reservoirs has advanced considerably in recent years, our knowledge of the processes, microorganisms involved, and quantitative understanding of the factors which control in-reservoir oil biodegradation remain far from complete. Petroleum geochemistry, geomicrobiology, and modeling have all contributed to our knowledge. Some basic questions have not yet been fully answered due to the difficulty in obtaining reliable or representative samples to ensure that indigenous microbial populations are really being examined! It is likely that nutrient supply exerts a control on in reservoir oil biodegradation, but what is the quantitative relationship between nutrient supply and degree of biodegradation? There are some indications that in-reservoir methanogenic crude oil degradation is driven through syntrophic acetate oxidation and dominated by methanogenic CO_2 reduction with acetoclastic methanogenesis assuming a secondary role. However, there is also evidence of more important roles for acetoclastic methanogenesis under some circumstances, so another important question is—what promotes subsurface methanogenic oil degradation through methanogenic CO_2?

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