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# Microorganisms and Crude Oil

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## 1. Introduction

Crude oil is one of the most important energetic resources in the world. It is used as raw material in numerous industries, including the refinery-petrochemical industry, where crude oil is refined through various technological processes into consumer products such as gasoline, oils, paraffin oils, lubricants, asphalt, domestic fuel oil, vaseline, and polymers. Oil-derived products are also commonly used in many other chemical processes.

Although crude oil is a natural resource, in some conditions its presence is unfavorable and causes devastation of the surroundings. Crude oil and formation water in oil reservoirs represent an extreme environment with many groups of autochthonous microorganisms strictly linked with this setting. The relationship between microorganisms and this extreme environment begins when crude oil is formed and it ends when these specialized microorganisms are applied for the bioremediation of the polluted environment by crude oil and oil-derived products.

It is common knowledge that crude oil is formed by biological, chemical, and geochemical transformations of organic matter accumulated in favorable locations. In the first stage crude oil is transformed during sediment diagenesis at moderate temperatures up to 50°C. Due to defunctionalization and condensation, kerogen, which is immature crude oil, is formed. Kerogen accumulations are considered to be the richest coal accumulation on Earth (Widdel & Rabus, 2001). Based on geochemical studies, immature crude oil contains higher volume of hydrocarbons with an odd number of carbon atoms, synthesized in plants. This fact has a practical meaning in determining the Carbon Preference Index (CPI). The organic origin of crude oil is also supplied by biomarkers, i.e. compounds whose carbon skeleton was not changed in geochemical processes, formed by living organisms, e.g. microorganisms. Such compounds include e.g. terpenes, porphyrines, and metalloporphyrines (Surygala, 2001).

The life activity of microorganisms occurring in crude oil has significant influence on its chemical composition and physical-chemical properties, and as a result often changes its economical value or exploitation conditions. This influence can be positive, e.g. decreased viscosity of heavy crude oil favors its exploitation, but also negative, e.g. corrosion of drilling equipment due to bacterial production of hydrogen sulphide. Products from the biological activity of autochthonous microorganisms or microorganisms introduced into the reservoir rock are the basis of biological methods applied to enhance the recovery of oil from already exploited (depleted) reservoirs. At specific conditions, crude oil may flow

uncontrolled onto the lithosphere surface and cause significant hazard to the environment. Such cases are often related to spilled oil and oil by-products during oil exploitation, processing, and transportation. This type of pollution is often removed by natural microorganisms occurring in crude oil; these microorganisms have the ability to biodegrade crude oil and oil-derived products (Mokhatab, 2006; Nazina et al., 2007; Wolicka et al., 2009; Wolicka et al., 2011).

## 2. Crude oil – Environment for microorganisms' growth

### 2.1 Crude oil composition

Crude oil is a mixture of thousand of various compounds, organic and inorganic, including aliphatic and aromatic hydrocarbons, which in average reaches 75 % of its content, as well as resins and asphalts. Non-hydrocarbon compounds include sulphur compounds (0.01–8 %), mainly as hydrogen sulfide (H<sub>2</sub>S), mercaptans (compounds containing the -SH group), sulfides and disulfides, thiophenes, as well as benzothiophenes and naphthothiophenes that prevail in oil fractions (Fig. 1). These compounds are unfavorable due to their chemical recalcitrance, therefore their presence is considered in evaluating crude oil quality (Surygała, 2001).

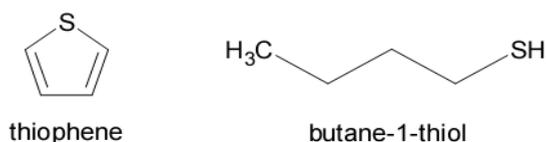


Fig. 1. Chemical structures of thiophene and 1-butanethiol (methyl mercaptan).

**Nitrogen compounds** represent non-hydrocarbon compounds that occur in crude oil at the level of 0.01–2 % weight, although over 10 % concentrations have been noted. Nitrogen occurs in alkaline and non-alkaline compounds. The first group includes pyridines, acridines, and quinolines, and the second group comprises pyrroles, carbazoles, indoles, and heterocyclic compounds (Fig. 2). Similarly, nitrogen also occurs as sulphur bonds, compounds from this group are concentrated in high-boiling fractions (Surygała, 2001).

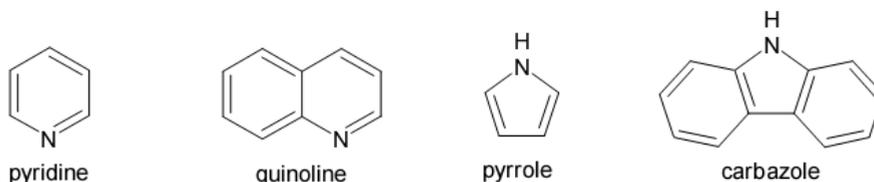


Fig. 2. Chemical structures of pyridine, quinoline, pyrrole, and carbazole

**Oxygen compounds** such as phenols, carboxylic acids (having the COOH functional groups), furans, and alcohols (Fig.3) occur in the heavy fractions of crude oil (Surygała, 2001).



Fig. 3. Structures of phenol, furan, and cyclohexanol.

**Porphyryns** often occur in crude oil. They are products of degradation of dyes produced by living organisms. They are composed of pyrrole rings connected by methine bridges. They often form chelate compounds of nickel, vanadium, and other metals. There is a relationship between the maturity of crude oil and the concentration of porphyryns, which decreases with oil maturity. Oil formed from marine organisms contains more vanadium than nickel porphyryns (Surygała, 2001).

Trace elements are present in crude oil in ppm quantities. Besides porphyryns, trace elements occur as soaps (particularly compounds of Zn, Ti, Ca, and Mg), as well as metal-organic bonds (V, Cu, Ni, Fe). The highest concentrations (up to 1500 ppm) of trace elements that have been determined correspond to vanadium, nickel, and iron (up to 1200 ppm), as well as calcium and cobalt (up to 12 ppm). These compounds are unfavorable during the refining process. Crude oil is naturally enriched with these elements during its migration within the reservoir rock. Particularly high contents of vanadium have been found in crude oils from Venezuela (Surygała, 2001; Swaine, 2000). Also trace quantities of phosphorus, arsenic, and selenium are found in most types of crude oil.

Resin-asphalt substances are present in crude oil, particularly with a low degree of maturity. They have a very complex chemical structure and include most heteroatoms, trace elements, and polycyclic aromatic hydrocarbons. They contain in average up to 80 % C, 10 % H, and 14 % heteroatoms; in which 1 to 2 % correspond to metal-organic compounds.

The majority of these organic compounds may serve as electron donors for various groups of autochthonous microorganisms present in crude oil.

## 2.2 Microorganisms present in crude oil

The conditions prevailing in a crude oil reservoir significantly differ from environmental settings typical to the occurrence of living organisms on Earth. The red-ox potential is very low, the pressure and temperature are very high, and the salt content may reach up to over 10%. Moreover, this setting lacks electron acceptors, such as oxygen, typical for most microorganisms; while sulfate and carbonate are present (Ortego-Calvo & Saiz-Jimenez, 1998) and the range of electron donors admissible for microorganisms is very wide.

Most hydrocarbons occurring in crude oil have toxic effects resulting mainly from their chemical structure (Gałuszka & Migaszewski, 2007). These toxic hydrocarbons include both aliphatic and aromatic compounds, such as polycyclic aromatic hydrocarbons (PAHs), whose toxicity increases proportionally to the number of carbon atoms in the compound. Particularly in the case of PAHs with more than four-member rings in its structure. Despite

the toxicity of the chemical compounds occurring in crude oil, several groups of microorganisms have been found in this setting (Magot et al., 2000; Stetter & Huber, 1999).

The main sources of carbon for microorganisms in crude oil are hydrocarbons, both aliphatic and aromatic, but also organic compounds that are often the products of crude oil biodegradation. These organic compounds include: organic acids such as acetic, benzoic, butyric, formic, propanoic, and naphthenic acids reaching up to 100 mM (Dandie et al., 2004). The electron donors may be  $H_2$ , and in the case of immature oil – resins and asphaltenes, whose metabolic availability is confirmed by the fact that anaerobic microorganisms may develop in cultures with crude oil without any modifications of its composition.

Recently, it was discovered that crude oil lenses contained not only bacteria that were supplied from the external environment to the reservoir by infiltration of surface water or introduced through fluid injection during oil recovery operations but also these bacteria which are autochthonous for environment of crude oil and formation waters. Distinguishing these autochthonous microorganisms from other surface microorganisms is very difficult, almost impossible, particularly in settings with low salt content and temperature, because in this environment surface bacterial strains can grow. Generally, only absolutely anaerobic microorganisms, whose physiological characteristics indicate adaptation to the *in-situ* conditions, are considered as really autochthonous for this setting and only these microorganisms demonstrate significant activity in these specific environments. However, so far it is not clear yet if these communities of microorganisms are characteristic of these ecosystems and the factors causing their activation or growth inhibition (Magot et al., 2000).

An important factor influencing microorganism activity is temperature (Stetter & Huber, 1999). Living organisms are not considered to occur theoretically at temperatures above 130–150°C due to the instability of biological compounds (Magot et al., 2000). Such conditions would correspond to deep oil reservoirs at depths ranging from 4030 to 4700 m having a geothermal gradient of 3°C per 100 m and a surface temperature of 10°C (unpublished data). So far, the presence of microorganisms has been detected at a depth of 3500 m (Stetter & Huber, 1999). Previous data indicates the presence of microorganisms at maximum temperatures of 80°C to 90°C, above which autochthonous bacteria do not occur. In some cases, microorganisms that are present at these high temperatures were introduced into the reservoir with sea water through rock fractures and faults. Crude oil is a setting characterized by the presence of many microorganism groups: fermentation bacteria and sulfate reducing bacteria (SRB) that causes complete oxidation of organic compounds to  $CO_2$  or incomplete oxidation of hydrocarbon compounds to acetate groups; as well as iron reducing bacteria and methanogenic archaea.

### 2.2.1 Fermentation bacteria

Numerous species of fermentation bacteria have been detected in crude oil (Nazina et al., 2007). Strains capable of thiosulfate ( $S_2O_3$ ) and elemental sulfur ( $S^0$ ) reduction were determined. Electron donors for these microorganisms may be sugars, proteins,  $H_2$ ,  $CO_2$ , and hydrocarbons. The products of metabolic reactions are organic acids and gases, such as  $H_2$  and  $CO_2$ , which may cause increase of reservoir pressure. These microorganisms have potential for their application in microbiological methods of oil production enhancement

(Magot et al., 2000; Nazina et al., 2007). Mesophilic fermentation bacteria are more uncommon than the thermophilic ones. The first group comprises such haloanaerobes as *Haloanaerobium acetoethylicum*, *H. congolense*, and *H. salsugo* that produce acetate or ethanol in the process of carbohydrate fermentation. These microorganisms differ also in the type of substrates used and their tolerance to salt content (up to 10 %). For example, *Spirochaeta smaragdinae* isolated from a Congo oil field prefers salt contents of up to 5 %. The same source, however, yielded also *Dethiosulfovibrio peptidovarans* with specific metabolism. These bacteria have the ability to biodegrade protein extracts and the products of its metabolism are organic acids such as: acetic, isobutyric, isovaleric, and 2-methylbutyric acids. Moreover, it has the ability to reduce thiosulfate and prefers salt contents up to 3 % of NaCl (Magot et al., 2000).

In a hydrocarbon reservoir together with crude oil, there is also water in the formation, in which different groups of microorganisms are known to occur. For example, from formation waters of the Tatarstan and western Siberia reservoirs, microorganisms such as *Acetoanaerobium romaskovii* were isolated (Magot et al., 2000). These microorganisms use acetates, H<sub>2</sub>, CO<sub>2</sub>, amino acids, and sugars as sources of energy and carbon. It has been reported in the literature that at these high reservoir temperatures a larger number of thermophilic bacteria has been detected than the number of mesophilic bacteria (Magot et al., 2000). Thermophilic microorganisms contain thermostable enzymes that are capable of enduring temperatures exceeding even 100°C. To this group of microorganisms belong species of *Thermotoga*: *T. subterranean*, *T. elfii*, and *T. hypogea*, which are capable of reducing thiosulfate to sulfides, as well as bacteria resembling *Thermotoga* that reduce elemental sulfur. Microorganisms as *Thermotoga* occur at low salinities up to 2.4% of NaCl and in the course of glucose degradation, these bacteria produce acetic acid and L-alanine (Magot et al., 2000).

Bacteria representing *Geotoga* and *Petrotoga* from the order *Thermotogales*, which are moderate thermophiles, occur also in a wide range of salt content conditions. They were detected for the first time in crude oil reservoirs in Texas and Oklahoma. Microbiological investigations of numerous high-temperature crude oil reservoirs supplied evidence on the significant biogeochemical role of these bacteria, which in morphologically and physiologically sense resemble representatives of the order *Thermotogales*, such as *Ferroidobacterium* and *Thermosiphon*. They include *Thermoanaerobacter* and *Thermoanaerobacterium* from the family *Thermoanaerobiaceae*, which are often isolated from hot but poorly salinated reservoirs. The first genus reduces thiosulfate to sulfides, and the second – thiosulfate to elemental sulfur (Davey et al., 1993).

Hyperthermophilic fermenting microorganisms were distinguished in high-temperature reservoirs. They include *Archaea*, such as *Thermococcus celer*, *T. litoralis*, and *Pyrococcus litotrophicus*. The first two species showed activity during incubation at 85°C, and the latter – above 100°C. These microorganisms used proteins or yeast extract as electron donors, and reduced elemental sulfur to sulfides (Magot et al., 2000; Stetter & Huber, 1999).

### 2.2.2 Sulfate reducing bacteria (SRB)

Sulfate reducing bacteria (SRB) are some of the oldest microorganisms on Earth. Their initial development and activity goes back to the Proterozoic Era (Rabus et al., 2000). The process

of dissimilative sulfate reduction is considered to be one of the few metabolic pathways that did not undergo mutations and horizontal gene transfer (Voordouw, 1992). This fact evidences also that the gene coding the enzyme catalyzing the first stage of dissimilative reduction is strongly conserved evolutionarily and occurs in unchanged form since its formation (Baker et al., 2003). Based on sulfur isotopic studies, bacterial sulfate reduction is believed to have developed earlier than oxygen photosynthesis (Kopp et al., 2005). The first studies on the metabolism and biology of these microorganisms were commenced in 1864. Meyer (1864) and Cohn (1867) first observed the production of hydrogen sulfide of biogenic origin in marine sediments. Bastin (1926) noted the undoubted presence of SRB in areas of crude oil exploitation, and Werkman & Weaver (1927) described the first sporing thermophilic SRB. In addition, these reports indicated the role of microorganisms in the corrosion of drilling equipment. The 1950s and 1960s brought the first attempts to understand metabolic processes conducted by SRB.

SRB are heterotrophic organisms and absolute anaerobes that use sulfates as well as other oxygenated sulfur compounds (sulfites, thiosulfites, trithionate, tetrathionate, and elemental sulfur) as final electron acceptors in respiration processes (Postgate, 1984; Gibson, 1990). All SRB are gram negative with the exception of the species of *Desulfonema*. This group of bacteria is very diverse and depending on the soil and water composition, different kinds of bacteria can be found within this group such as psychro-, meso- and thermophilic, halo- and barophilic. Some species of SRB like *Desulfosporosinus orientis* (Stackebrandt et al., 1997), *Desulfotomaculum halophilum* sp. nov. (Tardy-Jacquenod et al., 1998), and *Desulfosporosinus meridiei* sp. nov. (Robertson et al., 2001) have the ability to develop spores.

The 1980-ties brought new discoveries with regard to the mechanisms of biological sulfate reduction. This allowed a different view on SRB metabolism. Reactions of the entire metabolic trail taking place during sulfate reduction were described in detail and two metabolic trails of SRB were confirmed. The first was linked to the partial oxygenation of organic compounds, i.e. to acetate, and the second metabolic trail corresponds to the complete oxygenation to CO<sub>2</sub> (Laanbroek et al., 1984). The second important scientific activity at that time was research on the SRB genome. Till the 1980s all SRB were classified based on their characteristic phenotype features such as feeding or morphology. However, with wider application of the 16S rRNA gene sequence analysis, a more detailed classification of SRB was possible. It indicated that the genus *Desulfotomaculum* was the only genus belonging to the group of gram positive bacteria, whereas the remaining SRB are gram negative (Madigan et al., 2006).

Stetter (1987) discovered that the ability to reduce sulfates is not only a feature of the SRB but also of some archaea. For instance, the thermophilic strain *Archaeoglobus fulgidus*, which can grow in environments at 83°C and is capable of sulfate reduction, was isolated. This strain showed larger similarity to Archaea than to the remaining SRB.

Pure strains of SRB capable of complete oxygenation of some hydrocarbons such as alkanes, xylenes, toluene, and naphthalene to CO<sub>2</sub> were isolated in the 1990s. It was also discovered that SRB may occur and develop in crude oil, whose components are a good source of carbon. This fact would explain the presence of hydrogen sulfide in crude oil reservoirs and in formation waters (Rabus et al., 2000; Wolicka & Borkowski, 2008a; Wolicka, 2008; Wolicka et al., 2010). Samples of isolated sulphidogenic bacterial communities from Carpathian's crude oil from Poland are presented in Fig. 4.

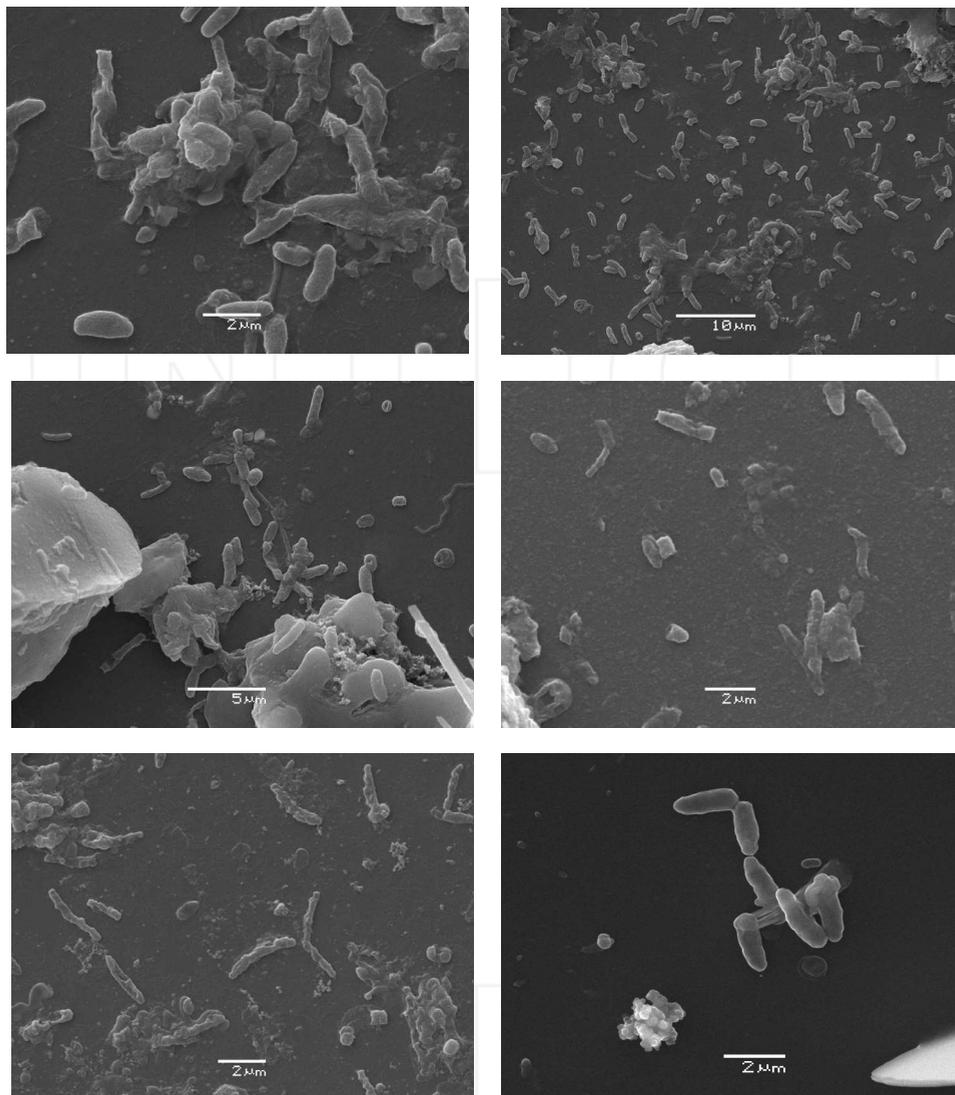


Fig. 4. Sulphidogenic bacterial communities from Carpathian's crude oil from Poland (Wolicka, own studies, not published).

For long time, SRB were thought to occur in environments polluted by crude oil and oil-derived products, and they always were considered to act as the producer of the toxic hydrogen sulfide and the main cause of bio-corrosion (O'Dea, 2007). Thus they were beyond scientific interest and their significant role in the biodegradation of organic compounds in anaerobic conditions was not known. Currently, the ability of SRB to metabolize many different organic compounds including crude oil and oil-derived products has been recognized; even the influence of the biological activity of SRB on oil quality and fluidity

(e.g. heavy oils) has been determined. Moreover, the activity of these microorganisms decreases the permeability of reservoir rocks caused by the precipitation of insoluble sulfides, as well as carbonates (Magot et al., 2000; Nemati et al., 2001).

SRB always accompany crude oil and therefore for long time were considered as indicator organisms when searching for new reservoirs (Postgate, 1984). Such cases were only possible when the natural environment was not polluted by oil-derived products as it is nowadays.

SRB are a group of microorganisms that play a significant role in the biodegradation of organic compounds in anaerobic conditions and in the biogeochemical cycle of many elements such as carbon or sulfur (Jørgensen, 1982a; Wolicka, Borkowski, 2007). The content of SRB in the terminal stages of organic matter mineralization in marine sediments exceeds 70% (Jørgensen, 1982b). SRB are introduced in the anaerobic biodegradation of organic compounds at the level of low-molecule compounds such as organic acids, e.g. acetic, propionic acid, formic, or alcohols, e.g. ethanol, propanol, butanol, etc., because most of them do not produce hydrolytic enzymes.

Due to the lack of chemical electron acceptors, such as oxygenated sulfur compounds (e.g. sulfates, sulfites, thiosulfates, or elemental sulfur), the transfer of electrons on a biological acceptor may take place by using hydrogen as is the case for methanogenic archaea. This mechanism enables the persistence of bacteria (e.g. reducing sulfates), in settings with poor availability of electron acceptors. This property is known as syntrophic growth (Nazina et al., 2007). Some evidences that confirm the biological activity of SRB are the concentration of hydrogen sulfide produced, the decreased concentration of  $\text{SO}_4^{2-}$  ions in relation to their concentration in the injected seawater; as well as the increased concentration of the isotopic sulfur in sulfates and its decreased in hydrogen sulfide occurring in the gas accompanying the reservoir (Rožanova et al., 2001).

The most common mesophilic SRB causing detrimental effects on drilling equipment and oil storage vessels include *Desulfovibrio longus*, *D. vietnamensis*, and *D. gabonensis*. These species incompletely oxidize organic compounds to acetate and use energy from the oxidation of hydrogen, lactate, and pyruvic acid. However, the *D. longus* bacteria are not considered autochthonous, as is the case of *Desulfotomaculum halophilum* (Magot et al., 2000).

In contrast to the *Desulfovibrio* bacteria, the *Desulfomicrobium apsheronum* bacteria, which belong to the SRB group and are tolerant to high salt content, are autotrophic (Rožanova et al., 1988). In the case of the *Desulfobacter vibrioformis* bacteria, which were isolated from the oil-water separation systems, use acetate as their only source of energy and carbon is used in sulfate reduction; while, the *Desulfobacterium cetonicum* bacteria have the ability to oxidize ketones to carbon dioxide. Research based on 56 samples of oil collected from several oil reservoirs using molecular techniques showed that the SRB may be grouped in communities preferring freshwater or brine (Magot et al., 2000).

Thermophilic SRB are mainly responsible for processes of *in-situ* oil transformation. An important genus from this group is the *Desulfotomaculum* bacteria. The *D. kuznetsovii* bacteria were found in a reservoir in the Paris Basin. In the Norwegian sector of the North Sea, the bacteria *D. thermocisternum* have been detected, which incompletely biodegrade hydrocarbons to compounds such acetate, lactate, ethanol, butanol, and carboxylic acids in the presence of sulfates. New subspecies, the *D. nigrificans - salinus* bacteria that incompletely oxidize lactate

and alcohols to acetate were identified in oil samples from western Siberia (Nazina et al., 2005). In oil samples from the North Sea, bacteria such as *Desulfacinum infernum*, *Thermodesulforhabdus norvegicus*, and *Thermodesulfobacterium mobile* were found. These first two bacteria the *Desulfacinum inferno* and *Thermodesulforhabdus norvegicus* oxidize completely acetate, butyrate, and palmitate to carbon dioxide. The *T. norvegicus* bacteria also utilize alcohols (Jeanthon et al., 2002). At high temperatures up to 80–85°C (optimum temperature range 60–65°C), the thermophilic bacteria *Thermodesulfobacterium* is capable of growing (Magot et al., 2000; Stetter & Huber, 1999). *T. mobile* bacteria were isolated from a reservoir in the North Sea and the *T. commune* bacteria were isolated from a reservoir located in the eastern part of the Paris Basin. Electron donors for these species include hydrogen, formate, lactate, and pyruvic acid (Magot et al., 2000). In turn, at higher temperatures (at an optimum temperature of 75°C) heterotrophic hyperthermophilic bacteria exist from the genus *Archaeoglobus*, which use lactate, pyruvic acid, and valerate in the presence of hydrogen as the carbon source (Stetter et al., 1987; Stetter & Huber, 1999). Genetically, these microorganisms are close to bacteria occurring near submarine vents. Thermophilic SRB have been discovered in the lower parts of the White Tiger oil reservoir. These SRB are probably autochthonous because they were not detected in the injected water or supplying boreholes. Nonetheless, the same samples yielded mesophilic aerobic bacteria and methanogenic archaea, suggesting the presence of a fractured system running through areas with low temperatures up to the productive horizon. It is thus not clear whether thermophilic SRB are derived from submarine vents or oceanic ridges, where bacterial sulfate reduction takes place at temperatures close to 100°C (Rozanova et al., 2001).

SRB play a significant role in oil reservoirs, mainly due to the ability to metabolize various organic compounds, including aliphatic, aromatic, and polycyclic aromatic hydrocarbons (PAHs). In anaerobic ecosystems, the process of organic matter mineralization is usually much more complex than in aerobic conditions and requires the co-operation of different microorganism groups. Each group has its own specific stage of substrate oxygenation and the final products are metabolized by the next link of the food chain until complete mineralization

Table 1 outlines several species of microorganisms reducing sulfates that have been isolated from crude oil exploitation areas.

Species	Salt Content (wt%)	T (°C)	Occurrence
<i>Desulfotomaculum nigrificans</i>	0–4	40–70	Oil field
<i>Desulfacinum infernum</i>	0–5	40–65	
<i>Thermodesulfobacterium mobile</i>	lack of data	45–85	
<i>Thermodesulforhabdus norvegicus</i>	0–5.6	44–74	
<i>Archaeoglobus fulgidus</i>	0.02–3	60–85	Formation water
<i>Desulfomicrobium apsheronum</i>	0–8	4–40	
<i>Desulfovibrio gabonensis</i>	1–17	15–40	
<i>Desulfovibrio longus</i>	0–8	10–40	
<i>Desulfovibrio vietnamensis</i>	0–10	12–45	Formation waters
<i>Desulfobacterium cetonicum</i>	0–5	20–37	
<i>Desulphomaculum halophilum</i>	1–14	30–40	Drill bit
<i>Desulfobacter vibrioformis</i>	1–5	5–38	Oil-water separator
<i>Desulfotomaculum thermocisternum</i>	0–5	41–75	Marine Sediments

Table 1. Species of sulfate reducing microorganisms isolated from different areas of crude oil exploitation (Magot et al., 2000)

According to literature data (Magot et al., 2000; Stetter&Huber, 1999) SRB isolated from crude oil and formation waters are characterized by a wide tolerance range in relation to salt content (0–17 %) and temperature (4–85°C).

### 2.2.3 Methanogenic archaea

Methanogenic archaea bacteria are the next important group of microorganisms occurring in crude oil reservoir settings (Magot et al., 2000; Nazina et al., 2007). The product of their activity is methane; therefore the biological activity of these microorganisms is measured by the methane production rate or by the volume of methane produced.

Methanogenic archaea bacteria occur in diverse settings. Their development and activity is influenced by physical and chemical factors such as temperature, salt content, and pH. Most methanogenes are mesophilic organisms, although extremophiles are also present. The latter include *Methanopyrus kandleri*, which can occur at temperatures of 110°C (Kurr et al., 1991) and *Methanococcus vulcanicus* (Jeanthon et al., 1999). There are very few publications on psychrophilic methanogenic archaea. Important conditions for the development and activity of methanogenic archaea are the salt content of the environment and the lack of oxygen. These microorganisms are very sensitive to very low concentrations of oxygen even in the range of several ppm (Elias et al., 1999).

Methanogenic archaea is physiologically nonuniform. Representatives of this group are known to produce hydrogen sulfide in the process of sulfur reduction (Mikesell & Boyd, 1990). Autotrophy is a common phenomenon among archaea. Many methanogenic archaea may bind carbon dioxide as well as use methanol or acetate as the carbon source to synthesize organic compounds.

Methanogenes include prototrophic species, requiring only CO<sub>2</sub>, H<sub>2</sub>, and mineral salts for growth. An example of this group is the *Methanobacterium thermoautotrophicum* (Zeikus & Wolfe, 1972). However, most methanogenes utilize hydrogen as the electron donor and carbon dioxide as the electron acceptor. The final product of this process is methane. The process of biogenic methane formation is known as methanogenesis, which is specific from anaerobic respiration with low energy yield. Most species, however, require very specific compounds for methanogenesis to take place such as vitamins (e.g. biotin or riboflavin). Methanogenic archaea take part in the final stage of organic matter degradation at strictly anaerobic conditions and very low reduction potential (–330 mV).

Mesophilic and thermophilic methanogenic archaea may occur in settings with various salt contents, however the simultaneous presence of high temperatures and high salt concentrations may hamper the activity of methanogenic archaea. *Methanococcoides* (*Methanohalophilus*) *euhalobius* was detected among methanogenic caryopsis utilizing methylamines in samples taken from oil reservoir drilling operations having salt contents of 140g NaCl/l. The presence of methanogenic archaea using methyl substituents in environments having intermediate to very high salt concentrations has been linked to the amine degradation that took part during osmoregulation; a similar process could occur within oil reservoir environments (Sowers & Ferry, 1983).

The next group of methanogenic archaea is represented by species utilizing acetate as the electron donor. Methane production was observed in samples from oil field reservoirs

located in the North Sea and western Siberia that were inoculated with yeast extract or acetate (Magot et al., 2000). In this work, the production of methane was used to detect the presence of methanogenic archaea, although they were not isolated (Gray et al., 2009; Kotsyurbenko et al. 2004). In these cases, a syntrophic bacterial community involving acetate oxidizing bacteria and methanogens (utilizing hydrogen) could be formed. Similar conclusions were drawn in relation to the Kongdian oil reservoir in China (Nazina et al., 2007). The situation may be explained by the competition between SRB and methanogenic archaea (MA). SRB utilize sulfates as the final electron acceptor in anaerobic respiration, a process that is energetically more favorable than methanogenesis. In fact, as a result of the SRB activity the electron donors available in the environment are utilized and methanogenesis is slowed down (Fig. 5).

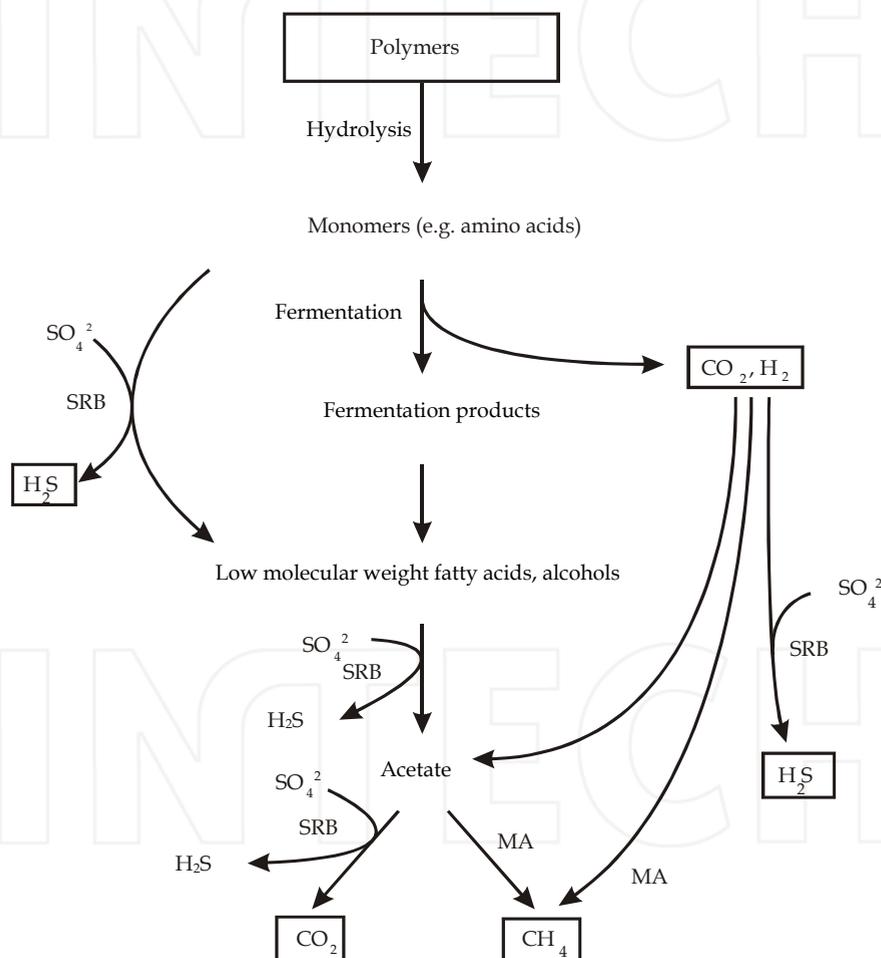


Fig. 5. Modified scheme of the biodegradation of organic compounds with the participation of various groups of microorganisms, based on Kalyuzhnyi et al. (1998); SRB - sulfate reducing bacteria, MA - methanogenic archaea.

The dominance of a group of microorganisms depends mainly on the concentration of sulfates in the environment and the organic carbon content (COD). The calculated COD/SO<sub>4</sub> ratio for the environment supplies information on the possible selection of a particular microorganism group (Hao et al., 1996). If the COD/SO<sub>4</sub> ratio reaches 0.67 or less, then according to the stoichiometry of the sulfate reduction process, the bulk of the organic compounds may be mineralized through production of CO<sub>2</sub> and H<sub>2</sub>S. If the COD/SO<sub>4</sub> ratio is higher than 0.67, then more organic compounds are decomposed during methanogenesis. Thus based on the known concentration of both components (organic carbon [C<sub>org</sub>] and S [SO<sub>4</sub>]) it is possible to determine (or create) conditions favorable for the development of selected group of microorganisms (Oude Elferinck et al., 1998).

The most common and often prevailing group of methanogenic archaea, particularly in formation waters having low salinity concentrations, corresponds to hydrogen oxidizing methanogenic species. They include the disc-shaped *Methanococcus termolittotrophicus*, *Methanoplanus petrolearius*, and *Methanocalculus halotolerans*; and the rod-like *Methanobacterium thermoautotrophicum*, *M. bryantii*, and *M. ivanovii*. Although hydrogen oxidation during methanogenesis at surface conditions may take place only at NaCl concentrations below 9 %, the species *Methanocalculus halotolerans* are capable of carrying out this metabolic activity at conditions of high salt content up to 12 %. This capability may be an evidence of their autochthonous character related to oil reservoir ecosystems (Ollivier et al., 1998).

#### 2.2.4 Iron III reducing bacteria

*Shewanella putrefaciens* is an iron reducing bacteria that also has the ability to reduce elemental sulfur, sulfites, and thiosulfates to sulfides. This bacteria is capable of withstand the harsh conditions of oil reservoirs. The electron donor may be H<sub>2</sub> or formate, and the acceptors - iron oxides and hydroxides. *Deferribacter thermophilus* is a bacterium that besides iron reduction also reduces manganese and nitrates using yeast extract or peptone. The source of energy is hydrogen and numerous organic acids. It is, however, not clear whether such type of metabolism occurs in conditions *in-situ* due to the lack of data on the content of iron and manganese ions (Grenne et al., 1997).

### 3. Microbiological biodegradation of crude oil components

The biodegradation of hydrocarbons *in-situ* (within the oil reservoir) significantly affects the composition of the crude oil. Peters & Moldowan (1993) proposed a 10-level biodegradation scale, in which 0 corresponds to unchanged material, 5 represent moderate biodegradation, and 10 corresponds to advanced biodegradation. The determination of the biodegradation stage may be also based on the relations of pristane and phytane (Pr+Ph) to nC17 + nC18, C30 alpha beta hopane to Pr+Ph, and 25-nor C29 alpha beta hopane to C30 alpha beta hopane. Depending on the stage of the process, different proportions of particular hydrocarbons are contained in the crude oil. Thus the composition of the crude oil is linked to the susceptibility of the specific crude oil to microbiological degradation. In crude oil reservoirs, a factor complicating the determination of the degree of oil biodegradation is the influx (migration) of oil from the source rock. If the initial fraction of oil was subjected to microbiological transformations and portions of unchanged oil were introduced later, then the total product will have very different composition, with biomarkers pointing to both

high and low microbiological evolution of the raw material. Moreover, there will be also differences in the physical properties of the oil such as viscosity. Complete homogenization of crude oil is not possible even in the geological time scale. Due to processes of oil mixing and diffusion its composition may become uniform only locally.

Generally, biodegradation of crude oil takes place in the contact zone between crude oil and water. The resulting solution gradient causes influx of substances prone to degradation such as *n*-alkanes and isoprene alkanes into this zone and outflow of reaction products in the opposite direction. Mixing of biodegraded oil with inflowing unchanged oil takes place also in this region. At the rise of 25-nor hopane concentration, a typical feature is the removal of *n*-alkanes, followed by isoprenoid hydrocarbons with the increase of biodegradation rate. At reservoir scale, advanced biodegradation is favored by moderate temperatures in shallow reservoirs.

The influence of biodegradation on the crude oil composition is reflected in the concentration of particular compound groups. For example, in the case of PAHs, three elements determine their susceptibility to microbiological degradation: the number of rings in the compound, the number of alkyl substituents, and the location of the bonds of these substituents. The percentage content of hydrocarbons with large number of rings is inversely proportional to the rate of biodegradation (Huang et al., 2004). Similar relationship takes place in the case of the number of substituents, e.g. dimethylnaphthalene is more susceptible to biodegradation than trimethylnaphthalene. In the case of methylnaphthalenes, the most thermodynamically stable isomers are very quickly decomposed. This indicates that the stereochemical configuration of the hydrocarbon compounds is the dominant factor affecting biodegradation of crude oil rather than thermodynamic effects. In the case of methylphenanthrenes, the highest resistance to biodegradation was observed in those with methyl groups in positions 9 and 10. In the case of steroid hydrocarbons such as mono-aromatics (MSHs) and tri-aromatics (TSHs) hydrocarbons, MSHs are more resistant to microbiological degradation than the TSHs. Branched TSHs with short side chains are more effectively biodegraded than branched TSHs with long chains. In turn, short chain pregnanes are rather resistant to biodegradation in comparison to long chain typical steranes. High thermal maturity is related to high values of the concentration ratios of these compounds, while the degree of biodegradation increases when the ratio  $[C_{21}/(C_{21}+C_{28})]$  decreases and when the ratio of  $[C_{21-22}\text{-pregnanes}/(C_{27-29}\text{-steranes})]$  increases. No trend has been identified for the ratios of short to long chains of MSHs. This is because thermally mature crude oils show higher concentrations of thermodynamically stable components. Thus, indicators for crude oil thermal maturity might be influenced by biodegradation because bacteria generally decompose thermodynamically stable components very quickly. In addition, thermal maturity trends and biodegradation indicators may differ between oil reservoirs due to different species within the bacterial population, red-ox conditions, crude oil composition, and nutrients availability (Huang et al., 2004).

*In-situ* biodegradation of the light hydrocarbon fraction, e.g. *n*-paraffins, iso-paraffins, cycloalkanes, as well as benzene and alkylbenzenes may also take place in crude oil reservoirs (Vieth & Wilkes, 2005). All these compounds are biodegraded under anaerobic conditions that typically occur in oil reservoirs. However, these bacteria are highly selective for metabolism and microorganisms capable of simultaneously biodegrade aromatic hydrocarbons and paraffins have not been found yet.

Generally within the non-aromatic hydrocarbons, bacteria degrade first *n*-paraffins and then iso-paraffins therefore an increase of the *i*-C5/*n*-C5 ratio may be used as an indicator of the biodegradation progress. In the case of methylcyclohexane/*n*-C7 ratio, the same trend also indicates a larger degree of oil degradation. Another method that allows the determination of oil biodegradation degree is the study of the isotopic ratios of particular compounds. Microorganisms' preference toward light isotopes causes the enrichment of heavier isotopes in the crude oil. This method may be, however, applied only in the case of low molecular weight carbon compounds. The reason is that the activation of the substrate takes place in most cases on only one carbon atom, thus its share in the bulk mass of the hydrocarbon compound would be very small and the isotopic effect would be immeasurable. The removal of light oil fractions due to biodegradation is an unfavorable event because it causes the decrease of the economical value of the crude oil (Vieth & Wilkes, 2005).

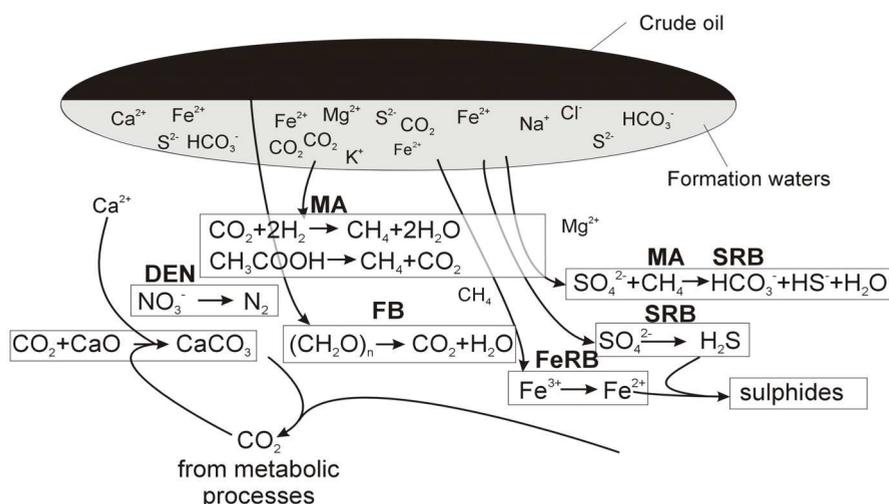
The biodegradation of oil within the reservoir rock may cause the formation of an upper gas cap, which is composed mainly of CH<sub>4</sub> that is formed during the reduction of CO<sub>2</sub> with H<sub>2</sub> or methyl acetate. If the oil contains high quantities of sulfur, the activity of SRB would result in the formation of H<sub>2</sub>S at concentrations that may reach over 10 % in the gas. Similarly, the activity of other microbes may result in the transformation of primarily wet gas into dry gas containing over 90 % methane. This process is linked to the preferential removal of C3 to C5 components by bacteria; thus the residual methane will be isotopically heavier and the isoalkane to *n*-alkane ratio will be increased. At the same time, the biodegradable oil fraction will become heavier; therefore the course of changes is two-directional. For instance, this type of situation has been observed in the Troll and Frigg reservoirs in the North Sea and in other reservoirs characterized by a dominant gas cap surrounded by an oil ring. Numerical simulation indicates that these gas reservoirs could be formed by the significant biodegradation of oil, condensate, and gas in cooperation with various physical and chemical processes with the simultaneous migration of part of the gas from the hydrocarbon trap through microfractures or communicating faults (Larter & Primio, 2004).

There is also a link between the acidity of the oil, which is measured as the content of KOH required for neutralizing this acidity, and the degree of oil biodegradation. This is observed particularly in oil with high acid value (>0.5 g KOH/g oil). According to Meredith et al. (2000), this fact is connected with the production of carboxylic acids during *in-situ* microbiological degradation of hydrocarbons. However, other factors also influence the total acid value of the oil, because some non-biodegraded oils have low pH, which is probably linked with high sulfur content. In oil with advanced biodegradation, the concentration of hopane acids increases, however this increase is not enough to affect the overall acidity of the crude oil.

#### 4. Mineral-forming processes

There is a closed relationship between crude oil, formation waters, and microorganisms (Wolicka et al., 2011). The physical and chemical conditions in oil reservoir environments selectively influence the development of particular microorganism groups; while simultaneously microorganisms may modify the chemical composition of the reservoir environment through their biological activity (Douglas & Beveridge, 1998; Onstott et al.,

2010). Nowadays, the role of microorganisms is an important subject in oil geology, due to the fact that they influence geological processes in hypergenic settings. Various mineral phases formed in biological processes may significantly influence microorganism activity and metabolism, as well as the physical and chemical properties of the environment. In many reports devoted to the crystallization of mineral phases, rock- and ore-forming processes and sedimentation in hypergenic conditions, there are evidences of the role of microorganisms in the formation of carbonates, sulfides or elemental sulfur in anaerobic conditions, however the information is insufficient and often the role of microorganisms in geological processes is just mention (Popa et al., 2004; Borkowski & Wolicka, 2007a, b; Wolicka & Borkowski, 2008b).



**MA - Methanogenic Archaea**    **SRB - Sulphate-Reducing Bacteria**    **DEN - Denitrifying Bacteria**

**FeRB - Iron-Reducing Bacteria**    **SB - Aerobic Sulphur Bacteria**    **FB - Fermentative Bacteria**

Fig. 6. Possible microbiological reactions in oil reservoirs from different groups of microorganisms.

Crude oil and oil refined products contain various organic compounds, mainly hydrocarbons, both aliphatic and aromatic. Biodegradation of crude oil and oil-derived products and formation of mineral phases are natural processes. Knowledge on these issues is, however, restricted, particularly in the case of anaerobic conditions. Particular attention is drawn to SRB, because they are the main link between the biogeochemical cycles of carbon and sulfur in anaerobic conditions. These microorganisms play an important role in the biodegradation of organic compounds and in mineral-forming processes. In some conditions, they are capable of forming mineral phases and to degrade minerals, e.g. aluminosilicates (Ehrlich, 2002). Mixed populations of SRB may begin the precipitation of many different minerals, e.g. carbonates such as calcite, dolomite, and siderite (Perry & Taylor, 2006; Wolicka & Borkowski, 2011), phosphates (e.g. apatite), elemental sulfur (Wolicka & Kowalski, 2006a, b), or sulfides (Labrenz et al., 2000). The process of dolomite,

calcite, and aragonite formation in settings typical of SRB and in conditions favoring sulfate reduction is commonly described from anaerobic zones of salinated lagoons (Warthman et al. 2000), in gypsum and anhydrite deposits (Peckmann et al. 1999), as well as in salinated natural lakes rich in sulfates (Wright, 1999a, b). Figure 6 summarizes possible microbiological reactions taking place in oil reservoirs from different groups of microorganisms.

Secondary iron sulfides (pyrite, marcasite) and other metals, e.g.: galena (PbS), sfalerite (ZnS), chalkopyrite (CuFeS<sub>2</sub>), chalcocite (Cu<sub>2</sub>S), covellite (CuS), cinnabar (HgS), and realgar (AsS) are formed during dissimilative sulfate reduction.

## 5. Microbial enhanced oil recovery

The presence of autochthonous microorganisms, as well as their persistence and reproduction within crude oil reservoirs is advantageous for the implementation of Microbial Enhanced Oil Recovery (MEOR) methods, which are also known as Microbial Improved Oil Recovery (MIOR), allowing additional oil recovery at relatively low cost and the extension of the exploitation life of mature reservoirs, otherwise abandoned.

There are several approaches to take advantage of the natural metabolic processes of autochthonous microorganism to boost oil recovery as follows.

1. Biosurfactant sources. Based on the fact that many microorganisms synthesize biosurfactants, a microorganism community with such properties may be selected for the controlled production of biosurfactants for injection into the reservoir to decrease the interfacial tension between water and oil, thus releasing oil trapped in the rock by capillary forces. Furthermore, the presence of biosurfactants in the reservoir environment aids the biodegradation of hydrocarbons by autochthonous microorganisms. Similarly, biosurfactants may also become an easily accessible carbon source for autochthonous microorganisms, which produce biogenic gases such as CO<sub>2</sub>, CH<sub>4</sub>, and H<sub>2</sub> that would increase the reservoir pressure, which favors the displacement of crude oil towards production wells.
2. Biogenic gas production. The direct introducing of selected microorganisms that are capable of crude oil biodegradation with the ultimate goal of producing biogenic gases to increase the reservoir pressure.
3. As fluid diverting agents by producing biofilms. Microorganisms develop biofilms on the reservoir rock surfaces, which cause plugging of watered pores, thus redirecting the injected water to upswept areas of higher oil saturations making waterflooding more effective in displacing oil toward the production wells.
4. Control of H<sub>2</sub>S production. The activity of SRBs can be hampered by the introduction of chemical compounds into the reservoir that would increase the activity of autochthonous microorganism groups to slow down the activity of the SRBs.

The main advantages of MEOR over conventional enhanced oil recovery (EOR) methods are much lower energy consumption and low or non-toxicity that makes MEOR an environmentally friendly process. Lower costs come from the fact that after the introduction of microorganisms into the reservoir their growth increases exponentially, therefore it is possible to obtain a large number of active substances from a small volume of initial organic

material. After the initial injection of microorganisms, it is only necessary to supply nutrients into the reservoir rock to enable the development of the existing bacterial population that will produce the required biosurfactants. Some of the common microorganisms used in MEOR processes include: *Pseudomonas aeruginosa*, *Bacillus licheniformis*, *Xanthomonas campestris* and *Desulfovibrio desulfuricans* (Singh et al., 2007).

Biotechnological approaches use microorganisms that oxidize oil to break-up asphaltene-resin-paraffin sludges that have been accumulated in the wellbore for years affecting the oil production due to plugging of the oil production zones. In mature reservoirs, it is common to find production wells rendering water cuts of 70 to 90 % of the total production. The microbial stimulation of aged production wells to break-up unwanted paraffin/asphaltene deposits is an economical way to reactivate old production zones.

In practice, the method may be difficult to implement because uncertainty in providing suitable conditions for the development of the microorganism groups taking part in the metabolic pathway. Furthermore, after microbial application it is essential to remove the bioproducts of microorganisms' activity, as well as the bacteria themselves in order to maintain the composition of the crude oil (it is not desirable to give up light fractions of the crude oil to biodegradation). For instance, *in-situ* bacterial cultivation may cause decrease of oil production. Another disadvantage of MEOR is the unpredictability of the process (Mokhatab, 2006). Other problems linked to MEOR include plugging of the reservoir rock by the bacterial mass in undesirable locations, *in-situ* biodegradation of the applied chemical compounds, and acidification of the crude oil by the bioproduction of hydrogen sulfide in the reservoir (Almeida et al., 2004).

### 5.1 Examples of Microbial Enhanced Oil Recovery applications

The application a MEOR process in the Dagang oil field, in China was reported by Liu Jinfeng (2005) In this MEOR application, zymogenic organisms (*Arthrobacter*, *Pseudomonas* and *Bacillus*) were introduced into the reservoir. These microorganisms have the ability to oxidize crude oil. *Arthrobacter* and *Pseudomonas* are highly effective oxidizing crude oil, whereas *Bacillus* bacteria produce surfactants in crude oil fermentation and consequently reduce the oil/brine interfacial tension. The field trial was initiated with the injection of nutrients for 3 days into the reservoir, followed by the injection of the suspension of *Arthrobacter* and *Pseudomonas*. The wells were shut in for 10 days to cultivate the introduced microorganisms. After 10 days, a suspension of *Bacillus* was injected in the reservoir and the wells were shut in again for another 10 days. Follow by an additional injection of nutrients (Jinfeng et al., 2005). Positive results were observed; significant changes in crude oil and gas properties were observed. The density of the oil decreased by 0.0024 g/cm<sup>3</sup>, oil viscosity by 4.1 mPas, paraffin content by 4.35% and asphaltenes by 2.31%. The content of methane in the accompanying gas increased from 85.4 % to 90.02 % and the content of CO<sub>2</sub> decreased from 5 % to 1.5 % – these favorable changes of gas composition are suggested to result from the activity of methanogenic archaea.

This field trial shows that MEOR may be successfully applied in high-temperature crude oil reservoirs. In this field trial, the reservoir temperature corresponded to the upper thermal boundary of 70°C for the development of thermophilic bacteria (Jinfeng et al., 2005).

Not all reservoir microorganisms can be applied in MEOR process; some such as the SRB may even be harmful for the exploitation and processing of crude oil. Very often applications of MEOR can result in the increase of hydrogen sulfide activity and production. This phenomenon is very unfavorable and cannot be controlled entirely due to the complex nature of biochemical and abiotic processes taking place in the reservoir and in the exploitation area. Figure 7 summarizes some of the negative and positive effects of microbial activity in the reservoir.

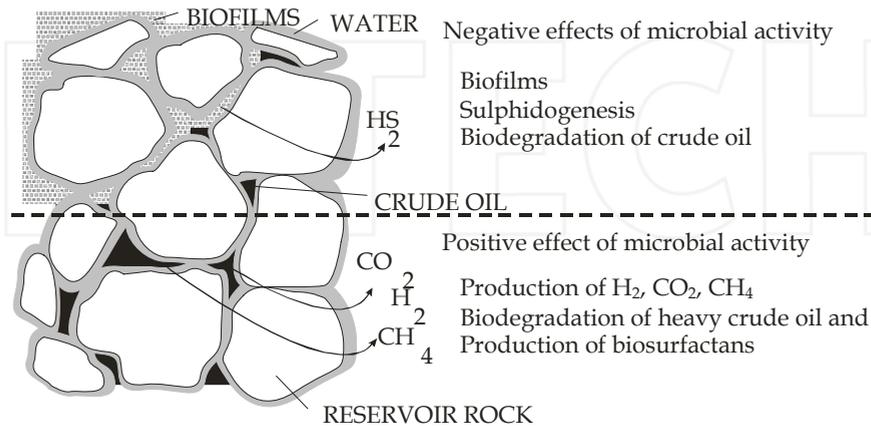


Fig. 7. Negative and positive effects of microbial activity in oil reservoirs

### 5.2 Changes of the reservoir rock permeability

A well-known and often studied MEOR method is the selective plugging of reservoir zones with the purpose of diverting injected water towards reservoir areas of high oil saturations. In the North Burbank in Oklahoma (1980) the growth of aerobic and anaerobic heterotrophic bacteria, SRB, and methanogenic halophilic archaea was stimulated by the addition of nutrients; the rapid growth of the bacteria population caused the decrease of permeability in the treated well by 33 %. Bacteria blocked highly permeable zones of the reservoir rock diverting water injected toward low permeability areas containing high saturation of crude oil. It is suspected that the microorganisms' mass and the products of their biological activity such as biopolymers, effectively blocked the flow of water within large pores and fractures. Biopolymers allow bacteria to connect and form biofilms, which improves nutrient gain and decreases their sensitivity to toxic substances (Mokhatab, 2006).

### 5.3 Biological demulsification of crude oil

MEOR applications also include the demulsification of crude oil. In crude oil processing there are two types of emulsions, e.g. oil in water and water in oil. These emulsions are formed at different stages of crude oil exploitation, production, and processing, causing significant operational problems to the industry (Singh et al., 2007). The release of oil from these emulsions is known as demulsification. Traditionally demulsification methods include: centrifuging, warming, electric current application, and the addition of chemical compounds to break up the emulsions.

However, the application of microbiological demulsification methods during drilling operations have been practiced with success, thus saving on transportation and on equipment costs (Leppchen, et.al.; 2006). There are many microorganisms with favorable demulsification properties including *Acinetobacter calcoaceticus*, *A. radioresistens*, *Aeromonas* sp., *Alcaligenes latus*, *Alteromonas* sp., *Bacillus subtilis*, *Corynebacterium petrophilium*, *Micrococcus* sp., *Pseudomonas aeruginosa*, *P. carboxydohydrogena*, *Rhodococcus aurantiacus*, *R. globerulus*, *R. rubropertinctus*, *Sphingobacterium thalophilum*, and *Torulopsis bombicola*. Microorganisms benefit from the double hydrophobic-hydrophilic nature of surfactants or the hydrophobic cell surface to remove emulsifiers from the interfacial surface between oil and water. Temperature increase is favorable for the demulsification process, because it decreases viscosity, increases the density difference between the phases, attenuates the stabilizing action of the interfacial surface, and increases the drop collision rate, which leads to coalescence (Singh et al., 2007).

## 6. Role of microorganisms in the biodegradation of crude oil

Microorganism activity serves to remove pollution by crude oil and oil-derived products from soil, groundwater, and seawater occurring near exploitation sites, leaking pipelines, and in dispersed locations such as petrol stations or roadsides.

The basic factors influencing the biodegradation of oil-derived products in soil include: chemical structure, concentration and toxicity of hydrocarbons in relation to the microflora, microbiological soil potential (biomass concentration, population variability, enzyme activity), physical-chemical environmental parameters (e.g. reaction, temperature, organic matter content, humidity), and availability of hydrocarbons for microorganism cells.

### 6.1 Biodegradation in aerobic conditions

Many soil microorganisms - bacteria and fungi - transform crude oil hydrocarbons into non-toxic compounds or conduct their complete mineralization to inorganic compounds. This natural microbiological activity is used in bioremediation to reduce the concentration and/or toxicity of various pollutants, including oil-derived substances. An unquestionable advantage of this technology is the low investment cost (complex and expensive technology is not required). Additionally, methods used in bioremediation do not involve introduction of chemical compounds that may negatively influence the biocoenosis to the soil and the soil may be used again after the remediation. The most important issue is that these processes are natural and the final products of the microbiological degradation are carbon dioxide and water.

The effectiveness of hydrocarbon degradation by bacteria does not depend on the number of carbon atoms in the compound (Klimiuk & Łebkowska, 2005; Stroud, 2007). Branched alkanes are biodegraded slower, although the process is similar to that in *n*-alkane degradation. The latter are preferred as the source of carbon by microorganisms and in their presence the degradation of branched alkanes is much slower (Klimiuk & Łebkowska, 2005). Cycloalkanes are degraded under aerobic conditions by bacterial consortia in co-metabolism as well as by pure microbial strains. However, biodegradation of cycloalkanes with high number of rings in the compound is much slower (Klimiuk & Łebkowska, 2005).

Small molecule (two or three ring) aromatic hydrocarbons are degraded by many soil bacteria as well as numerous fungi genera e.g. *Rhizopus*, *Aspergillus*, *Candida*, *Penicillium*, *Psilocybe*, and *Smittium*. In turn, the ability to degrade large-molecule (4 or more rings) of PAHs is rather rare among bacteria (e.g. *Pseudomonas putida*, *P. aeruginosa*, *P. saccharophila*, *Flavobacterium* sp., *Burkholderia cepacia*, *Rhodococcus* sp., *Stenotrophomonas* sp. and *Mycobacterium* sp.). These compounds are rather degraded by ligninolytic fungi, such as: *Phanaerochaete chrysosporium*, *Trametes versicolor*, *Bjerkandera* sp., *Pleurotus ostreatus*, and non-ligninolytic fungi, such as *Cunninghamella elegant*, *Penicillium janthinellum*, and *Syncephalastrum* sp. (Austin et al., 1977; Kirk & Gordon, 1988; Wolicka et al., 2009).

Degradation of compounds with five or more aromatic rings depends largely on the activity of the mixed microorganism populations. The metabolism of symbiotic systems may be the only form of metabolism of these compounds. So far, it has been reported that the biotransformation of benzo[a]pyrene by bacteria took place in co-metabolic conditions (Bogan et. al, 2003).

The microorganism's ability to grow on large-molecules of polycyclic aromatic hydrocarbons is probably a common feature of *Mycobacterium*. This property was noted in several species, e.g. *M. flavescens* and *M. vanbaalenii* sp. and strains such as: AP-1, PYR-1, BB1, KR2, GTI-23, RJGII-135, BG1, CH1. Many bacteria from the genus *Mycobacterium* that biodegrade PAHs also have the ability to degrade aliphatic hydrocarbons.

## 6.2 Biodegradation at anaerobic conditions

Biodegradation of hydrocarbons by microorganisms at anaerobic conditions is common knowledge since the end of the 1980s (Widdel & Rabus, 2001; Meckenstock et al., 2004). Anaerobic microorganisms utilize monocyclic aromatic hydrocarbons, such as benzene, toluene, ethylbenzene, xylene (BTEX), hexadecane, and naphthalene as the sole carbon source. Strains RCB and JJ of *Dechloromonas* ( $\beta$ -*Proteobacteria*) completely oxidize benzene in anaerobic conditions using nitrate as the electron acceptor. *Geobacter metallidurans* and *G. grbicum* are capable of anaerobic toluene oxidation to CO<sub>2</sub> with reduction of Fe(III). Some organisms were reported to link anaerobic toluene degradation with nitrate respiration (*Thauera aromatica* strains K172 and I1, *Azoarcus* sp. strain T, *A. tolulyticus* strains To14 and Td15, *Dechloromonas* strains RCB and JJ), perchlorate respiration (*Dechloromonas* strains RCB and JJ) and sulfate respiration (*Desulfobacterium cetonicum*, *Desulfobacula toluolica*) (Chakraborty & Coates; 2004). A methanogenic consortium composed of two archaea species related to *Methanosaeta* and *Methanospirillum* and two bacterial species, of which one is linked to *Desulfotomaculum*, were reported to degrade toluene (Beller et al., 2002). Fluorescence *in-situ* hybridization (FISH) of denitrifiers degrading alkylbenzenes and *n*-alkanes indicated that the bacteria belong to the *Azoarcus/Thauera* group. SRB are known to utilize benzene, toluene, ethylbenzene, and xylene as the sole carbon source (Coates et al., 2002; Kniemeyer et al., 2003; Ribeiro de Nardi et al., 2007).

Polycyclic aromatic hydrocarbons may also be degraded at anaerobic conditions. So far, only few 2-ring PAHs have studied with regard to biodegradation under sulfate reduction conditions. Metabolism studies were mainly focused on naphthalene and 2-methylnaphthalene; while less attention was given to the anaerobic metabolism of phenanthrene. Among 3-ring PAHs, phenanthrene may be degraded through an initiating

reaction step that could be carboxylation. Anaerobic degradation of 3-ring PAHs also takes place through co-metabolism, for instance, benzothiophene is biodegraded in the presence of naphthalene as the subsidiary substrate. Anaerobic degradation is also possible for heterocyclic compounds such as indole or chinoline (Meckenstock et al., 2004; Widdel & Rabus, 2001).

### 6.3 Hydrocarbon biodegradation by psychrophilic microorganisms

Bacterial oil degradation takes place in extreme conditions e.g. in Polar Regions (Aislabie et al., 2006). Populations of microorganisms utilizing hydrocarbons are dominated in this case by psychrotolerants microorganisms rather than by psychrophiles. *Rhodococcus*, *Sphingomonas*, *Pseudomonas*, *Acinetobacter*, *Shewanella*, and *Arthrobacter* are present at conditions of low temperatures (Aislabie et al., 2006; Kato et al., 2001). Some microorganisms that biodegrade alkanes include: *Rhodococcus*, *Acinetobacter*, and *Pseudomonas*. It seems that alkane degradation is conducted by microorganisms of the genus *Rhodococcus*, and *Pseudomonas* have been often found in areas where elevated hydrocarbon concentration existed.

Aromatic hydrocarbons may be degraded both by *Pseudomonas* and *Sphingomonas*. Obviously, temperature is a factor hampering the bioremediation process in polar settings, because defrosting of soil as a thin layer above the permafrost takes place only for 1 to 2 months. Additionally, there are significant daily and annual temperature oscillations. The maximum temperature of the exposed soil horizon may reach 20°C during the day. Low temperature results in decrease of oil viscosity that hampers microbial metabolism. Moreover, the evaporation of light compounds with toxic properties is restricted, which prolongs the time required by microorganisms to adapt to the toxic conditions.

Low temperature unfavorably influences the degradation of mixtures comprising various oil-derived compounds, whereas the course of degradation of uniform pollution composition decreases to a lesser degree. Twenty-four hour cycles of defrosting and frosting may increase the bioavailability of the required compounds due to changes in soil structure (Aislabie et al., 2006).

In Polar Regions, the difficulties faced by microorganisms during hydrocarbon bioremediation processes in soil are not only connected with low temperature conditions, but also with the presence of relatively low concentrations of biogenic elements. The successive pollution of already polluted environments causes further impoverishment of the required elements. A factor that could also restrict the bioremediation process under these conditions is the lack of water in the soil, as well as high pH values, particularly in seaside areas (Aislabie et al., 2006). So far in the published literature, only hydrocarbon biodegradation by aerobic action of microorganisms have been described for Polar Regions.

## 7. Bioremediation – a natural method of removing crude oil and oil-derived products pollution from the environment

Bioremediation is a biological method that uses living organisms to reduce or completely remove pollutants (organic and inorganic compounds) from polluted areas. In the case of crude oil and oil-derived products pollution, the most effective way of biological

purification of water-soil environments is the application of microbiological methods. Such approach to bioremediation of areas polluted by crude oil can be conducted by the separation of groups of microorganisms capable of the effective biodegradation of many of the organic compounds occurring in oil-derived products and crude oil. In addition, microorganisms have the most complex and comprehensive metabolism among living organisms.

Understanding the metabolic interactions between diverse hydrocarbons and microorganisms requires knowledge on the various forms of occurrence and diverse properties of hydrocarbons in the environment.

Laboratory and field studies have allowed the determination of the optimal parameters for the bioremediation of soil polluted by oil hydrocarbons: biomass content over  $10^5$  cells/g dry mass, relative humidity 20–30 %, temperature 20–30°C, pH 6.5–7.5, oxygen content at least 0.2 mg/l hydrocarbons, Carbon:Nitrogen:Phosphorous (C:N:P) = 100:10:1 or 70:7:1 (Szlaski & Wojewódka, 2003; Sztompka 1999).

### 7.1 Strategies for bioremediation

Soil bioremediation may be carried out *in-situ* at the polluted site, or *ex situ* through the removal of the polluted soil from the site and placing it in a specially prepared location. Important ways of *in-situ* purification include: soil cultivation, bioventilation, bioextraction, and *in-situ* biodegradation. Bioremediation methods *ex situ* include: soil cultivation, composting, biostacks, and bioreactors.

Three types of soil remediation are distinguished: natural bioremediation (natural attenuation), biostimulation, and bioaugmentation.

Natural bioremediation is based on the process of natural biodegradation (known also as natural attenuation) carried out by microorganisms; it requires only regular monitoring of pollution concentration. The method uses microorganisms naturally occurring in the environment without stimulating their development in any way. Parallel monitoring of the polluted area is carried out to evaluate the effectiveness of decontamination and recognition of process mechanisms (Klimiuk & Łebkowska, 2005; Tiehm & Schulze, 2003). An important element in bioremediation analysis is the determination of the range and location of zones of red-ox potential. This may be carried out through hydrogeochemical methods that are based on the measurement of relative impoverishment in terminal electron acceptors and the enrichment in typical reaction products such as  $\text{Fe}^{2+}$ . The disadvantage of this method is the influence of independent abiotic processes and interactions between the resulting metabolites (e.g.  $\text{Fe}^{2+}$  and  $\text{H}_2\text{S}$ ) on the target measurement. Another possible analytical technique is the electrochemical determination of the red-ox potential, despite the fact that the final measurement may be the sum of potentials from many different reactions that do not reach equilibrium state fast enough to ensure precise measurements.

In the case of native populations in a given polluted area that do not reveal the expected pollutant degradation activity, which could be the result of the toxic effect of the pollutants on the native microorganisms and if the number and quality of microorganisms is deficient, then bioaugmentation is applied. This method is most commonly used for bioremediation of soils polluted with oil-derived products. In this method, microorganism communities,

which are capable of biodegradation of oil-derived products, are isolated from the polluted area, cultivated at laboratory conditions, and later introduced into the polluted environment. Previous research indicates that a 4-ply increase of microorganism activity is reached by the application of this procedure (Suthersan, 1999).

Another approach to bioaugmentation is the application of genetic engineering to optimize the biodegradation effectiveness of these microorganisms, by increasing the content of enzymes important for the biodegradation of particular substances. At present, genetically modified microbes are typically used in bioremediation (Stroud et al., 2007).

Microorganisms are introduced into soils as free cells or as immobilized form on stable media. The latter method has many advantages. In the stable media there is high concentration of bacteria, which allows obtaining high biomass density; furthermore, the method prolongs the time of biochemical activity because of the protective effect of the medium. In these conditions, the excess of substrates and products does not hamper the enzymatic processes. Additionally, the medium improves soil structure, particularly in clay soils; the soil may also be recovered and reused. Expanded clay aggregates, woodchips, volcanic rocks, and plastic materials are used in the immobilization process (Klimiuk & Lebkowska, 2005).

Another means to increase bioremediation in polluted soils is to take advantage of the fact that the pollutant (hydrocarbon) itself could be the main source of energy (carbon) for bacteria survival. Yet another method for increasing the bacteria's accessibility to substrates in the environment is the addition of surfactants or surface-active substances (SAS), which can be produced chemically or formed naturally by microorganisms. SAS increase the mobility of hydrocarbons even at lower concentrations because the decrease of interfacial tension at the oil-water boundary. However, some downsides of the use of SAS are that at high concentrations, these compounds are toxic to microorganisms and may hamper their growth. The use of natural SAS (biosurfactants) to modify bacteria's accessibility to substrates (bioavailability) is recommended. In contrast to chemically synthesized surfactants, biosurfactants properties include high surface activity, biodegradability, and low toxicity. These substances belong to different groups, including lipopeptides, glycolipids, neutral lipids, and fatty acids.

Singh et al. (2007) and Stroud et al. (2007) have shown that the application of biological SAS accelerates the time for bacteria adaptation to the polluted site and render a 5 times increase of bioremediation effectiveness in comparison to the application of chemical surfactants.

Application of beta-cyclodextrins to increase bioavailability of hydrocarbons is an interesting method. Cyclodextrins (CD) have many advantages, e.g. they may form soluble complexes with hydrophobic substances and they are non-toxic. Thus cyclodextrins may accelerate bioremediation by increasing bioavailability. In an experiment conducted by Bardi et al. (2007), a 10-ply increase on the biodegradation of PAHs was obtained after 42 days of treatment in comparison to a sample without CD.

Similarly, the stimulation of the growth of autochthonous microorganisms was more effective with the simultaneous application of biosurfactants (natural SAS) and nutrients in comparison to a case when only nutrients were introduced to the environment (Klimiuk & Lebkowska, 2005). In some situations, despite the fact that biodegradation action has

previously taken place, differences in bioavailability cause that some hydrocarbons remain in the environment, while others are removed completely (Stroud et al., 2007).

## 7.2 Advantages and disadvantages of bioremediation

Bioremediation is an effective method for removing hydrocarbon pollution from various environments. Stimulation of microorganisms at polluted sites may solve the pollution problem at low cost. Conventional remediation methods usually generate higher costs or move the pollution problem to another location. In contrast, bioremediation is a natural and non-hazardous process to the environment. Bioremediation can be carried out at the polluted site without changing the existing land management plans (Vidali, 2001).

As every method, bioremediation has its drawbacks. Not all compounds are susceptible to relatively fast and complete degradation. In some cases, products of degradation of oil-derived products have toxic effects on microorganisms. Furthermore, sometimes bioremediation requires the application of complex techniques in the presence of mixtures of compounds that are randomly dispersed in the medium. Bioremediation lasts longer than chemical methods, usually several years; moreover, its exact duration cannot be precisely determined. *In-situ* bioremediation methods are not always easy to monitor, whereas *ex-situ* bioremediation methods are more expensive due to transportation costs and soil storage (Zieńko & Karakulski, 1997; Vidali, 2001).

## 8. Conclusions

The role of bacteria in crude oil reservoirs is linked to numerous interactions between oil and the environment. Bacteria influence the chemical composition of crude oil, the conditions of its *in-situ* exploitation through the decomposition of some oil fractions, through the production of metabolism products such as biopolymers, biosurfactants, organic acids, and gases ( $\text{CH}_4$ ,  $\text{CO}_2$ ,  $\text{H}_2\text{S}$ ,  $\text{H}_2$ , among others), and by the presence of microorganism's cells that may change the properties of the reservoir rock.

The controlled influence of parameters such as oil-water interfacial tension, oil viscosity, and rock permeability through the use of microorganisms are the basis of Microbial Enhanced Oil Recovery, which has great potential as an EOR process. MEOR methods can be applied in a variety of oil reservoir conditions such as low or high temperature or at high salinity concentrations. The versatility of MEOR applications is explained by the natural adaptation of bacterial species to oil reservoir conditions. Microorganisms play also a positive role in the removal of pollutants in crude oil fields or along transportation routes. Some microorganisms can biodegrade many of the chemical compounds contained in crude oils. Bioremediations process may occur naturally (without human intervention); however natural bioremediation process may take a long time (years) to be completed.

The complex nature of biochemical processes, which take place in harsh environments such as crude oil reservoirs, causes several interpretation problems during the analysis of the models used to describe the microbiological interactions in these settings.

Although a complete description of the bio-interactions taking place in crude oil - microorganisms systems is still pending, the existing knowledge, even in a restricted mode, can be applied for the recovery of additional oil in mature oil fields (MEOR) and in

bioremediation and biotechnology for the remediation of soil-water environments polluted by crude oil and oil-derived products.

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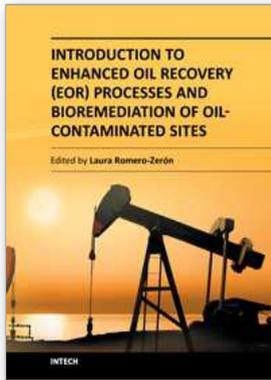
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## **Introduction to Enhanced Oil Recovery (EOR) Processes and Bioremediation of Oil-Contaminated Sites**

Edited by Dr. Laura Romero-Zerón

ISBN 978-953-51-0629-6

Hard cover, 318 pages

**Publisher** InTech

**Published online** 23, May, 2012

**Published in print edition** May, 2012

This book offers practical concepts of EOR processes and summarizes the fundamentals of bioremediation of oil-contaminated sites. The first section presents a simplified description of EOR processes to boost the recovery of oil or to displace and produce the significant amounts of oil left behind in the reservoir during or after the course of any primary and secondary recovery process; it highlights the emerging EOR technological trends and the areas that need research and development; while the second section focuses on the use of biotechnology to remediate the inevitable environmental footprint of crude oil production; such is the case of accidental oil spills in marine, river, and land environments. The readers will gain useful and practical insights in these fields.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Dorota Wolicka and Andrzej Borkowski (2012). Microorganisms and Crude Oil, Introduction to Enhanced Oil Recovery (EOR) Processes and Bioremediation of Oil-Contaminated Sites, Dr. Laura Romero-Zerón (Ed.), ISBN: 978-953-51-0629-6, InTech, Available from: <http://www.intechopen.com/books/introduction-to-enhanced-oil-recovery-eor-processes-and-bioremediation-of-oil-contaminated-sites/microorganisms-and-crude-oil>

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