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THE STUDY OF INFLUENCE OF BIOSTIMILATION FACTORS ON MICROBIOLOGICAL DEGRADATION OF MAIN COMPONENTS IN PETROLEUM-TYPE POLLUTANT

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PROUČAVANJE UTICAJA BIOSTIMULACIONIH FAKTORA NA MIKROBIOLOŠKU RAZGRADNJU GLAVNIH KOMPONENATA U ZAGAĐIVAČU NAFTNOG TIPA

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ABSTRACT

The aim of this thesis was the investigation of the influence of biostimulation factors on degradation of the main components of petroleum pollutant. As the most significant component, from the environmental chemistry point of view, the aromatic hydrocarbons in oil pollutant were chosen.

The heavy fuel oil polluted soil was excavated contaminated soil from an energy power plant. Due to a breakdown of the energy power plant facilities, the soil had been polluted with heavy fuel oil (mazut) and sediment from a heavy fuel oil reservoir for a year.

The mazut polluted soil was uniformly distributed over not rinsed ungraded sand from the Sava River. River sand was added as a bulking and porosity increasing material. The sawdust from poplar, beech, and oak was added in order to increase the retention water capacity, but as alternative additional carbon source as well. To ensure homogeneity, the components were mixed. The entire homogenized material was then formed into a biopile shape.

A consortium of microorganisms was isolated from the polluted soil. Analytical profile index tests were used for identification of microorganisms. The number of microorganisms was determined by plating appropriate serial dilutions on agar plates incubated at 28 °C.

After formation the biopile was sprayed once a month with the biomass of microbial consortia isolated from the heavy fuel oil – contaminated soil (re-inoculation) and nutritive substances (biostimulation). Biosurfactant of biosolve type was applied on the biopile to solubilize the oil pollutant. During bioremediation, the biopile was watered, turned and mixed each 2 weeks to maintain the required moisture and aeration levels.

At the beginning of the study, immediately after mixing, but before the addition of sawdust, biomass, nutrient substances, and biosurfactant, approximately 10 m^3 of the biopile mixture was set aside on the same waterproof asphalt surface, to be used as a control pile. The complete analytical procedure that was applied to the samples was also

applied to the control samples during an independent parallel non-biostimulated biodegradation experiment.

During the 6-month interval, the samples were taken five times.

Organic substance from in total 5 soil samples was extracted and separated into fractions of saturated hydrocarbons, aromatic hydrocarbons, alcohols and fatty acid methyl esters using column chromatography.

Hydrocarbons were analyzed by the gas chromatography – mass spectrometry (GC–MS) techniques. Preliminary analyses of the investigated samples were conducted in the full-scan mode. Detailed analyses of the target compounds were conducted in the single-ion monitoring mode (SIM), comprising the following ion chromatograms: 178 (phenanthrene), 192 (methylphenanthrenes), 206 (dimethyl-phenanthrenes), and 220 (trimethyl-phenanthrenes).

Fraction of saturated hydrocarbons isolated by column chromatography was separated into the fractions of cyclic and branched alkanes and *n*-alkanes by karbamide addukt. *n*-Alkanes were analyzed by the gas chromatography.

The mazut polluted soil chosen for this research was shown to be potentially a good substrate for monitoring the bioremediation of petroleum pollutant. Due to the addition of sawdust and raw river sand to the mazut polluted soil some parameters improved as a consequence of dilution. Changes in basic microbiological parameters during bioremediation showed that the level of the active bacterial consortium, particularly active hydrocarbon degrading microorganisms, was maintained and increased. The composite sample and the individual samples were homogenous and satisfactory reflected the properties of the whole biopile.

Analyses of the total saturated fraction showed that the investigated oil pollutant from the biopile at the beginning of the experiment was classified to be at the boundary between the third and the fourth Head's biodegradation level (Head *et al*., 2003). It was shown that in the bioremediation experiment under the conditions applied in this research, monitoring the changes in the fraction of total saturated hydrocarbons could not lead to any precise conclusions about the intensity of microbial degradation of oil pollutant investigated. Based on the results of the analysis of the fraction of total saturated hydrocarbons, a precise comparison between the microbial degradation intensity of oil pollutant investigated in the present biostimulated experiment and a paralel non-

biostimulated, could not be made. Accordingly, a precise conclusion about the influence of biostimulation factors on degradation of the fraction of total saturated hydrocarbons could not be drawn.

The stimulated biodegradation process under the conditions used (re-inoculation, biostimulation, aeration and the addition of sawdust and biosurfactant) resulted in a preferable biodegradation of higher homologues of methyl-phenanthrenes comparing to the lower ones. This effect was the most pronounced in the case of trimethyl-phenanthrenes and the least in the case of methyl-phenanthrenes. As a result of specific biodegradation pattern during the stimulated biodegradation process described in this research, an increase in the relative abundance of phenanthrene compared to its methyl isomers occured. This increase was the most pronounced in comparison of phenanthrene with trimethyl-phenanthrenes and the least comparing phenanthrene with methyl-phenanthrenes.

The biodegradation pattern observed in this research was explained as a consequence of better interaction of reactive methyl groups with the active centers on the surface of bacterial cells and, in this way, promoted decomposition of methyl-phenanthrene derivatives. The presence of biosurfactant that increased solubility and thus the availability of products with a higher degree of alkylation could have also contributed this process. Re-inoculation, biostimulation, aeration and the addition of sawdust and biosurfactant promoted the process of decomposition of methyl-phenanthrene derivatives, by favoring some bacterial strains in the consortium.

The biodegradation trend among phenanthrene and its methyl-, dimethyl- and trimethyl-homologues observed in this research was opposite to the typical biodegradation trend of phenanthrene and its methyl isomers during the natural "unstimulated" biodegradation.

The distribution of *n*-alkanes during this experiment did not change. The abundance of *n*-alkanes remained at the initial low level, even at end of the experiment, after six months of the intensive stimulated bioremediation. According to that result it was concluded that individual components of oil pollutants must be present in soil in some minimum "threshold" amount to be biodegraded. Otherwise, their biodegradation will not proceed even in conditions of intensive stimulated bioremediation. This applies even on *n*-alkanes which are the most biodegradable hydrocarbons in oils.

Low biodegradability of *n*-alkanes during the investigated six month period of applied stimulated bioremediation was quite similar to the low biodegradability of *n*-alkanes during the parallel nonstimulated biodegradation experiment. These results supported the explanation from the previous research (Ramadan *et al*., 2012) that low biodegradability of *n*-alkanes might have been a consequence of their capture by incorporation with nonbiodegradable components in soil such as humic substances or fulvic acids.

Keywords:

Biostimulation, Biodegradation, Bioremediation, Soil, Residual fuel oil, Phenanthrene, Methyl-phenanthrene isomers, *n*-Alkanes, Degradability.

Scientific field: Chemistry
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REZIME

Cilj ove teze bio je ispitivanje uticaja biostimulacionih faktora na degradaciju glavnih komponenti naftnog zagadjivača. Kao najznačajnije komponente, sa stanovišta hemije životne sredine, odabrani su aromatični ugljvodonici u naftnom zagadjivaču.

Zemljište zagađeno mazutom je iskopano zagađeno zemljište iz kruga jedne termoelektrane. Usled kvara objekata termoelektrane zemljište je bilo zagađeno mazutom i sedimentom iz rezervoara za mazut tokom godinu dana.

Zemljište zagađeno mazutom je ravnomerno raspoređeno preko neispranog i nesortiranog peska iz reke Save. Rečni pesak je dodat kao materijal za povećanje zapremine i poroznosti. Piljevina od topole, bukve i hrasta je dodata kao materijal za povećanje retencionog kapaciteta vode ali i kao dodatni izvor ugljenika. U cilju homogenizacije komponente su promešane. Čitav homogenizovan materijal je zatim formiran u oblik halde.

Konzorcijum mikroorganizama je izolovan iz zagađenog zemljišta. „Analytical profile index" testovi su korišćeni za identifikaciju mikroorganizama. Broj mikroorganizama je određivan zasejavanjem odgovarajućih serijskih razblaženja na agrne ploče inkubirane na 28 °C.

Nakon formiranja halda je jednom mesečno prskana biomasom mikrobiološkog konzorcijuma izolovanog iz zemljišta kontaminiranog mazutom (reinokulacija) i hranljivim supstancama (biostimulacija). Biosurfaktant tipa „biosolve" je dodat haldi radi rastvaranja naftnog zagađivača. Tokom bioremedijacije halda je kvašena, okretana i mešana svake dve sedmice radi održavanja potrebne vlage i aerisanosti.

Na početku ispitivanja, odmah nakon mešanja ali pre dodatka piljevine, biomase, hranljivih supstanci i biosurfaktanta, približno 10 m^3 smeše sa halde je odvojeno sa strane za potrebe korišćenja kao kontrolna halda. Celokupana analitički postupak koji je primenjen na uzorke halde primenjen je i na kontrolne uzorke tokom nezavisnog paralelnog eksperimenta nebiostimulisane biodegradacije.

Tokom perioda od šest meseci, uzorci su prikupljeni pet puta.

Organska supstanca iz 5 uzoraka zemljišta je ekstrahovana i hromatografijom na koloni razdvojen na frakcije zasićeni ugljovodonika, aromatični ugljovodonika, alkohola i estara masnih kiselina.

Ugljovodonici su analizirani gasno hromatograsko – maseno spektrometrijskom (GC-MS) tehnikom. Preliminarne analize ispitivani uzoraka su rađene u „full-scan" režimu rada. Detaljne analize najznačajnijih jedinjenja rađene su u „single-ion monitoring" režimu rada (SIM) obuhvatajući sledeće jonske hromatograme: 178 (fenantren), 192 (metilfenantreni), 206 (dimetil-fenantreni), and 220 (trimetil-fenantreni).

Frakcija zasićeni ugljovodonika koja je izolovana hromatografijom na koloni razdvojena je na frakcije ciklični i razgranatih alkana i *n*-alkana pomoću karbamidnog adukta. *n*-Alkani su analizirani gasnohromatografski.

Pokazano je da je zemljište zagađeno mazutom, odabrano za ovo ispitivanje potencijalno dobar supstrat za praćenje bioremedijacije naftnog zagađivača. Nakon dodatka piljevine i rečnog peska ovom zemljištu neki parametri su poboljšani usled razblaženja. Promene osnovnih mikrobiološki parametara tokom bioremedijacije pokazale su da je nivo aktivnog bakterijskog konzorcijuma, naročito aktivni degradera ugljovodonika, uspešno održan ili čak i povećan. Kompozitni uzorak i pojedinačni uzorci su bili homogeni i zadovoljavajuće predstavljali osobine čitave halde.

Analize ukupne zasićene frakcije pokazale su da se ispitivani naftni zagađivač na početku eksperimenta nalazio na granici između trećeg i četvrtog stepena biodegradacije na Headćovoj skali (Head *et al*., 2003). Pokazano je da u uslovima koji su primenjeni u okviru ovog istraživanja, praćenje promena u frakciji ukupnih zasićeni ugljovodonika ne može dovesti do precizni zaključaka o intenzitetu mikrobiološke degradacije ispitivanog naftnog zagađivača. Na osnovu rezultata analize frakcije ukupnih zasićeni ugljovodonika, ne može se precizno uporediti intenzitet mikrobiološke degradacije naftnog zagađivača ispitivanog u ovom biostimulisanom eksperimentu i paralelnom nebiostimulisanom. U skladu s tim, nije moguće dati precizni zaključak o uticaju biostimulacioni faktora na degradaciju ukupnih zasićenih ugljovodonika.

Proces stimulisane biodegradcije pod uslovima primenjenim u okviru ovog rada (re-inokulacija, biostimulacija, aeracija i dodatak piljevine i biosurfaktanta) rezultovao je intenzivnijom biodegradacijom viših homologa metil-fenantrena u poređenju sa

nižim. Ovaj efekat je bio najizraženiji u slučaju trimetil-fenantrena, a najmanje izražen u slučaju metil-fenantrena. Kao rezultat specifičnog redosleda biodegradacije tokom procesa stimulisane biodegradacije opisanog u ovom radu, uočeno je povećanje relativne obilnosti fenantrena u poređenju sa njegovim metil-izomerima. Ovo povećanje je bilo najizraženije u poređenju obilnosti fenantrena sa obilnošću trimetilfenantrena, a najmanje izrađeno u poređenju obilnosti fenantrena sa obilnošću metil-fenantrena.

Specifičan redosled biodegradacije uočen u ovom radu objašnjen je kao posledica bolje interakcije reaktivnih metil-grupa sa aktivnim centrima na površini bakterijskih ćelija i, na ovaj način, favorizovanom razgradnjom meyil-fenantrenskih derivata. Prisustvo biosurfaktanta koji je povećao rastvorljivost, a samim tim i dostupnost proizvoda sa višim stepenom alkilovanja, takođe je mogao doprineti ovom procesu. Re-inokulacija, biostimulacija, aeracija i dodatak piljevine i biosurfaktanta olakšali su proces razgradnje metil-fenantrenskih derivata favorizujući neke bakterijske vrste u konzorcijumu.

Redosled biodegradacije fenantrena i njegovih metil-, dimetil- i trimetil-homologa koji je uočen tokom ovog istraživanja bio je suprotan u odnosu na tipičan redosled biodegradacije fenantrena i njegovih metil izomera tokom prirodn „nestimulisane" biodegradacije.

Raspodela *n*-alkana tokom ovog biodegradacionog eksperimenta se nije menjala. Obilnosti *n*-alkana ostle su na početnom niskom nivou čak i na kraju eksperimenta, posle šest meseci intenzivne stimulisane biodegradacije. Na osnovu ovog rezultata zaključeno je da pojedine komponente naftnog zagađivača moraju biti prisutne u zemljištu u nekoj minimalnoj, graničnoj količini da bi bile biodegradovane. U suprotnom do njihove biodegradacije neće doći, čak ni u uslovima intenzivno stimulisane bioremedijacije. Ovo važi čak i za *n*-alkane koji su najbiodegradabilniji ugljovodonici u nafti.

Niska biodegradabilnost *n*-alkana tokom ispitivano šestomesečnog perioda primenjene stimulisane bioremedijacije je bila vrlo slična niska biodegradabilnosti *n*-alkana tokom paralelnog nestimulisanog biodegradacionog eksperimenta. Ovi rezultati potvrđuju objašnjenje iz prethodnog istraživanja (Ramadan *et al*., 2012) da niska biodegradabilnost *n*-alkana može biti posledica njihove vezanosti inkorporacijom sa

nebiodegradabilnim komponentama i zemljištu kao što su huminske supstance ili fulvo kiseline.

Ključne reči:

Biostimulacija, Biodegradacija, Bioremedijacija, Zemljište, Mazut, Fenantren, Metil-fenantrenski izomeri, *n*-Alkani, Degradabilnost.

Content

1. Introduction

The contamination of soil and water with petroleum and its products occurs due to accidental spills during exploitation, transport, processing, storing and use. In order to control the environmental risks caused by petroleum products a variety of techniques based on physical, chemical and biological methods have been used. Biological methods are considered to have a comparative advantage as cost effective and environmentally friendly technologies.

Bioremediation, defined as the use of biological systems to destroy and reduce the concentrations of hazardous waste from contaminated sites, is nowadays undoubtedly considered one of the most effective approaches for removal of organic pollutants from different parts of the environment, first of all recent sediments, soils and surface waters. The efficiency of bioremediation processes is proved on the example of chlorinated organic solvents, polycyclic aromatic hydrocarbons, and pesticides. However, in case of oil type pollutants (crude oil and refinery products of petroleum refining), their biodegradation and removal from the environment are difficult to be classified in one category. Oil is a very complex mixture of hydrocarbons, but also nitrogen, sulfur, and oxygen compounds (NSO). Each class of compounds and often individual compounds as well, require special study aimed to define the type of microorganisms and optimal conditions for microbial degradation.

The aim of this thesis is investigation of the influence of biostimulation factors on degradation of main components of petroleum pollutant. As the most significant component, from the environmental chemistry point of view, the aromatic hydrocarbons in oil pollutant were chosen.

This research is based on the analysis and comparison of the results of bioremediation of soil during the period of six months.

After application of classic methods for extraction and separation of isolated oil pollutants from samples, the obtained fractions were analyzed using instrumental methods (gas chromatography and gas chromatography – mass spectrometry).

In this research the results of bioremediation of soil, i.e. results of microbial degradation of phenanthrene and its methyl-, dimethyl- and trimethyl- isomers in processes

where different biostimulation agents were added were compared with results of bioremediation processes where these agents were not added.

The main biostimulation agents used were: addition of biomass, addition of biosurfactants and addition of nutrients.

Additionally, the intensity of degradation of these aromatic hydrocarbons is compared with the intensity of degradation of *n*-alkanes which are the most abundant class of compounds in oils and, accordingly, in oil pollutants, and which are the most susceptible to microbiological degradation.

The proposed subject of research gain in importance if we take into account that aromatic hydrocarbons are very often dominant compounds in oils and that bioremediation is one of the most often used methods for cleaning the environment from organic pollutants including the oil.

2. Theoretical part

2.1. Petroleum as a main fossil fuel

Petroleum is a homogeneous, highly complex mixture of many chemical compounds that have high energy potential and is thermodynamically metastable under geological conditions (Tissot, Welte, 1984). Composition and properties of petroleum depend on its origin, environmental conditions of deposition, degree of thermal maturity, length of the migration path, lithological composition of the source and reservoir rocks, biodegradation, water washing and deasphalting.

Generation of petroleum is a natural process of maturation of buried organic matter in sediments. Summary of this process is shown in Figure 1. The primary sources of organic matter in sediments are living organisms. They contribute to the sedimentary organic matter deposits during their life processes (biosynthesis, secretion and excretion) and later with their dead bodies (Salomons, Stigliano, 1995; Tissot, Welte, 1984).

Once when it gets into the sediments, organic matter is further buried due to the effect of continuous sedimentation. Upon burial, organic matter in sedimentary rocks undergoes numerous compositional changes that are dictated initially by microbial activity and later mainly by thermal stress. This process is termed *thermal maturation* and is divided into three consecutive stages: *diagenesis*, *catagenesis*, and *metagenesis* (Tissot, Welte, 1984).

During early diagenesis, one of the main transformation agents is microbial activity. The organic material from primary production is broken down by microbial action into smaller constituents, which then undergo condensation reactions, giving rise to humic substances. With increasing time and burial depth most of this humic material becomes progressively insoluble due to increasing defunctionalization, polycondensation and insolubilization. By the end of diagenesis kerogen formation is complete (Figure 1). Kerogen is considered the major precursor of petroleum. It consists of selectively preserved, resistant, cellular organic materials (algal, pollen, spores, and

leaf cuticle) and the degraded residues of less resistant biological organic matter (amorphous material) in variable proportions. (Killops, Killops, 2005).
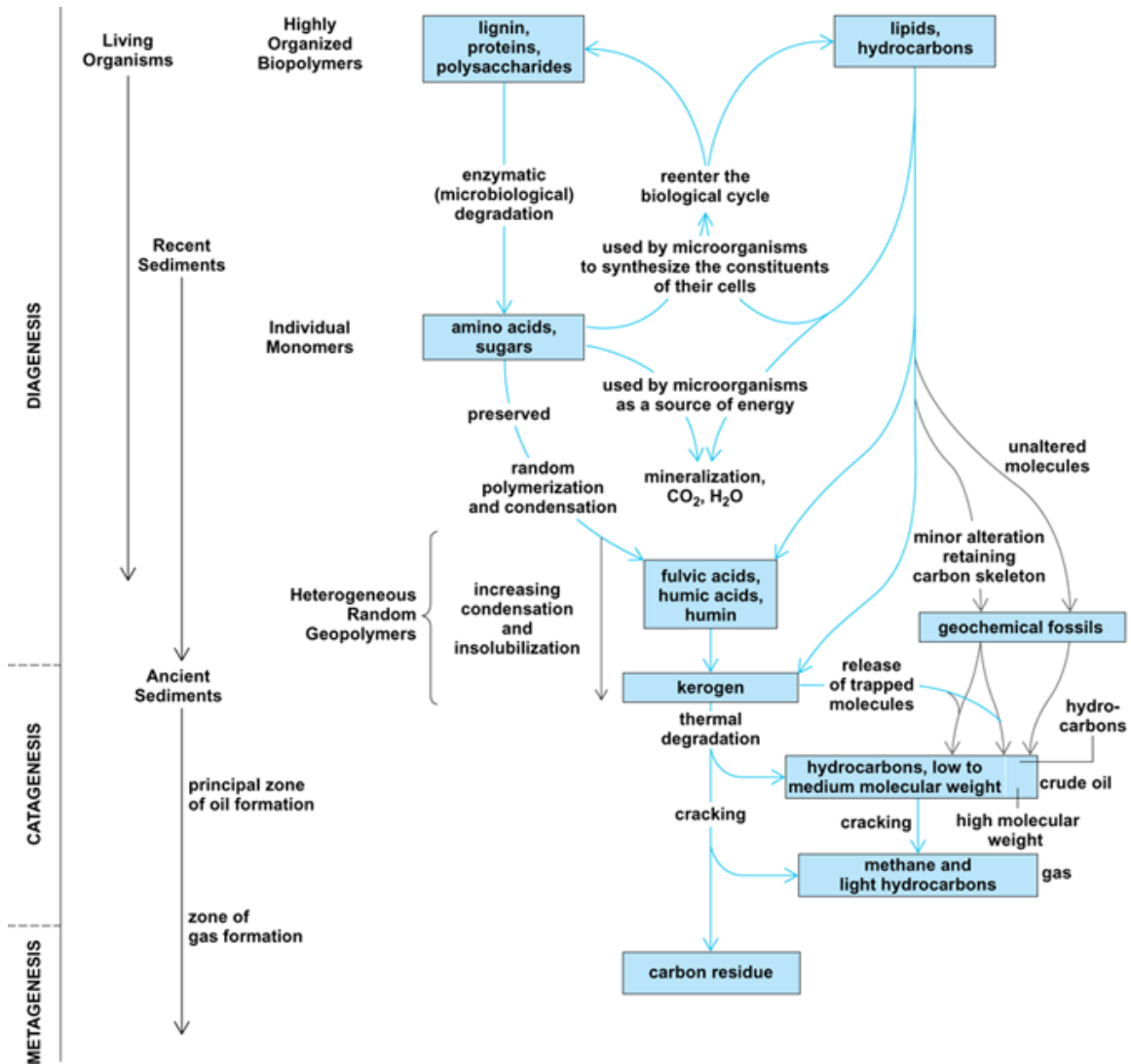
Figure 1. Transformation of organic matter during sedimentation and maturation (Tissot, Welte, 1984).

The general scheme of evolution of the organic fraction and the hydrocarbons produced is depicted in Figure 2 (Tissot, Welte, 1984). In young sediments, at shallow depths, during diagenesis, small amounts of hydrocarbons are present (geochemical fossils). The only new hydrocarbon generated is methane. In special cases, microbial activity may result in abundant methane generation (biogenic gas; Figure 2). As

temperatures and pressures increase (deeper burial) the process of catagenesis begins. The increase in temperature results in thermal degradation of kerogen which eliminates hydrocarbon chains. Most of the newly formed hydrocarbons are of medium to low molecular weight and they are the source of crude oil. Catagenesis is the principal stage of oil formation. Corresponding depth and temperature ranges are referred to as oil window. In addition, catagenesis also corresponds to the beginning of cracking of oil to gas, which produces wet gas with a rapidly increasing proportion of dry gas. The last stage of evolution of organic matter is called metagenesis and it is reached only at great depths. During metagenesis no significant amounts of hydrocarbons are generated from kerogen except from some methane. However, large amounts of methane may result from the cracking of previously generated liquid hydrocarbons. In terms of hydrocarbons exploration, the stage of metagenesis corresponds to the dry gas zone (Verweij, 1993).
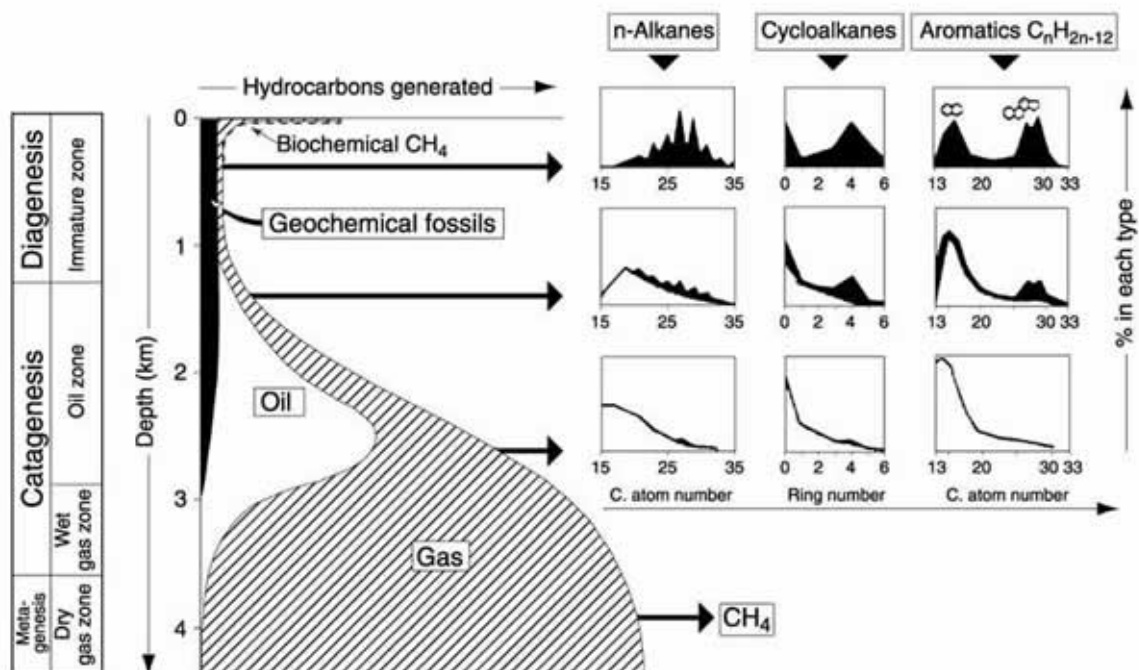
Figure 2. Evolution of the organic fraction in the subsurface and the hydrocarbons produced (Tissot, Welte, 1984).

The hydrocarbon-rich fluids (liquids and gases), evolved from kerogen during catagenesis and metagenesis, are collectively termed petroleum. When sufficient amount of hydrocarbons is generated in thermally mature source rock they are expelled, along with other pore fluids, due to the effects of internal source rock over pressuring caused by hydrocarbon generation as well as by compaction. This first phase of the migration process is called *primary migration*. Once released into porous and permeable carrier beds or into faults planes, oil and gas then move upwards the surface, from areas of higher pressure and higher temperature to the area of lower pressure and lower temperature. This overall buoyancy driven process is known as *secondary migration*. Secondary migration can involve distances of 10 – 100 km and occasionally more. Finally, migration stops when fluids reach the rock which is able to accumulate large volumes of petroleum. These rocks are termed reservoir rocks and petroleum can be preserved in them over long periods of time (Killops, Killops, 2005; Waples, 1985).

2.2. The composition of petroleum (biological markers)

Composition and properties of oil depend on the origin, environmental conditions of deposition, degree of thermal maturity, the length of the migration path, lithological composition of the source and reservoir rocks, biodegradation, water washing and deasphalting.

Oil is a homogeneous, complex mixture, made from large number of chemical compounds. The highest percentage of oil, 95 - 98 % represents hydrocarbons, and the remaining 2 - 5 % is NSO-compounds (polar compounds with nitrogen, sulfur and oxygen). Content of mineral substances in oil is extremely low. However, vanadium, nickel, copper and molybdenum are almost always present in ash (Višekruna, 1988).

Although there is a considerable variation between the ratios of organic molecules, the elemental composition of petroleum is well-defined: 83 - 87 % carbon and 11 - 14 % of hydrogen, while the rest consists of sulfur, nitrogen and oxygen. The mass ratio of the basic elements, carbon and hydrogen, is variable and ranges from 6:1 to 8:1. Most crude oils contain less than 0.5 % sulfur. However, in some samples the content of this element can reach 7 %.

Dominant compounds in crude oil are liquid hydrocarbons. In this dominant liquid fraction, gaseous hydrocarbons are absorbed while solid hydrocarbons are dispersed (Višekruna, 1988; Kamyanov, 1996).

Petroleum is usually separated into several fractions containing different types of chemical compounds. Table 1 shows petroleum fractions and their most important components (Vitorović, Jovančićević, 2005).

In analytical organic geochemistry, the most informative compounds present in oils are biologic markers or biomarkers. These compounds, primarily hydrocarbons, are structurally similar to, and are diagenetic alteration products of, specific natural products. During the geologic history, they retain all or most of the original carbon skeleton and as such are incorporated in the sediments. Because of that, they are usually called "molecular fossils". From analytical point of view, the most important among them are *n*-alkanes, isoprenoid aliphatic alkanes, steranes, terpanes, naphthenoaromatics and porphyrins (Waples, 1985). The most important biological markers and their biological precursors are listed in Table 2.

Table 1. Petroleum fractions and their most important components
(Vitorović, Jovančićević, 2005).

Fraction	Components
Saturated fraction	Normal alkanes Isoprenoids and other branched alkanes Alicyclic hydrocarbons (including steranes, diterpanes and triterpanes)
Aromatic fraction	Alkylaromatic hydrocarbons Naphthenic-aromatic hydrocarbons Lighter aromatic compounds containing sulphur (thiophenes)
Resins (NSO-compounds)	Higher fatty acids and alcohols Aliphatic and cyclic ketones Aliphatic and aromatic sulphur compounds Porphyrins
Asphaltenes	Condensed high-molecular-weight aromatic compounds with a large number of heteroatoms

Table 2. The most important biological markers and their biological
precursors (Vitorović, Jovančićević, 2005).

Biological markers	Precursors
n-Alkanes (> C_{22})	Waxes of terrestrial plants
n-Alkanes (C_{17}, C_{22})	Lipids of algae
Isoprenoids (< C_{20})	Chlorophyll
Isoprenoids (> C_{20})	Lipids or chlorophyll of hypersaline algae
Porphyrins	Chlorophyll
Steranes	Steroids
Triterpanes	Bacterial triterpenoids
Diterpanes	Constituents of plant resins
Naphthenoaromatic compounds	Steroids, triterpenoids

2.2.1. *n-Alkanes*

n-Alkanes are the most abundant hydrocarbons in all nonbiodegraded oils and mature bitumens. Due to their distribution in geological samples, but also to relatively simple analytical procedures, *n*-alkanes are definitely the most investigated class of organic compounds. Therefore, most of the applied data in many organic-geochemical studies are based on this class of compounds and they are very useful to solve many geochemical problems. In addition, the knowledge about the composition of *n*-alkanes in fossil organic matter enabled the use of the *n*-alkane distribution in solving environmental problems (e.g. Matsumoto 1982; Colombo *et al*. 1989; Theobald *et al*. 1995; Wang *et al*. 1995; Tran *et al*. 1997; Rogers, Savard, 1999).

n-Alkanes were among the first biomarkers to be studied extensively. Their potential biological precursors can be found in all extant organisms. The high concentration of *n*-alkanes in sedimentary organic matter is best explained by their existence in plant and algal lipids. However, despite the ubiquity of straight-chain lipids in the biosphere, some *n*-alkane profiles can be environmentally and taxonomically diagnostic, especially if combined with carbon-isotopic and micropaleontological analysis (Hoffmann *et al*., 1987; Rieley *et al*., 1991). For example, higher concentrations of *n*-alkanes with odd carbon numbers between *n*-C_{15} and *n*-C_{19} in

Ordovician rocks point to the presence of the marine cyanobacterium or alga *Gloeocapsomorpha prisca* (Fowler, 1992; Hoffmann *et al*., 1987). Long chain *n*-alkanes with more than 27 carbon atoms and a predominance of odd numbered homologues are frequently derived from plant waxes indicating a post-Silurian age and organic matter input from terrestrial sources (Hedberg, 1968; Tissot, Welte, 1984).

n-Alkanes have been investigated since the earliest period of development of instrumental techniques in organic geochemistry (Smith, 1954, Henderson *et al*., 1968). They can easily be identified and analyzed in saturated fractions of nonbiodegraded crude oils using gas chromatography (GC) method with flame ionisation detector (FID; Figure 3).
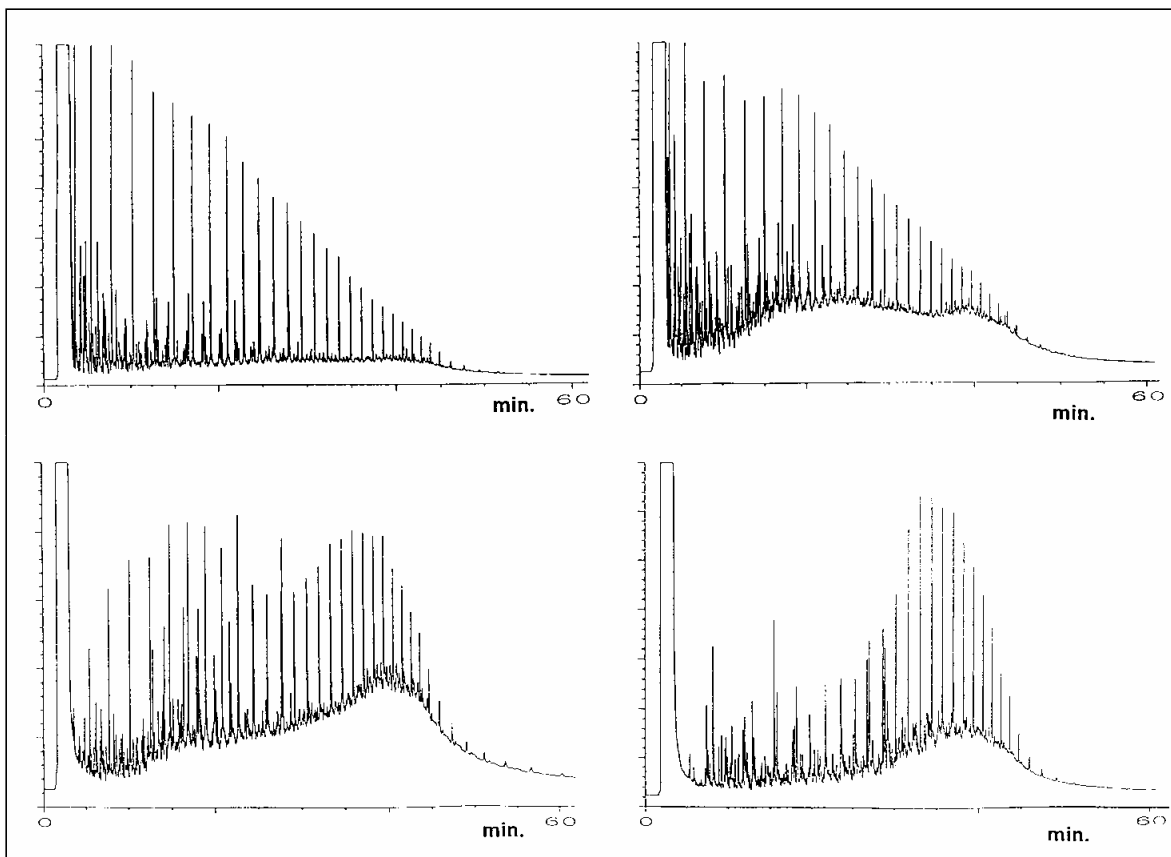
Figure 3. GC-FID chromatograms of saturated hydrocarbon fractions
isolated from different crude oils (Wang *et al*., 1999).

In bitumens where *n*-alkanes are predominant in alkane fraction, homologous series of *n*-alkanes can also be successfully identified with GC-analysis of the total alkane fraction. Figure 4 for example, shows gas chromatograms of alkanoic fractions of bitumen of recent sediments, mainly marine (a) and mostly of terrestrial origin (b).
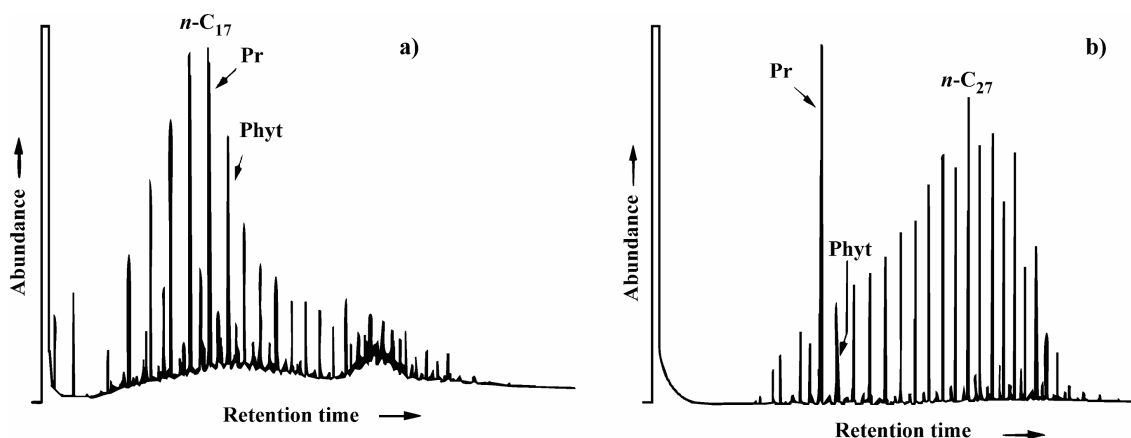
Figure 4. Gas chromatograms of alkane fractions of bitumen of recent sediments of marine (a) and terrestrial origin (b; Vitorović, Jovančićević, 2005)

Conventional gas chromatography (GC) and gas chromatography - mass spectrometry (GC-MS) methods have identified *n*-alkanes up to C_{40} in the oil. The development of computerized instrumental techniques allowed the identification of *n*-alkanes containing up to 120 C-atoms (del Rio, Philp, 1999; Hsieh *et al*., 2000; Killops *et al*., 2000; Mueller, Philp, 1998).

Specific correlation parameters and diagnostic ratios which are calculated from the distribution and abundance of *n*-alkanes are: Carbon Preference Index, the range of *n*-alkanes, *n*-alkane maximum and the isotopic profile of the individual *n*-alkanes.

Carbon Preference Index (CPI) or Odd-Even Preferences (OEP) is a parameter which is related to dominance of *n*-alkanes with odd or even number of C-atoms. Originally, CPI was developed as a measure of the strength of the odd-carbon predominance in *n*-alkane group (Waples, 1985). Several ways to calculate the CPI or OEP are suggested in the literature:

1) Bray and Evans in 1961. calculated the CPI in the C_{24}-C_{33} range of *n*-alkanes according to the following equation:

$$CPI = \frac{1}{2}\left(\frac{C_{25} + C_{27} + C_{29} + C_{31} + C_{33}}{C_{24} + C_{26} + C_{28} + C_{30} + C_{32}} + \frac{C_{25} + C_{27} + C_{29} + C_{31} + C_{33}}{C_{26} + C_{28} + C_{30} + C_{32} + C_{34}} \right)$$

2) Philippi (1965) suggested the relationship given by:

$CPI = 2 \times n\text{-}C_{28}/(n\text{-}C_{29} + n\text{-}C_{30})$

3) When the oil is dominated by lower homologous n-alkanes, Petrov (1984) proposed following calculation of the CPI:

$CPI\ 1 = 1/2 \times (n\text{-}C_{15} + n\text{-}C_{17})/n\text{-}C_{16}$

4) Scalan and Smith (1970) proposed two ways to calculate the OEP:

$OEP\ 1 = (n\text{-}C_{21} + 6 \times n\text{-}C_{23} + n\text{-}C_{25})/4 \times (n\text{-}C_{22} + n\text{-}C_{24})$
$OEP\ 2 = (n\text{-}C_{25} + n\text{-}C_{27} + n\text{-}C_{29})/4 \times (n\text{-}C_{26} + n\text{-}C_{28})$

The average of two ranges is taken to minimize bias produced by the generally decreasing *n*-alkane concentration with increasing number of carbon atoms.

On the basis of *n*-alkane abundances in sedimentary organic matter, the CPI (Carbon Preference Index) values, as well as on the maxima in their homologous series, it is possible to assess the type of the biological precursor material, the type of the environment it was deposited in, the degree of thermal maturity and sometimes microbial degradation (e.g. Tissot, Welte, 1984; Waples, 1985; Philp 1985; Bordenave, 1993; Engel, Macko, 1993; Peters, Moldowan 1993; Table 3).

Table 3. CPI and *n*-alkane maximum of biolipid main fractions
(a) geolipid fractions of recent sediments (b) and geolipid fractions
of the ancient sediments (c; Vitorović, Jovančićević, 2005)

		CPI	Maximum
a	High terrestrial plants	> 10	C_{25}-C_{29}
	Some marine algae	~ 1	C_{17}, C_{22}
	Many marine organisms	~ 1	C_{17}
	Planktons, bacteria	~ 1	C_{17}
b	Terrestrial recent sediments	1.5-2	C_{25}
	Marine recent sediments	~ 1	C_{17}, C_{22}
c	Terrestrial ancient sediments	1-1.5	C_{17}-C_{22}
	Marine ancient sediments	1	C_{17}

Carbon-isotopic content of organic matter carries information about the environment of an organism, its primary carbon assimilation pathways and processing of its metabolic products in the environment. While isotopic measurements of organic materials (e.g., biomass, kerogen, bitumen, petroleum) allow some correlations between precursor and product, measurements at the molecular or intramolecular level can reveal detailed information about the biosynthetic pathways and sources of individual carbon skeletons (Brocks, Summons, 2004).

Because the organic matter from which kerogen is formed is relatively enriched in ^{12}C and depleted in ^{13}C, so is the kerogen in sedimentary rocks. Accordingly, the hydrocarbons evolved from kerogen reflect this source-related, relatively light isotopic signature. This is because they are generated by cleavage of C–C bonds in kerogen and it is needed less energy to break a ^{12}C–^{12}C bond than a ^{13}C–^{12}C bond. Methane generally exhibits an even lighter isotopic signature. Hence, during hydrocarbon cracking in the subsurface isotopically light methane is preferentially formed. As might be expected, with increasing maturity the residual kerogen becomes isotopically heavier (Galimov 1980; Killops, Killops, 2005)

2.2.2. Isoprenoids

Aliphatic alkanes isoprenoids are hydrocarbons consisting of repeating C_5 isoprene units. In ancient sediments organic matter and petroleum they are the most abundant hydrocarbons after *n*-alkanes (Kamyanov, 1996).

The largest number of geolipid isoprenoids has head-tail isoprenoid bonding. They are called regular isoprenoids. Among them, the most widespread are C_{15}-farnesane, C_{18}-norpristan, C_{19}- pristane and C_{20} – phytane (Figure 5a). In addition to regular, irregular isoprenoids were identified in oils as well. The most ubiquitous are those with a tail-tail bonding in a series of head-tail linkage, such as lycopene, squalane and perhydro-β-carotene (Albaigés *et al*., 1985; Figure 5b). Irregular isoprenoids with a head-head bonding in a series of head-tail linkage were identified in oils in the C_{32}-C_{40} range (Petrov *et al*., 1990). Isoprenoid of this type, identified in bitumens, is 3,7,11,15,18,22,26,30-octamethyldotriacontan (Figure 5c; Vitorović, Jovančićević, 2005).
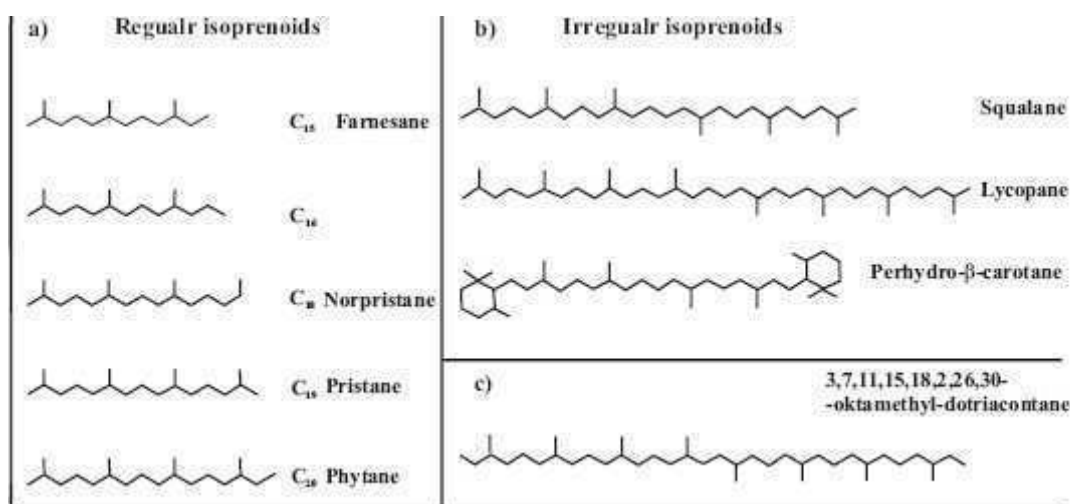
Figure 5. The most important petroleum isoprenoids: regular (a), irregular with tail-tail bonding in a series of head-tail linkage (b) irregular with a head-head bonding in a series of head-tail linkage (c; Vitorović, Jovančićević, 2005)

Farnesane, C_{15} regular isoprenoid, and isoprenoids having less then fifteen carbon atoms originate either from chlorophyll *a* or from bacterial chlorophylls that have C_{15} isoprenoids as side chains. C_{16}-C_{20} regular isoprenoids are transformation products of

chlorophyll *a*. Regular isoprenoids C_{2l}-C_{25} cannot be related with certainty to the corresponding precursor, while C_{25}-C_{45} isoprenoids have been shown to originate from long series of isoprenoids from higher terrestrial plants. The irregular isoprenoids of type b (Figure 5), squalane, perhydro-β-carotene and lycopene, are considered to be of algal origin. Isoprenoids in a C_{32}-C_{40} range with a "head-to-head" isoprenoid coupling, (the third type), were identified in different species of bacteria (Vitorović, Jovančićević, 2005).

Apart from the isoprenoids inherited from living organisms, which are incorporated in bitumen during the diagenetic phase, bitumen is enriched during the catagenesis with new isoprenoid molecules that are formed by thermal degradation of kerogen. In most cases there is no simple accumulation of biolipid isoprenoids in sediments, but during the geological history, especially during diagenesis, they are structurally transformed into hydrocarbon shapes as identified in geolipid fractions. These changes are rarely minimal, and most often lead to significant structural changes (Vitorović, Jovančićević, 2005).

An example of biolipid isoprenoid which undergoes extreme changes in the geosphere is botryococcen, C_{34} isoprenoid of algae *Botryococcus braunii*. During diagenetic and catagenetic changes of organic matter, botryococcen is converted to botryococcan (Vitorović, Jovančićević, 2005).

The most important isoprenoids in geolipid fractions are pristane (Pr) with 19 carbon atoms and phytane (Ph) with 20 carbon atoms. They derive from phytol, isoprenoid alcohol of chlorophyll *a*. Changes in phytol in the geosphere are example of significant structural changes of isoprenoids in sediments (Vitorović, Jovančićević, 2005). Phytol has two chiral centers C_7 and C_{11}, which are due to the selectivity of enzymatic reactions in the biosphere characterized by R-configuration. During defunctionalization and hydrogenation of phytol in the geosphere, C_7 and C_{11} positions in the precursor transform into C_{10} and C_6 positions in pristane and phytane (Figure 6; Patience *et al*., 1980). Pristane in immature sediments is intermediate form with 6(R),10(S) configuration. Maturation processes involves the isomerization of the chiral centers, where following reaction takes place 6(R),10(S)-pristane $\rightarrow$ 6(R),10(R)-pristane + 6(S),10(S)-pristane. Therefore, the ratios 6(R),10(R)-pristane / 6(R),10(S)-pristane and 6(S),10(S)-pristane / 6(R),10(S)-pristane can be used as indicators of

maturation, as long as the equilibrium is not reached. The equilibrium of the ratio 6(R),10(S)-pristane : 6(R),10(R)-pristine : 6(S),10(S)-pristane is 2:1:1 (Patience *et al*., 1980). Direct analysis of the eight possible stereoisomers of phytane, which result from three chiral centers in the molecule, is not possible, because they can not be separated by the columns that are commonly used in gas chromatography (Peters, Moldowan, 1993).

phytol
(tetramethyl-hexadec-2-enol)

6(R), 10(S) - pristane (intermediate)   6(S), 10(R) - pristane (intermediate)

6(R), 10(R) - pristane   6(S), 10(S) - pristane
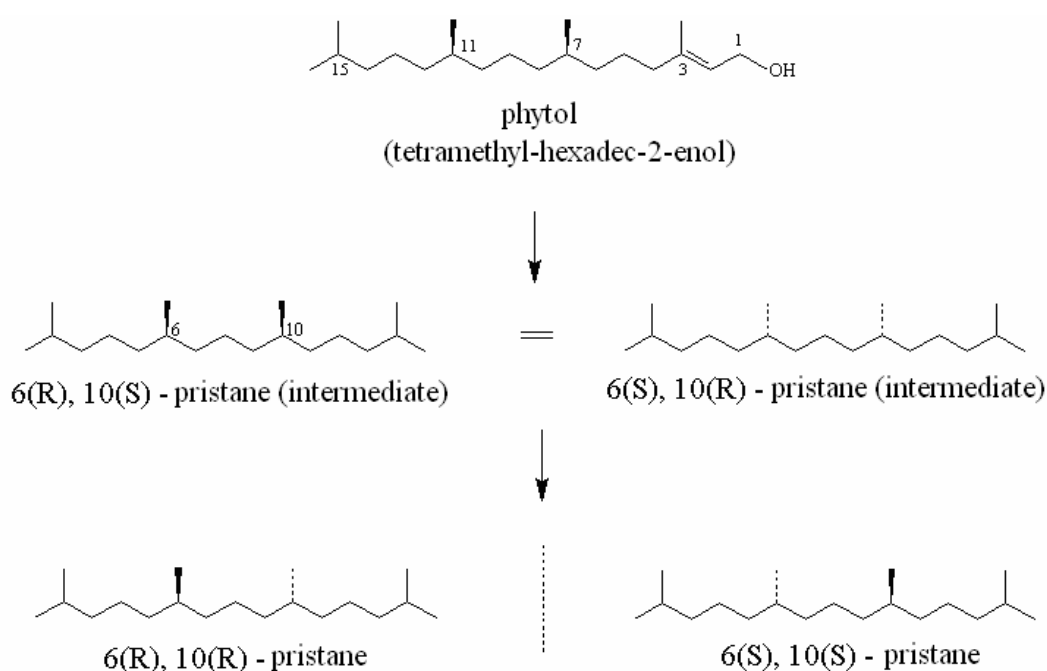
Figure 6. Stereoisomers of the pristane generated from phytol.

In oxidizing environment, phytol is converted to phytenic acid; by decarboxylation it is converted to pristene, and finally by hydrogenation, to pristane. In the reducing environment phytol is converted to dihydrophytol, followed by dehydration and hydrogenation to phytane.

In addition to chlorophyll *a*, pristane and phytane precursors can be chlorophyll b, α - and β-tocopherol, carotenoid pigments and membrane components of archaebacteria (Chappe *et al*., 1982; Illich, 1983; Goosens *et al*., 1984; Rowland, 1990).

Pristane and phytane are usually identified by GC or GC-MS analysis using ion fragmentograms *m/z* = 183. The lack of these techniques is the coelution of pristane and irregular oil isoprenoid 2,6,10-trimethyl-7-(3-methyl-butyl)-dodecane from the most of

capillary columns (Volkman, Maxwell, 1986). Ion fragmentogram $m/z = 168$ allows precise identification of the aforementioned irregular isoprenoid, even if the presence of large quantities of pristane, and the correction of the results obtained by GC or GC-MS analysis of the $m/z = 183$ ion (Peters, Moldowan, 1993).

The best known and most widely used isoprenoid parameter in organic geochemical research is Pr/Ph ratio. This parameter can be used as an indicator of the maturity and as an indicator of the depositional environment (Peters, Moldowan, 1993).

Numerous authors indicated that the Pr/Ph ratio can be applied as a maturation parameter in the investigation of oils from similar sedimentation environments, which source rocks did not reach the maximum of the "oil window" in diagenetic/catagenetic evolution (Albrecht *et al*., 1976; Borrego *et al*., 1999; Connan, 1974; Jovančićević *et al*., 1994, 1998; Li *et al*., 2003; Pang *et al*., 2003a; Radke *et al*., 1980). However, a good agreement of Pr/Ph ratio with diasterane/sterane and Ts/(Ts+Tm) ratios may not be indicative of the early catagenesis (Peters, Moldowan, 1993). Because of this influence of thermal maturity, Pr/Ph ratio is not considered to be a very reliable indicator of environment of deposition in immature oils. However, it was found that if an immature sample has a value of Pr/Ph > 3.0, it can be concluded with a relatively high degree of certainty that it is dominantly of terrestrial origin and is deposited in oxidative environment. On the other hand, the values of Pr/Ph < 0.6 are typical of marine oils from highly reductive environments (Volkman, Maxwell, 1986).

The beginnings of oil investigations at the molecular level were associated with the determination of ratios of pristane and phytane with n-alkanes C_{17} and C_{18} (Pr/n-C_{17} and Fit/n-C_{18}, Tissot *et al*., 1971). These parameters are inversely proportional to the maturity of the nonbiodegraded oils of similar origin, deposited in similar oxidation/reduction conditions (Alexander *et al*., 1981). Analoguos ratio, (Pr + Ph) / (n-C_{17} + n-C_{18}), is also applied in organic-geochemical studies (Petrov, 1984; Kamyanov, 1996).

Because of the significant influence of numerous factors on the maturation parameter values calculated from the distribution and abundance of n-alkanes and aliphatic isoprenoid alkanes, it can be concluded that they give only rough information on the thermal maturity of a sample.

Isoprenoids are good indicators that bitumen and petroleum are of biogenic origin, but, similarly to porphyrins, they are of very limited value in assessing the contribution of any particular organism (Waples, 1985).

2.2.3. Steranes

Steranes in crude oils are found in trace amounts and their concentration is usually expressed in ppm (parts per million). Therefore, qualitative and quantitative analyses of polycyclic alkanes require most modern methods of instrumental analysis, like computer gas chromatography – mass spectrometry analysis (C-GC-MS; Vitorović, Jovančićević, 2005). Although their abundance in oil is low, steranes are among structurally and stereochemically most detailed studied biomarkers (Peters, Moldowan, 1993).

Petroleum steranes originate from stanols, stenols, stanons, and stenons from live organisms. The most widespread in the animal world is C_{27}-cholesterol, while ergosterol (C_{28}) is typical of yeasts and fungi. Large amounts fucosterol (C_{29}) were detected in diatomaceous organisms and other algae. C_{29}-sterols, campesterol, sitosterol and stigmasterol, have been identified in higher plants. Significant amounts of C_{30}-sterols were identified in the photosynthetic algae (Mackenzie *et al*., 1982, Volkman, 1986). Although the presence of sterols is a characteristic of eukaryotes it was found that some prokaryotic species contain sterols as well (Kohl *et al*., 1983, McCaffrey *et al*., 1989).

Based on the results of a large number of organic-geochemical investigations of bitumen and crude oils of different maturity, the transformation paths of steranes were revealed: different steroids (stanols, stenols, stanons and stenons) of extinct organisms are decomposed by microorganisms during early diagenesis, at shallow depths and low temperatures and pressures, and through various processes of defunctionalisation are converted into sterenes and steranes (Figures 7 and 8; Mackenzie *et al*., 1982, Moldowan *et al*., 1985, Volkman, 1986; de Leeuw *et al*., 1989, Rubinstein *et al*., 1975; Sieskind *et al*., 1979).

On the other hand, the same conditions may lead to aromatization of A or C rings. Finally, at greater depths and at higher temperatures, diasterenes convert to diasteranes, and steroids with aromatic A or C ring, convert to triaromatic steroids, thus ending the process of aromatisation. Triaromatic steroids may eventually convert into phenanthrene or its alkyl derivatives.

Steranes, diasteranes, steroids with aromatic C-ring, triaromatic steroids and phenanthrene (and its alkyl derivatives), were identified in bitumens and oils. In addition to series of C_{27}-C_{30} steranes (Mackenzie *et al*., 1982, Moldowan *et al*., 1985, Volkman, 1986; de Leeuw *et al*., 1989), C_{27}-C_{29} diasteranes were identified in oil.
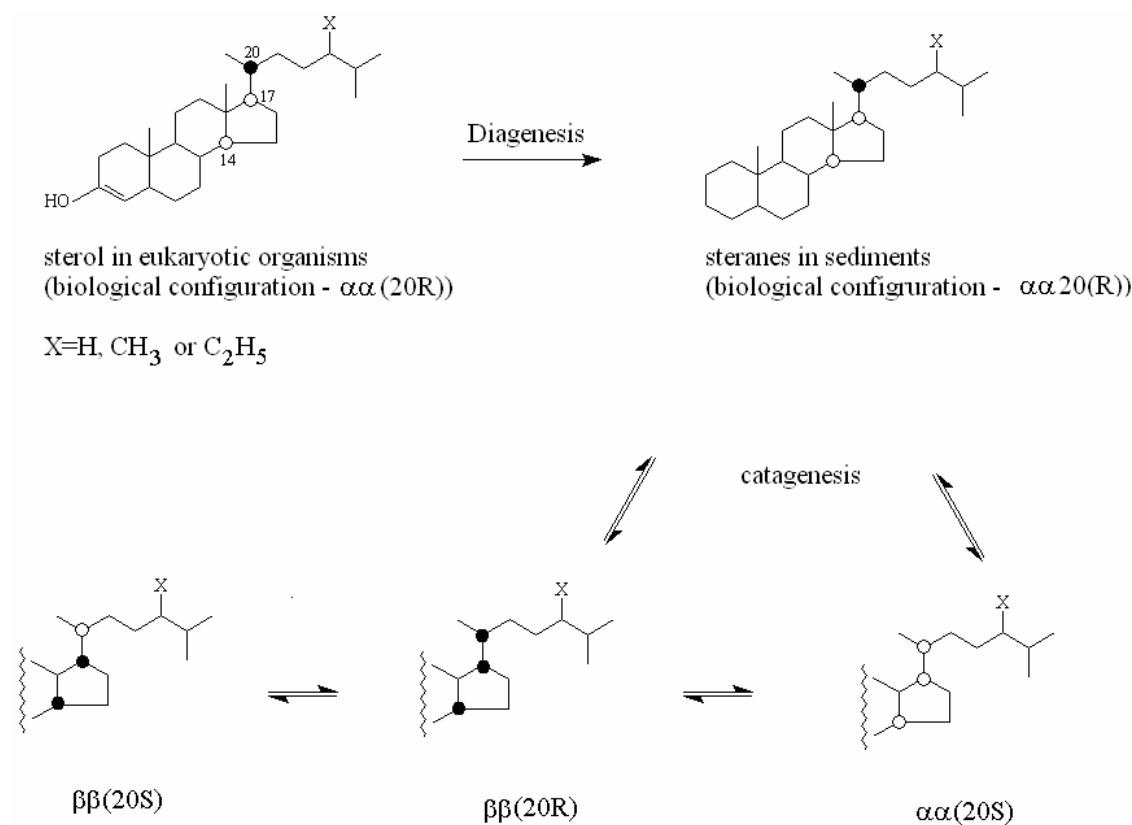
Figure 7. Scheme of the origin of petroleum steranes (Mackenzie *et al*., 1982, Moldowan *et al*., 1985, Volkman, 1986; de Leeuw *et al*., 1989)

Table 4 provides an overview of identified steranes in oil, with the appropriate precursors and conditions in sedimentary environment where they are formed (Peters, Moldowan, 1993).
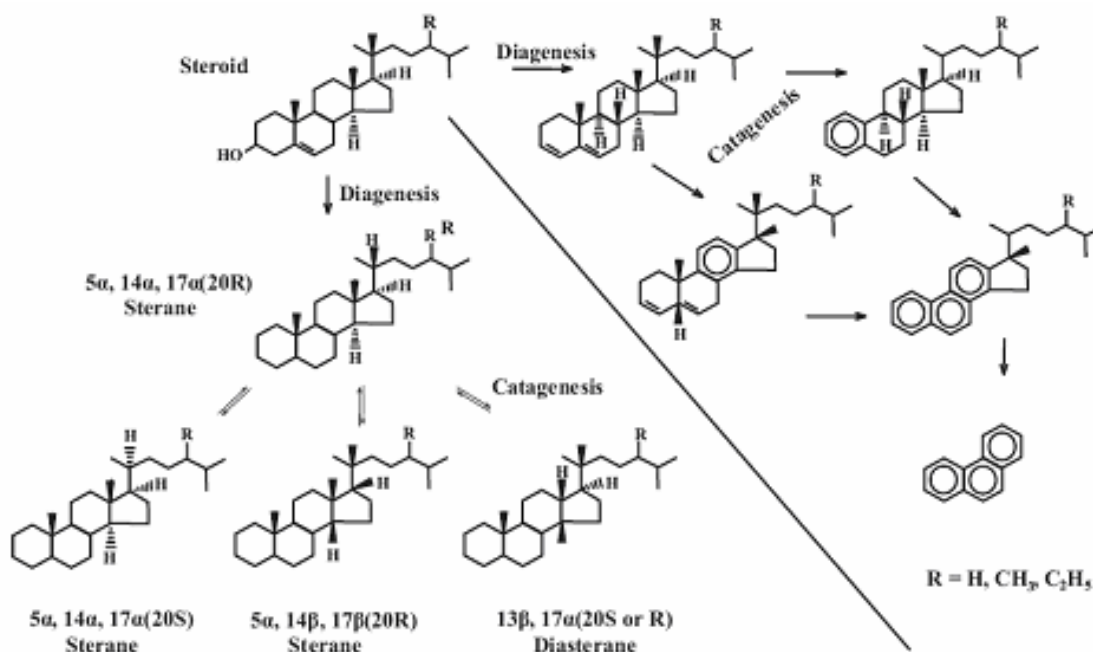
Figure 8. The most important structural and stereochemical changes of steroids in the geosphere (Al-Arouri *et al*., 1998, George *et al*., 1998).

Table 4. Summary of petroleum sterane biological precursors, and characteristics of the environment of deposition (Peters, Moldowan, 1993).

Compound	The biological precursor	Environments of deposition	Literature
C_{27}-C_{29}- steranes	Algae and higher plants	different	Moldowan *et al*., 1985; Volkman, 1986
C_{27}-C_{29}- diasteranes	Algae and higher plants	The rich clays	Rubinstein *et al*., 1975
C30-4-desmethyl 24-n-propylcholestanes	Chrysophyta algae	Marine, Triassic age or younger	Moldowan *et al*., 1985; Peters *et al*., 1986
C_{28}-C_{30}- 4- methylsteranes	Dinoflagellates and some bacteria	Lake or marine, Triassic age or younger	Brassell *et al*., 1986; Wolff *et al*., 1986
C_{30}- dinosteranes	dinoflagellate	Marine, Triassic age or younger	Summons *et al*., 1987; Goodwin *et al*., 1988
C_{21}-pregnane and C_{22}-homopregnane	unknown	hypersaline	ten Haven *et al*., 1986

Due to the low abundance of sterane biomarkers in crude oils, their identification and quantification requires the application of gas chromatographic-mass spectrometric analysis (fragmentogram *m/z* 217 ions for steranes and diasterane or fragmentograms *m/z* 218, 232 and 259 ions for diasteranes; Philp, 1985, Peters, Moldowan, 1993). A typical example of a sterane mass fragmentogram from ancient sediment bitumen is shown in Figure 9. However, when these instrumental conditions are used, some isomers of C_{27}-C_{28}-steranes coelute with C_{28} and C_{29} diasteranes.

GC-MS-MS technique allows the separation of the sterane carbon number and completely accurate quantification of all isomers (Al-Arouri *et al*., 1998, George *et al*., 1998). Using this instrumental technique C_{26} steranes and different series of C_{30}-sterane were identified in crude oils.
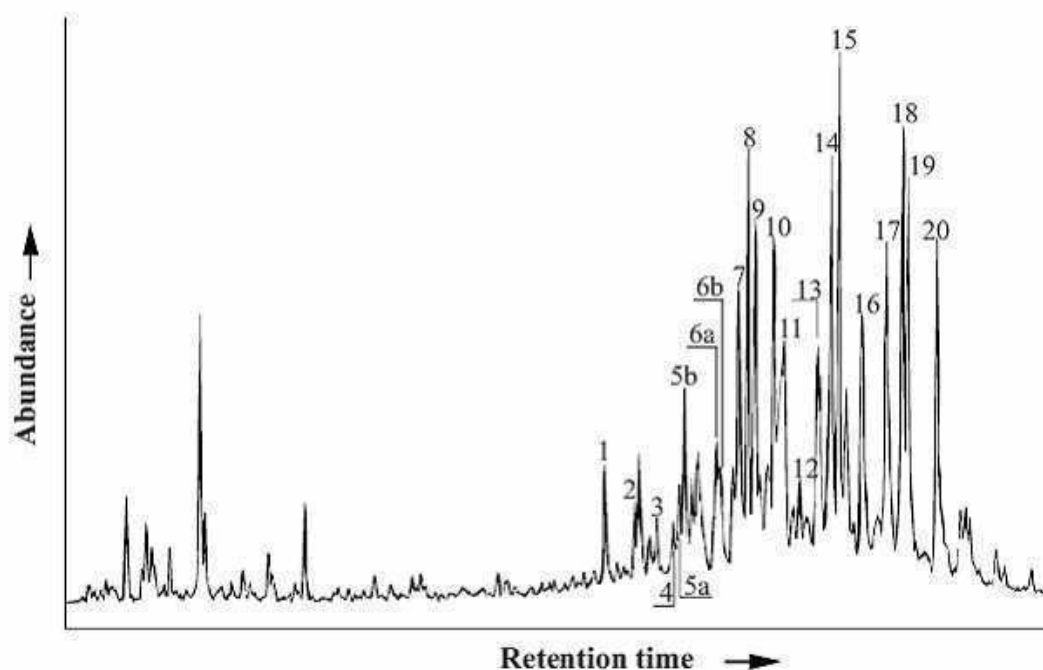
Figure 9. Typical example of a sterane fragmentogram of an ancient sediment bitumen (Vitorović, Jovančićević 2005; Peak identification is shown in Table 5).

Table 5. Identification of the peaks in the chromatogram shown in Figure 9 (sterane fragmentogram; Vitorović, Jovančićević, 2005).

Peak	Compounds
1	C_{27} 13β(H)17α(H)20(S)-diasterane
2	C_{27} 13β(H)17α(H)20(R)-diasterane
3	C_{27} 13α(H)17β(H)20(S)-diasterane
4	C_{27} 13α(H)17β(H)20(R)-diasterane
5a	C_{28} 13β(H)17α(H)20(S)24(S)-diasterane
5b	C_{28} 13β(H)17α(H)20(S)24(R)-diasterane
6a	C_{28} 13β(H)17α(H)20(R)24(S)-diasterane
6b	C_{28} 13β(H)17α(H)20(R)24(R)-diasterane
7	C_{28} 13α(H)17β(H)20(S)-diasterane + C_{27} 14α(H)17α(H)20(S)-sterane
8	C_{29} 13β(H)17α(H)20(S)-diasterane + C_{27} 14β(H)17β(H)20(R)-sterane
9	C_{28} 13α(H)17β(H)20(R)-diasterane + C_{27} 14β(H)17β(H)20(S)-sterane
10	C_{27} 14α(H)17α(H)20(R)-sterane
11	C_{29} 13β(H)17α(H)20(R)-diasterane
12	C_{29} 13α(H)17β(H)20(S)-diasterane
13	C_{28} 14α(H)17α(H)20(S)-sterane
14	C_{29} 13α(H)17β(H)20(R)-diasterane + C_{28} 14β(H)17β(H)20(R)-sterane
15	C_{28} 14β(H)17β(H)20(S)-sterane
16	C_{28} 14α(H)17α(H)20(R)-sterane
17	C_{29} 14α(H)17α(H)20(S)-sterane
18	C_{29} 14β(H)17β(H)20(R)-sterane
19	C_{29} 14β(H)17β(H)20(S)-sterane
20	C_{29} 14α(H)17α(H)20(R)-sterane

2.2.4. Terpanes

For the analysis of terpanes in bitumens of recent and ancient sediments, and in oils, the same analytical methods and the same instrumental techniques are applied as for the analysis of steranes.

Terpanes are, similarly to steranes, found in oils in trace amounts, and their concentration is expressed in ppm. By examining a large number of oil from reservoirs Hamilton Dome, Wyoming, it was found that the average amount of total triterpanes in these crudes was 1773 ppm, while the average content of the most abundant terpane biomarker in petroleum, C_{30}17 α(H)β(H)-hopane was 145 ppm.

In the alkane fraction of recent sediments tricyclic terpanes are present in traces, or not present at all. The largest amount of these hydrocarbons is incorporated into kerogen or is in the form of polar fractions of bitumens, resins and asphaltenes, from which it is released during thermal degradation of the sediment. Therefore, only in the alkane fractions of mature sediments and crude oils tricyclic terpanes are found in the range from $C_{19}H_{34}$ to $C_{45}H_{86}$. It is believed that the precursor of C_{19}-C_{30} tricyclic terpanes is C_{30} –tricyclohexaprenol (Figure 10) However, this alcohol was not found in living organisms, suggesting that it was formed by abiogenic cyclisation of hexaprenol, which is found in the cells and membranes of algae and bacteria (Vitorović, Jovančićević, 2005).

Precursors of C_{30}-C_{45} tricyclic terpanes are probably higher unsaturated isoprenoid alcohols. An example is solanesol (C_{45}), isoprenoid alcohol of higher terrestrial plants.

Based on the analyses of tricyclic terpanes in a number of geological samples it was concluded that organic matter from saline lacustrine environment and marine organic matter usually contain a greater amount of tricyclic terpanes, where C_{23} terpane is most abundant. In the organic matter of terrestrial origin, these polycyclic hydrocarbons are much less present, and the most abundant among them is C_{20} tricyclic terpane. Regardless the origin, the least abundant among the tricyclic terpanes are C_{22} and C_{27} terpanes. Compounds with more than 24 C-atoms exist in the form of a mixture of 22R and 22S diastereoisomers. During catagenesis, higher members of tricyclic

terpanes (C_{23}-C_{25}) are transformed into C_{19}-C_{23} tricyclic terpanes. Having in mind this fact, it is possible to use higher to lower tricyclic terpane ratio as maturation parameters.
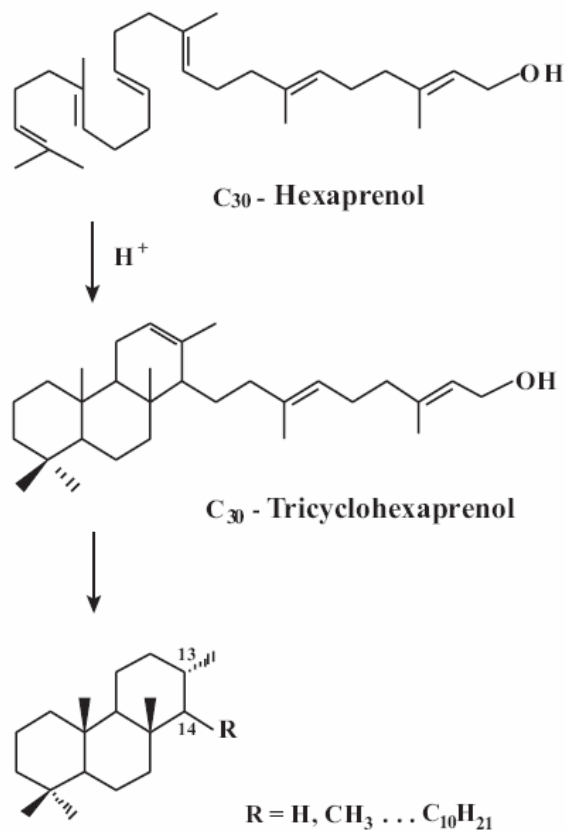
Figure 10. The probable mechanism of genesis of tricyclic triterpanes from hexaprenol (Vitorović, Jovančićević, 2005).

Tetracyclic terpanes were also identified in the range C_{24}-C_{27} in bitumens of a number of sedimentary rocks of different origin and age, and in the oils (Figure 11; Vitorović, Jovančićević, 2005).
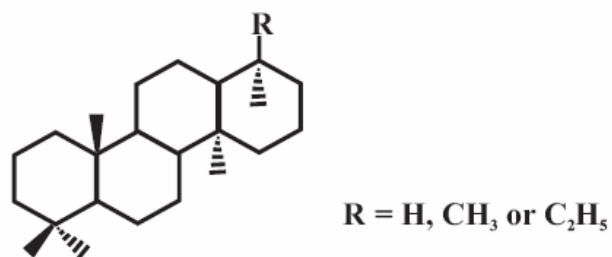
Figure 11. Structural formula of tetracyclic terpanes.

Comparing to tricyclic terpanes, tetracyclic terpanes are less abundant in various forms of organic matter of the geosphere (Vitorović, Jovančićević, 2005).

There are two theories on the origin of these hydrocarbons in geolipids. According to the first one, tetracyclic terpanes are formed by thermocatalytic degradation of hopanoids of microbiological origin. According to the second theory, tetracyclic terpanes are formed by opening of the E-ring in hopanoids by microbial activity during early diagenesis, and then defunctionalization during maturation. (Vitorović, Jovančićević, 2005).

Contrary to steranes which originate from sterols, mainly represented in eukaryotes, the precursors of triterpanes are biolipids from prokaryotic organisms, bacteria and algae. Tri-tetra- and pentacyclic triterpanes are found in oils. Summary of most triterpanes identified in oil with their biological precursors, and characteristics of their depositional environments is given in Table 6.

Table 6. Summary of oil triterpanes with biological precursors, and characteristics of their environments of deposition (Peters, Moldowan, 1993).

Compound	Biologic precursor	Environments of deposition	Literature
C_{35}-hopane	bacteria	Marine, reductive conditions	Peters i Moldowan, 1991; Moldowan *et al*., 1992
C_{29}-hopane	prokaryotes	Carbonate, salt	Clark and Philp, 1989
Gammaceran	Protozoa, bacteria	Marine, evaporite	Fu *et al*., 1986; Venkatesan, 1989
C_{30}*17α(H)-diahopane	unknown	Continental, Oxic or oxidative	Peters and Moldowan, 1991
Oleanane	vascular plants, angiosperms	Continental Cretaceous age and younger	Ekweozor and Udo, 1988; Riva *et al*., 1988
C_{24}-Tatracyclic terpane	unknown	different	Connan *et al*., 1986; Philp i Gilbert, 1986
Tricyclic terpane	Bacteria, algae Tasmanites	Marine or lake	Albrecht, 1986; Simoneit *et al*., 1993; Revill *et al*., 1994

In alkane fractions from geological samples, besides hopane, some other structural isomers of pentacyclic triterpanes, such as oleanane, ursane, lupane and gammacerane were identified (Figure 12; Vitorović, Jovančićević, 2005).

Due to their low abundance in crude oils, identification and quantification of triterpanes requires the application of gas chromatographic-mass spectrometric analysis using $m/z = 191$ ion fragmentogram. Typical example of triterpane fragmentogram of ancient sediment bitumen is shown in Figure 13, and the corresponding peak identification is given in Table 7. (Vitorović, Jovančićević, 2005).
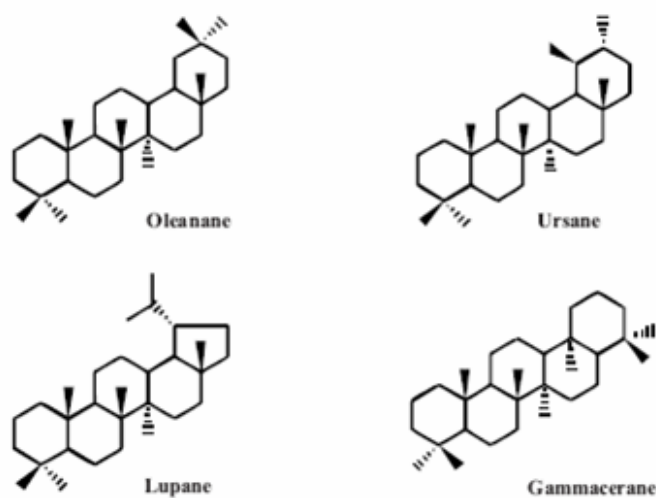
Figure 12. Some pentacyclic terpanes identified in bitumens and oils.
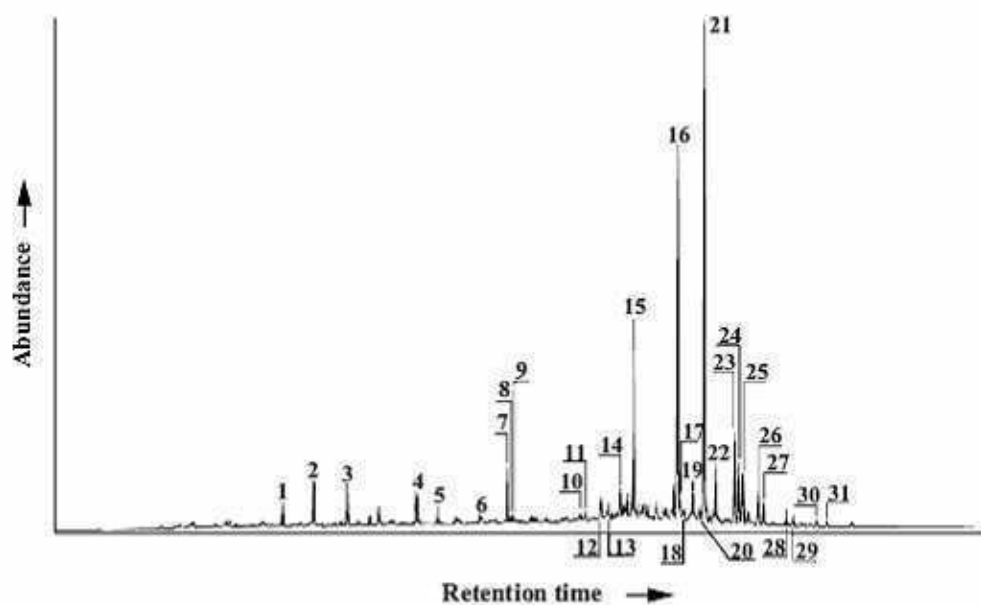
Figure 13. GC-MS fragmentograms of triterpanes from an ancient sediment bitumen (Vitorović, Jovančićević, 2005).

Table 7. Identification of the peaks in the chromatogram shown in Figure 13 (terpane fragmentogram; Vitorović, Jovančićević, 2005).

Peak	Compounds
1	C_{19}-tricyclic terpane
2	C_{20}- tricyclic terpane
3	C_{21}- tricyclic terpane
4	C_{23}- tricyclic terpane
5	C_{24}- tricyclic terpane
6	C_{25}- tricyclic terpane
7	C_{24}-tetracyclic terpane
8	$C_{26}22(S)$- tricyclic terpane
9	$C_{26}22(R)$- tricyclic terpane
10	$C_{28}22(S)$- tricyclic terpane
11	$C_{28}22(R)$- tricyclic terpane
12	$C_{29}22(S)$- tricyclic terpane
13	$C_{29}22(R)$- tricyclic terpane
14	C_{27} 18α(H),22,29,30-trisnorneohopane, Ts
15	C_{27} 17α(H),22,29,30-trisnorhopane, Tm
16	C_{29} 17α(H)21β(H)-hopane
17	C_{29} 18α(H),30-norneohopane
18	C_{30} 17α(H)-diahopane
19	C_{29} 17β(H)21α(H)-moretane
20	Oleanane
21	C_{30} 17α(H)21β(H)-hopane
22	C_{30} 7β(H)21α(H)-moretane
23	C_{31} 17α(H)21β(H)22(S)-hopane
24	C_{31} 17α(H)21β(H)22(R)-hopane
25	Gammacerane
26	C_{32} 17α(H)21β(H)22(S)-hopane
27	C_{32} 17α(H)21β(H)22(R)-hopane
28	C_{33} 17α(H)21β(H)22(S)-hopane
29	C_{33} 17α(H)21β(H)22(R)-hopane
30	C_{34} 17α(H)21β(H)22(S)-hopane
31	C_{34} 17α(H)21β(H)22(R)-hopane

2.2.5. Naphthenoaromatic hydrocarbons

These compounds are usually the major constituents of the high boiling fraction of hydrocarbons in oils. Naphthenoaromatic hydrocarbons contain mixtures of aromatic and saturated rings, usually one or several condensed aromatic rings fused with naphthenic rings and a saturated hydrocarbon chain. However, naphthenoaromatics hydrocarbons with various structural arrangements have been found in petroleum so far. Bicyclic (1 aromatic, 1 saturated cycle), indane, tetralin (tetrahyronaphthalene) and their methyl derivatives are usually abundant and have been determined in several crude oils (Tissot, Welte, 1984). From the organic-geochemical point of view, among the geolipid naphthenoaromatic compounds, aromatic steroids are the most important.

So far, in aromatic fractions of geological samples, C_{20}, C_{21} and C_{26}-C_{30} monoaromatic steroids (aromatic A or C ring), and C_{20}, C_{21} and C_{26}-C_{28} triaromatic steroids have been identified. A typical C27 monoaromatic steroid structure is shown in Figure 14.
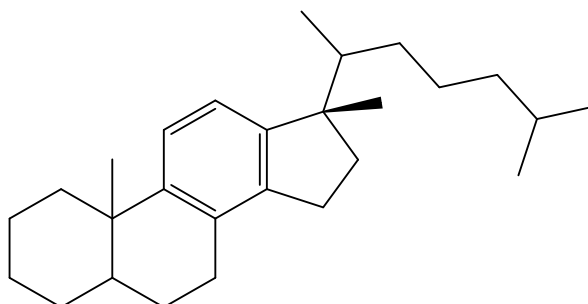
Figure 14. $C_{27}H_{42}$ monoaromatic steroid.

2.2.6. Aromatic hydrocarbons in petroleum

Aromatic hydrocarbons (aromatics or arenes) represent one of the most abundant groups of compounds in petroleum and bitumen. Their concentrations range from 15 % to 35 % and sometimes even reach the value of 50 % (Hunt, 1982; Tissot, Welte, 1984). Statistical analysis of the data acquired from 400 analyses of crude oils worldwide

showed that content of the aromatic hydrocarbons is the highest in naphthenic oils (average 37 %) and the lowest in paraffinic oils (average 20 %; Kuklinski *et al.*, 1983).

Various groups of aromatic hydrocarbons are identified in crude oils: mono-, di-, tri- and polycyclic aromatic hydrocarbons (with more than three condensed aromatic rings). Predominant part of the aromatic fraction of crude oils is formed from the molecules without any naphthenic rings. In organic geochemistry they are labeled as alkylaromatics (Radke, 1987). On the other hand, naphthenoaromatics (which, beside aromatic, consist of 1 to 8 saturated or naphthenic rings) are found in crude oils just as trace components (Golovko, 2001; Kamyanov, 1996; Kamyanov *et al.*, 1982). Usually the most abundant components of the aromatic fraction of crude oils are monoarenes.

On the basis of spectral analysis, average distribution of aromatic rings in crude oils is calculated as follows: 67 % of benzene, 18 % naphthalene, 8 % phenanthrene, 3 % chrysene and benzofluorene, 2 % pyrene and less than 1 % of anthracene rings (Kuklinski *et al.*, 1983). The samples of Central Asian crude oils showed that the increase of thermal maturity of petroleum contributes to the increase of concentrations of aromatic hydrocarbons with lower number of condensed aromatic rings (1-3), which is followed with the simultaneous methanization of petroleum (Kamyanov, 1996; Kamyanov *et al.*, 1982).

Many studies considering aromatic hydrocarbons emphasize relation of these compounds with molecules from the biosphere, first of all lipids. Based on a large number of experimental facts, the possibility of polygenetic formation of aromatic compounds in crude oils is suggested (Borrego *et al.*, 1997; Kawka, Simoneit, 1994; Petrov, 1984; Püttman, Villar, 1987; Radke, 1987). However, although the transformation paths from biological precursors into arenes are well known, for many aromatic compounds identified in crude oils, kinetic, thermodynamic and catalytic aspects of these transformations are not completely clarified yet. Also, according to suggested mechanisms, the direct products of biomolecule transformations are naphthenoarenes, which are just trace components of crude oils. The biggest portion of aromatic hydrocarbons in crude oils (alkylaromatics) is produced by catagenetic transformation of kerogen, and these molecules keep just fragments of precursor structures (Alexander *et al.*, 1984, 1985; Ishiwatari, Fukushima, 1979; Radke, 1987; Radke, Welte, 1983; Requejo, 1994; Simons *et al.*, 2003).

Many authors stated that catagenetic changes which lead to intensive production of liquid hydrocarbons (petroleum) are always followed by significant increase in alkylaromatics quantity (Radke, 1987; Radke *et al*., 1998; Shimoyama *et al*., 2000). In other words, alkylaromatics are products of the maturation processes in catagenetic sequence and therefore the major portion of aromatic hydrocarbons in crude oils does not belong to the category of biomarkers. Because of that their distribution and abundance is still most often used for assessment of thermal maturity level (Alexander *et al*., 1984, 1985; George *et al*., 1998, 2001; Golovko, 1997; Ivanov, Golovko, 1992; Radke *et al*., 1982a, b; Radke, 1987; Simons *et al*., 2003).

Regardless the fact that monocyclic aromatic hydrocarbons are usually the most abundant aromatics in crude oils, di- and triaromatics showed much greater applicability in organic-geochemical studies (Alexander *et al*., 1984, 1985, 1994; George *et al*., 1998, 2001; Golovko, 1997; Radke, 1987; Radke *et al*., 1982a, b).

2.2.6.1. *Dicyclic aromatic hydrocarbons*

Content of dicyclic aromatic hydrocarbons in crude oils ranges from 1 % to 10 % (Golovko *et al*., 2002). Previous studies indicated distinct domination of alkylnaphthalenes (up to 90 %; Figure 15) in diaromatic fraction of crude oils. Besides naphthalene derivates, crude oils contain in substantially lower concentrations biphenyls, biphenyl-alkanes and naphthenic-diarenes with maximum 6 - 8 saturated rings (Cumbers *et al*., 1987; Golovko, 2001; Golovko & Koržov, 1992; Grice *et al*., 1999; Kamyanov *et al*., 1982).
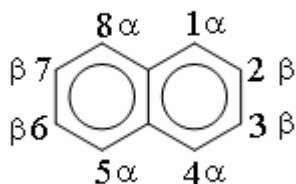
Figure 15**.** Structural formula of naphthalene
with corresponding α- and β- substituent positions.

Alkylnaphthalenes in petroleum are usually represented as methyl-isomers and can contain one to five CH_3-groups directly attached to the aromatic rings (van Aarssen *et al*., 1999; Alexander *et al*., 1984, 1985; Bastow *et al*., 1998; Radke *et al*., 1982b, 1984; Strachan *et al*., 1988). In previous studies 1- (α) and 2- (β) methylnaphthalenes (MN), as well as almost all theoretically possible isomers of dimethylnaphthalene (DMN) and trimethylnaphthalene (TMN) are identified in crude oils (Table 8). It was found that among dimethylnaphthalenes, compounds with the substituents on different rings predominate (> 80 %) in crude oils (Golovko, 1997). Development of instrumental techniques enabled identification of 12 isomers of tetramethylnaphthalene (TeMN) and 4 isomers of pentamethylnaphthalene (PMN; Table 9; van Aarssen *et al*., 1999; Bastow *et al*., 1998). Besides methylnaphthalenes, ethylnaphthalenes (EN, 1- and 2-) are also present in crude oils, usually making 7 – 10 % of total C_{12}-naphthalenes (Table 8; Golovko, 1997; Radke *et al*., 1982b). In a recent study, homologous series of $C_{13} - C_{32}$ monoalkylnaphthalenes with the normal side chain is identified in Australian crude oils (Ellis *et al*., 1999).

Besides tetramethylnaphthalenes and *n*-butylnaphthalene, some other isomers of C_{14}-naphthalene are present in petroleum - dimethylethylnaphthalenes and isopropylmethylnaphthalenes, often labeled as norcadalenes (Singh *et al*., 1994). Moreover, besides *n*-pentylnaphthalene and the series of pentamethylnaphthalenes, 4 isomers of C_{15}-naphthalene are also identified in crude oils (Alexander *et al*., 1994; Singh *et al*., 1994). Among all of them, 1,6-dimethyl-4-isopropylnaphthalene (cadalene) rouses the special interest of many researchers. This compound is found in the resins of higher plants (Weiss, Edwards, 1980) and there is evident structural connection of cadalene with sesquiterpenoids in the biosphere - farnesol and cadinene (Baset *et al*., 1980; Noble *et al*., 1991). In addition to alkylnaphthalenes, naphthenic naphthalenes are also found in crude oils, but in substantially smaller quantities (Golovko, 2001; Kamyanov, 1996; Petrov, 1984; Simoneit, 1977).

Biphenyl and alkylbiphenyls represent extremely low portion of dicyclic aromatic fraction of crude oils (Figure 16; Cumbers *et al*., 1987; Grice *et al*., 1999; Trolio *et al*., 1999). For instance, in Ponka City petroleum the concentration of $C_{12}+C_{13}$ biphenyls is 50 times less than the total concentration of $C_{12}+C_{13}$ naphthalenes (Cumbers *et al*., 1987). The low content of biphenyls in crude oils can be explained by

their low thermodynamical stability. Aromatic rings in these molecules are not co-planar, so their conjugation is reduced and therefore they are less stable than naphthalenes. Their concentrations in petroleum usually follow this decreasing trend: 3-MBPh > BPh > 4-MBPh > 2-MBPh (BPh - biphenyl, MBPh - methylbiphenyls) (Cumbers *et al*., 1987; Grice *et al*., 1999; Trolio *et al*., 1999).

Table 8. Review of the most abundant alkylnaphthalenes in
dicyclic aromatic fraction of crude oils.

Compounds	Isomers
Methylnaphthalenes (MN)	1-, 2-MN
Dimethylnaphthalenes (DMN)	1,2-, 1,3-, 1,4-, 1,5-, 1,6-, 1,7-, 1,8-, 2,3-, 2,6-, 2,7-DMN
Trimethylnaphthalenes (TMN)	1,2,4-, 1,2,5-, 1,2,6-, 1,2,7-, 1,3,5-, 1,3,6-, 1,3,7-, 1,6,7-, 2,3,6-TMN
Tetramethylnaphthalenes (TeMN)	1,2,3,5-, 1,2,3,6-, 1,2,3,7-, 1,2,4,6-, 1,2,4,7-, 1,2,5,6-, 1,2,5,7-, 1,2,6,7-, 1,3,5,7-, 1,3,6,7-, 1,4,6,7-, 2,3,6,7-TeMN
Pentamethylnaphthalenes (PMN)	1,2,3,5,6-, 1,2,3,5,7-, 1,2,3,6,7-, 1,2,4,6,7-PMN
Ethylnaphthalenes (EN)	1-, 2-EN
n-Alkylnaphthalenes C_{13}-C_{32}	1-, 2-*n*-propylnaphthalene – 1-, 2-*n*-dodecylnaphthalene
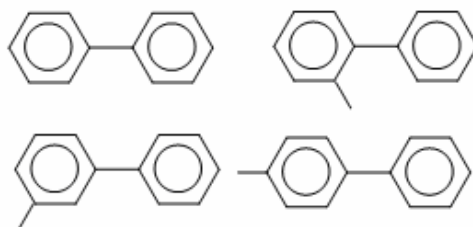
Figure 16. Structural formulas of biphenyl and methylbiphenyls
identified in crude oils(Cumbers *et al*., 1987; Grice *et al*.,
1999; Trolio *et al*., 1999).

2.2.6.1.1. Identification of dicyclic aromatic hydrocarbons

The classical methods for extraction and purification of dicyclic aromatic fraction of crude oils are column- and thin layer chromatography. Hydrocarbons in this fraction show absorption maximums around 250 nm in UV-spectra, which enables control for merging of effluents that get off the column (Stojanović, 2004).

Diarene concentrates, obtained by classical chromatography methods, can successfully be separated into alkylnaphthalenes and derivates of biphenyl and biphenyl-alkanes using the high performance liquid chromatography - HPLC (Alexander *et al*., 1985; Golovko, Koržov, 1992). Additionally, depending on substituent positions on aromatic rings, di-, tri- and tetramethylnaphthalenes can successfully be separated using the molecular sieves (Ellis *et al*., 1994).

GC and GC-MS analyses are the methods used for identification and quantification of dicyclic aromatic hydrocarbons (Figure 17; George *et al*., 1998; Golovko, 1997). Methyl-, dimethyl- and trimethylnaphthalenes are efficiently separated both by GC- and GC-MS techniques. However, for identification of tetra- and pentamethylnaphthalenes application of GC-MS method is necessary. Characteristic *m/z* values of ion fragmentograms for the most important petroleum diarenes are listed in Table 9 (Alexander *et al*., 1994; Bastow *et al*., 1998; Grice *et al*., 1999; Singh *et al*., 1994).

Table 9**.** Characteristic *m/z* values for identification of alkylnaphthalenes by GC-MS analysis

Compounds	*m/z*
Methylnaphthalenes	142
Dimethyl- and ethylnaphthalenes	156
Trimethylnaphthalenes	170
Tetramethyl-, dimethylethyl- and isopropylmethylnaphthalenes	184
Pentamethyl- and dimethylisopropylnaphthalenes	198
Biphenyl	154
Methylbiphenyls	168
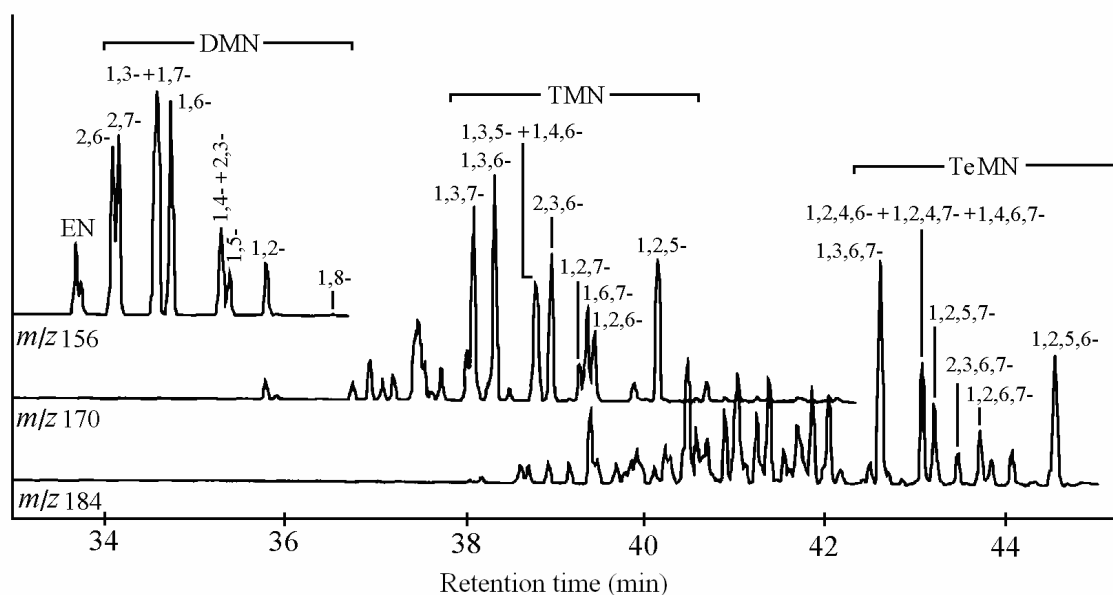
Figure 17. GC-MS analysis of dicyclic aromatic fraction of the crude oil
from South Pepper basin, Australia (George *et al*., 1998).

2.2.6.2. Tricyclic aromatic hydrocarbons

Although the abundance of tricyclic aromatic hydrocarbons in petroleum is lower than the abundance of mono- and diaromatic hydrocarbons, tricyclic aromatic hydrocarbons are equally important in organic-geochemical and environmental investigations.

Depending on the form that aromatic rings are fused in, angular-type molecules (phenanthrene and its alkyl-derivates) and linear-type molecules (anthracene and its alkyl-derivates) are distinguished among triaromatics (Figure 18).

Fusing character determines physical and chemical properties of these compounds and therefore their presence in crude oils and other forms of organic matter in the Earth's crust.

Phenanthrene and anthracene were identified for the first time in products of coal pyrolysis, and later in source rock and oil shale extracts, as well as in crude oils (Klar, 1971). Phenanthrene molecule possesses two aromatic sextets, while anthracene

possesses one and consequently is thermodynamically less stable. Therefore it is understandable fact that the total concentration of phenanthrene isomers in crude oils can be up to 50 times grater than that of anthracene isomers (Kamyanov *et al*., 1982). Consequently, basic components of tricyclic aromatic fraction of crude oils are phenanthrene-type compounds.
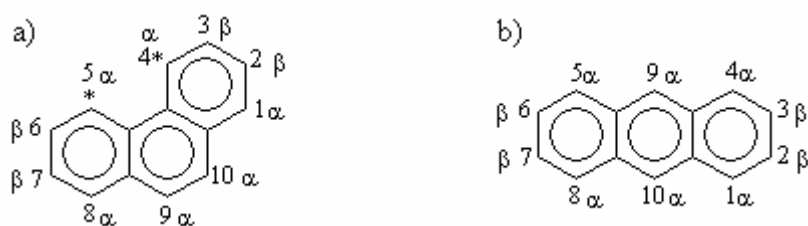
Figure 18. Structural formulas of phenanthrene (a) and anthracene (b) with corresponding α- and β- substituent positions.

Condensed system of aromatic rings in phenanthrene molecule provides a large number of possible alkyl-isomers. Theoretically there are 5 isomers of monosubstituted phenanthrene, 30 isomers of disubstituted phenanthrene and even 115 possible isomers of trisubstituted phenanthrene (Vitorović, Jovančićević, 2005). In tricyclic aromatic fraction of crude oils, the most abundant are phenanthrene itself and its methyl-derivates (Table 10). From methylphenanthrene (MP) isomers, 1-, 2-, 3- and 9-MP are found in significant concentrations in crude oils.

In previous organic-geochemical studies, 16 isomers of dimethylphenanthrene (DMP) are identified in crude oils, as well as 4 isomers of ethylphenanthrene (EP) (Table 10; Golovko, 1997; Radke, 1987). Although 14 isomers of trimethylphenanthrene (TMP) are identified in petroleum, their difficult separation during GC and GC-MS analyses gives them minimal importance in petroleum correlation studies (Table 10; Golovko, 1997; Radke, 1987). Among C_4-substituted phenanthrenes, 1-methyl-7-isopropyl-phenanthrene (retene) is also identified in crude oils (Golovko, 1997). It rouses the special interest of many researchers, owing to the

evident structural connection with diterpenoids identified in the resins of higher plants (Alexander *et al.*, 1987; Ellis *et al.*, 1996; Wakeham *et al.*, 1980).

Table 10. Review of the most abundant alkylphenanthrenes in tricyclic aromatic fraction of crude oils.

Compounds	Isomers
Methylphenanthrenes (MP)	1-, 2-, 3-, 4-, 9-MP
Dimethylphenanthrenes (DMP)	1,2-, 1,3-, 1,6-, 1,7-, 1,8-, 1,9-, 2,3-, 2,6-, 2,7-, 2,9-, 2,10-, 3,5-, 3,6-, 3,9-, 3,10-, 4,9-DMP
Trimethylphenanthrenes (TMP)	1,2,3-, 1,2,8-, 1,3,6-, 1,3,7-, 1,3,8-, 1,3,9-, 1,7,10-, 2,3,6, 2,3,7-, 2,3,10-, 2,6,10-, 2,7,10-, 2,8,10-, 3,8,10-TMP
Ethylphenanthrenes (EP)	1-, 2-, 3-, 9-EP

Anthracene and its alkyl-derivates are usually found in very low concentrations in crude oils (for example, 0.07 – 0.30 % in Sakhalin oils, Russia), which makes their extraction and identification difficult (Ivanov, Golovko, 1994). Nevertheless, 12 alkyl-derivates of anthracene are successfully identified (Golovko, 1997; Ivanov, Golovko, 1994; Jiang, Guan, 1999; Matveeva *et al.*, 1991).

In addition to alkylphenanthrenes, naphthenic phenanthrenes are also found in crude oils, but in substantially smaller quantities (Golovko, 2001; Kamyanov, 1996; Petrov, 1984; Simoneit, 1977). The most abundant among them are alkyl-derivates of cyclopentanophenanthrene, called triaromatic steroids - because of the structural similarities with the steroids from the living world (Peters, Moldowan, 1993; Radke, 1987).

2.2.6.2.1. Identification of tricyclic aromatic hydrocarbons

Classical methods for extraction and purification of tricyclic aromatic fraction of crude oils are column- and thin layer chromatography. Triarenes show characteristic absorption maximums in λ = 252 - 263 nm and λ = 292 - 307 nm areas in UV-spectra,

which enables control for merging of effluents that get off the column (Ivanov, Golovko, 1992; Ivanov *et al*., 1991).

Besides classical chromatography methods (column- and thin layer chromatography), mid-pressure liquid chromatography (MPLC) and high-pressure liquid chromatography (HPLC) are also used for extraction and purification of triaromatic hydrocarbons (Radke, Welte, 1983; Radke *et al*., 1990). Additionally, depending on the substituent positions on phenanthrene molecule, methyl- and dimethylphenanthrenes can successfully be separated using the molecular sieves (Ellis *et al*., 1994).

GC and GC-MS analyses are techniques used for identification and quantification of tricyclic aromatic hydrocarbons (Figure 19; Ellis *et al*., 1994; Golovko, 1997; Radke *et al*., 1982a&b; Simons *et al*., 2003; Stojanović, 2000).
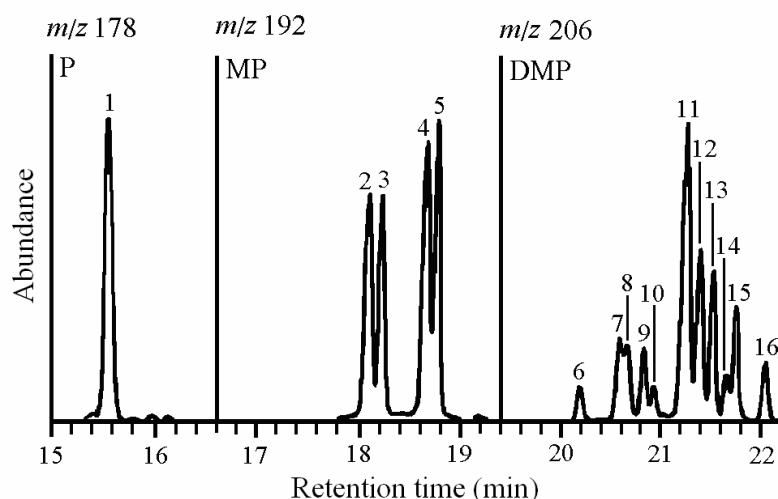
Figure 19**.** GC-MS analysis (ion fragmentograms *m/z* 178, 192, 206) of tricyclic aromatic fraction of source rock extract from the Western Interior Seaway formation, Canada (Simons *et al*., 2003).

1 – P; 2 – 3-MP; 3 – 2-MP; 4 – 9-MP; 5 – 1-MP; 6 – 3-EP; 7 – 2-+9-EP; 8 – 1-EP; 9 – 2,6-+3,5-DMP; 10 – 2,7-DMP; 11 – 1,3-+2,10-+3,9-+3,10-DMP; 12 – 1,6-+2,5-+2,9-DMP; 13 – 1,7-DMP; 14 – 2,3-DMP; 15 – 1,9-+4,9-DMP; 16– 1,2-+1,8-DMP; P – phenanthrene; MP – methylphenanthrene; DMP – dimethylphenanthrene; EP – ethylphenanthrene.

Application of GC-MS method does not contribute to the significant improvement of alkylphenanthrene and alkylanthracene separation, comparing to GC

method. The reason is that methylphenanthrenes and methylanthracenes, similarly to dimethylphenanthrenes and ethylphenanthrenes, have the same molecular masses and they are hardly subjected to fragmentation (Ivanov, Golovko, 1992; Radke *et al*., 1986). Therefore, characteristic *m/z* values of ion fragmentograms are: 178 for phenanthrene, 192 for methylphenanthrenes and 206 for dimethylphenanthrenes and ethylphenanthrenes, which actually corresponds to their molecular masses (Figure 19; Simons *et al*., 2003).

2.3. Biodegradation of petroleum as a native organic matter in the reservoir sediment rock

Biodegradation is one of the most important processes that alter the composition of the oil in the reservoir rocks, and refers to the selective consumption of certain compounds in the oil by microorganisms (Peters *et al*., 2005).

It has been considered for a long time that aerobic biodegradation is a dominant process in reservoir rocks (Milner *et al*., 1977; Blanc, Connan, 1994). However, recent studies showed that anaerobic processes may be even more important. (Zengler *et al*., 1999). Mechanisms of aerobic and anaerobic biodegradation are still not completely understood, but it is known that the following conditions are necessary for the biodegradation of large amounts of oil in the reservoir rocks (Connan, 1984, Palmer, 1993; Blanc, Connan, 1994, Head *et al*., 2003):

1. The temperature in reservoir rocks must be lower than 80 °C, which corresponds to the depths lower than 2000 m at usual geothermal gradients. At higher temperatures, biodegradation can proceed, but its speed is significantly reduced;

2. The influx of sufficient amounts of nutrients and electron acceptors (molecular oxygen, nitrate and phosphate) is necessary. This is usually provided by circulation of meteoric water into the deeper parts of the basin;

3. The presence of H_2S is unfavorable for aerobic microorganisms. However, for the proper activity of anaerobic sulfate reducers, H_2S content should not exceed 5 %;

4. The salinity of water in the formation must be less than 100 – 150 ‰.

The effect of biodegradation on the physical properties and molecular composition of petroleum in reservoir rocks is well-known (Volkman *et al*., 1983; Connan, 1984; Palmer, 1993; Peters *et al*., 1996; Peters *et al*., 2005; Muller *at al*., 1987). With increase in the degree of biodegradation, oil becomes more viscous, has lower value of API density, and higher content of sulfur, resins, asphaltenes and metals (Ni and V).

Biodegradation of hydrocarbons is usually described as quasi-sequential, meaning that more resistant classes of compounds can be attacked by microorganisms before a complete consumption of less resistant compounds (Peters *et al*., 2005). Biodegradation level of oils (on the 0 – 10 scale) is usually determined on the basis of composition and distribution of hydrocarbons present, according to the scheme proposed by Peters and Moldowan (1993). However, in recent years, more and more is used a biodegradation scale proposed by Wenger *et al*., (2002), which is based on changes in composition of oil at lower biodegradation levels (Figure 20).

2.4. Petroleum as a main organic type pollutant in the environment

Pollution is the introduction into the air, water or ground of toxic substances that are damaging or harmful to living organisms and ecosystems. It can occur naturally, for example through volcanic eruptions. However, it is mainly linked with human activities such as the spillage of oil, disposal of domestic, industrial and agricultural waste, application of pesticides by farmers, leakage of radioactive materials or gas emissions into the atmosphere.

Petroleum is a naturally occurring substance that can be assimilated into the environment when present in small quantities. However, large volumes of spilled oil and refined products can harm organisms directly or impact their environment and cause indirect harm. Direct effects may be physical or chemical and can cause acute and/or chronic illness or death. Physical damage can occur when oil coats feathers or fur, resulting in decreased thermal insulation and buoyancy, and by closing off respiratory

passages. Chemical effects may cause acute poisoning, tissue and organ damage, and mutations or cancer. Indirect harm results from habitat destruction, interference of nutrient flow, and disruption of food chain.

	Light oils				Heavy oils	
	Major mass loss ~50% →				Structural rearrangement/ minor mass loss ~<20%	
Total acid number (mg KOH per g oil)	0.2	0.5	1.0	1.5	2.0	2.5+
API gravity (°API)	36	32	31	28	20	5–20
Gas:oil ratio (kg gas per kg oil)	0.17	–	0.12	0.08	0.06	<0.04
Gas wetness (%)	20		10		5	2
Sulphur content (wt%)	0.3	0.4	0.5	–	1.0	1.5+
C_{15+} saturated hydrocarbon content (%)	75	70	65	60	50	35
Level of biodegradation						
Scale of Peters and Moldowan	0	1	2	3	4 5	6–10
Scale of Wenger *et al.*	None	Very slight	Slight	Moderate	Heavy	Severe

C_1–C_5 gases: Methane‡, Ethane, Propane, Isobutane, *n*-Butane, Pentanes

C_6–C_{15} HCs: *n*-Alkanes, Isoalkanes, Isoprenoid alkanes, BTEX aromatics, Alkylcyclohexanes

C_{15}–C_{35} HCs: *n*-Alkanes, isoalkanes; Isoprenoid alkanes; Naphthalenes (C_{+10}); Phenanthrenes, dibenzothiophenes; Chrysenes

C_{15}–C_{35} biomarkers: Regular steranes; C_{30}–C_{35} hopanes; C_{27}–C_{29} hopanes; Triaromatic steroid hydrocarbons; Monoaromatic steroid hydrocarbons; Gammacerane; Oleanane; C_{21}–C_{22} steranes; Tricyclic terpanes; Diasteranes; Diahopanes; 25-Norhopanes‡

N†: Alkylcarbazoles

O: Carboxylic acids‡

Legend:
- – – – – Minor removal
- ──── Major alteration
- ━━━➤ Removal
- ·······➤ Methane generation and possible destruction
- ‡ Produced and destroyed during biodegration

Figure 20. Biodegradation scale after Wenger *et al.*, (2002).

In spite of remarkable advancement of petroleum exploitation and improvements in technology and equipment used for oil drilling, transport and processing by the petroleum industry and refining technologies (Jovančićević, 2002), oil and oil derivatives represent a significant source of environmental contamination, and its products continue to be one of the most abundant environmental pollutants. Consequently, transformation processes of petroleum-type pollutants in soil, recent sediments, alluvial sediments, ground and surface water were studied by numerous authors (Vaajasaari *et al*., 2002; Booth *et al*., 2005).

When oil pollutant enters the environment its composition begins to change immediately due to the simultaneous effects of different abiotic and biotic processes (Figure 21). These processes, called weathering, change the chemical composition of the pollutant and determine its behavior and fate in the environment (Barakat *et al*., 2001, Douglas *et al*., 2002, Kaplan *et al*., 1997).

The fate of the pollutant in the environment is never controlled by one single process. Quite often several physical, chemical and biological processes act simultaneously. Which one of them will dominate depends on the composition and quantity of contaminant that entered the environment, but on the specific characteristics of the environment as well.
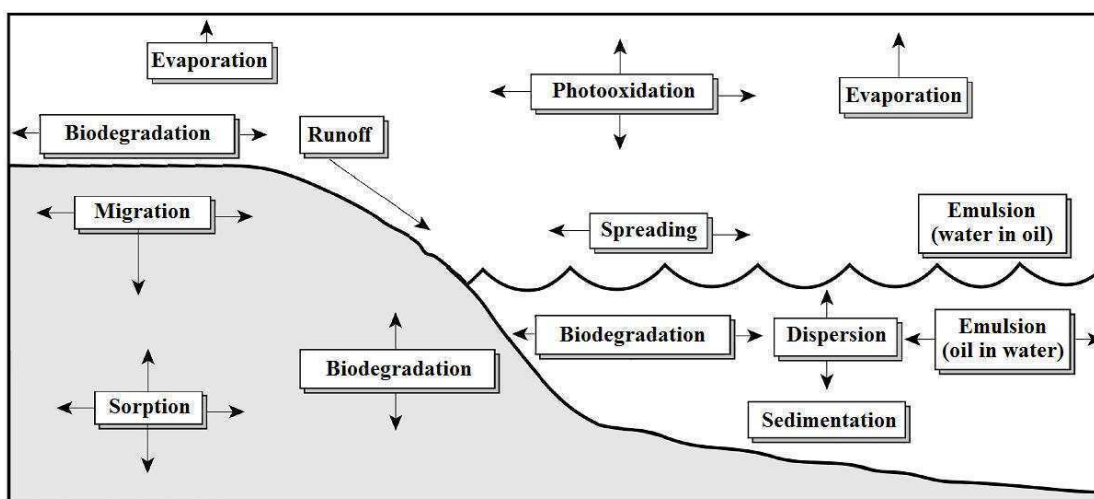
Figure 21. Transformations of oil pollutant in the environment
(modified from EPA 1999.)

As stated before, weathering process changes the composition and the amount of the oil pollutant due to the simultaneous effect of different abiotic and biotic processes. Abiotic losses result from physical removal of certain classes of chemical compounds (evaporation, sorption, runoff, dissolution). From the point of view of the environmental chemistry these processes are considered the most undesirable because they transfer the pollutant from one matrix to another (soil – water, soil – air, water – air; Rodgers *et al.*, 1999).

Abiotic transformations, different from physical abiotic losses include reactions such as hydrolysis, photodegradation and oxidation/reduction. These reactions chemically modify or completely degrade pollutants (Rodgers *et al.*, 1999). Depending of the type of a chemical reaction and structure of a compound to be transformed, they can give products which can be more or less toxic than starting molecules (ATSDR, 1999; EPA, 1996).

Biotic processes include ingestion by live organisms and microbial degradation. Although seam similar, these processes have completely opposite final effects. Ingestion by live organisms introduces some molecules of pollutants into the food chain. As a result, these compounds due to bioconcentration may have much higher toxic effect than the original pollutant (Metcalf, 1977). On the other hand, biodegradation, a process when microorganisms consume some compounds present in the oil pollutant as a source of energy, is actually a process of self-purification of the environment.

2.5. Biodegradation of petroleum as an anthropogenic organic matter in the environment

The ability to utilize hydrocarbons as a source of energy and carbon is widely distributed among the diverse microbial populations. Hydrocarbons are naturally occurring organic compounds so microorganisms evolved the ability to utilize them. Hydrocarbon degrading bacteria and fungi are widely distributed in marine, freshwater, and soil habitats. In unpolluted ecosystems, they generally constitute less then 0.1 % of microbial community. However, in petroleum polluted ecosystems they can constitute up to 100 % of viable microorganisms (Atlas, 1981).

Growth of hydrocarbon decomposing microorganisms and hence the rate of microbiological degradation of petroleum and petroleum derivatives contaminants depends on a number of factors. The main chemical factors are nature of hydrocarbon substrate to be decomposed and its effective concentration, possible presence of toxic substances either from petroleum or in the medium itself and presence and concentration of appropriate nutrients (sources of N, P and other necessary biogenic elements). Additionally, biodegradation process is largely influenced by factors in the environment such as: temperature, oxygen availability, salinity and pH of the system.

Compounds that are constituents of petroleum pollutants vary significantly according to their susceptibility to transformation by microorganisms. Some compounds are readily biodegradable, while others are resistant during biodegradation. The most biodegradable compounds have simple molecular structure (often similar to the structure of organic compounds that occur in nature), they are soluble in water, non-toxic and can be transformed aerobic metabolism (Atlas, 1981). On the other hand, compounds that are stable during biodegradation often have a complex molecular structure (usually structures which are not normally found in nature), low solubility in water, or inability to support microbial growth, or they may be toxic to microorganisms. Complex molecules (those with branched and condensed rings) are more resistant to biodegradation, which means that a smaller number of microbial species can degrade them, that biodegradation rates are lower than for simple molecules and that is more likely to accumulate partially-oxidized intermediary metabolites (Atlas , 1981).

Biodegradation of oil is usually described as quasi-sequential because some of the more resistant compound classes can be attacked before complete degradation of less resistant classes (Peters *et al*., 2005). For biomarkers which are analytically the most important compounds present in crude oils, its derivatives and therefore in petroleum contaminants, the order of biodegradability of certain classes of compounds corresponds to the following sequence: *n*-alkanes (the most biodegradable) > acyclic isoprenoids > hopanes (25-norhopanes present) ≥ steranes > hopanes (25-norhopanes absent) ≈ diasteranes > aromatic steroids > porphirin (the least degradable) (Peters *et al*., 2005).

Crude oils in the environment are never completely degraded. After extensive weathering, a complex residue remains which often appears as a black tar and contains a

very substantial portion of branched aliphatic compounds, polycyclic aromatic hydrocarbons with a large number of condensed rings and asphaltene compounds. Fortunately, toxicity and bioavailability of these remaining compounds is very low, and it can persist in the environment for a long time as an inert pollutant without any toxic effects (Atlas, 1981).

2.6. Bioremediation as a tool for removal of petroleum-type pollutants from the environment

Environmental pollution is an adverse side effect of technological development and human population growth. It is generally considered as a problem of 20 and 21 century.

Nowadays is widely recognized that contaminated land is a potential threat to human health. The estimated number of contaminated sites in the world is significant (Cairney, 1993) which have led to the international efforts to remedy them.

The conventional techniques used for remediation have been to dig up contaminated soil and remove it to another location, or to cap and contain the contaminated areas of a site. These methods have some consequences. The first method simply moves the contamination elsewhere and may create significant risks in the new location. Additionally, it is very difficult and expensive to find new locations for the final disposal of the contaminated material. The cap and contain method is only a temporary solution since the contamination remains on site, requiring monitoring and maintenance long into the future, with all the associated costs and potential liability (Vidali, 2001).

Much better approach than these methods is to completely destroy the pollutants if possible, or at least to transform them to nonharmful substances. Some technologies that have been used so far can be very effective at reducing levels of some contaminants. However, they can also be technologically complex and expensive (Vidali, 2001).

Bioremediation is a cost-effective, green technology that offers the possibility to reduce the amount of various contaminants through their transformation into non-toxic

compounds or to completely degrade them into carbon dioxide and water. Naturally occurring microorganisms are commonly used as biological agents. As such, bioremediation uses relatively low-cost techniques, which can easily be applied on the contaminated sites.

However, bioremediation is not always suitable. First of all, the list of contaminants on which bioremediation is effective is limited. Additionally, the time scales needed for proper bioremediation can be relatively long. Finally, the residual contaminant levels can sometimes be quite high.

Although the methodologies employed for bioremediation are usually technically very simple, significant experience and expertise may be required to design and implement a successful bioremediation program. Additionally, it is always needed to optimize conditions to achieve satisfactory results.

Bioremediation has been used at a number of sites worldwide, with varying success. With time, greater knowledge and experience are gained, and techniques are being more and more improved

The list of contaminants potentially suitable for bioremediation is given in Table 11 (Vidali, 2001).

Table 11. List of contaminants potentially suitable for bioremediation (Vidali, 2001).

Class of contaminants	Specific examples	Aerobic	Anaerobic	More potential sources
Chlorinated solvents	Trichloroethylene Perchloroethylene		+	Drycleaners Chemical manufacture
Polychlorinated biphenyls	4-Chlorobiphenyl 4,4-Dichlorobiphenyl		+	Electrical manufacturing Power station Railway yards
Chlorinated phenol	Pentachlorophenol		+	Timber treatment Landfills
"BTEX"	Benzene Toluene Ethylbenzene Xylene	+	+	Oil production and storage Gas work sites Airports Paint manufacture Port facilities Railway yards Chemical manufacture
Polyaromatic hydrocarbons (PAHs)	Naphthalene Antracene Fluorene Pyrene Benzo(a)pyrene	+		Oil production and storage Gas work sites Coke plants Engine works Landfills Tar production and storage Boiler ash dump sites Power stations
Pesticides	Atrazine Carbaryl Carbofuran Coumphos Diazinon 2,4-D Glycophosphate Parathion Propham	+	+	Agriculture Timber treatment plants Pesticide manufacture Recreational areas Landfills

2.6.1. *In situ* bioremediation

In situ bioremediation techniques are defined as those that are applied to soil and groundwater at the site with minimal disturbance.

These techniques are generally the most desirable options due to lower cost and fewer disturbances since they provide the treatment in place avoiding excavation and transport of contaminants.

In situ treatment is limited by the depth of the soil that can be effectively treated. In many soils effective oxygen diffusion for desirable rates of bioremediation extend to a range of only a few centimeters to about 30 cm into the soil, although depths of 60 cm and greater have been effectively treated in some cases. The most important land treatments are (Vidali, 2001):

2.6.1.1. Bioventing

Bioventing is the most common *in situ* treatment and involves supplying air and nutrients through wells to contaminated soil to stimulate the indigenous bacteria. Bioventing employs low air flow rates and provides only the amount of oxygen necessary for the biodegradation while minimizing volatilization and release of contaminants to the atmosphere. It works for simple hydrocarbons and can be used where the contamination is deep under the surface (Vidali, 2001).

2.6.1.2. In situ biodegradation

In situ biodegradation involves supply of oxygen and nutrients by circulating aqueous solutions through contaminated soils to stimulate naturally occurring bacteria to degrade organic contaminants. It can be used for soil and groundwater. Generally, this technique includes conditions such as the infiltration of water-containing nutrients and oxygen or other electron acceptors for groundwater treatment (Vidali, 2001).

2.6.1.3. Biosparging

Biosparging involves the injection of air under pressure below the water table to increase groundwater oxygen concentrations and enhance the rate of biological degradation of contaminants by naturally occurring bacteria. Biosparging increases the mixing in the saturated zone and thereby increases the contact between soil and groundwater. The ease and low cost of installing small-diameterair injection points allows considerable flexibility in the design and construction of the system (Vidali, 2001).

2.6.1.4. Bioaugmentation

Bioaugmentation techniques involve the addition of microorganisms with the ability to degrade pollutants. Two factors limit the use of added microbial cultures in a land treatment unit (Vidali, 2001):

1. nonindigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels and

2. most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degrades if the land treatment unit is well managed.

2.6.2. *Ex situ* bioremediation

Ex situ techniques are those that are applied to soil and groundwater at the site which has been removed from the site via excavation (soil) or pumping (water). These techniques involve the excavation or removal of contaminated soil from ground (Vidali, 2001).

Landfarming is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded. The goal is to stimulate indigenous biodegradative microorganisms and facilitate their

aerobic degradation of contaminants. In general, the practice is limited to the treatment of superficial 10 – 35 cm of soil. Since landfarming has the potential to reduce monitoring and maintenance costs, as well as clean-up liabilities, it has received much attention as a disposal alternative (Vidali, 2001).

Composting is a technique that involves combining contaminated soil with nonhazardous organic amendants such as manure or agricultural wastes. The presence of these organic materials supports the development of a rich microbial population and elevated temperature characteristic of composting (Vidali, 2001).

Biopiles (Fahnestock, 1998) are hybrid of landfarming and composting. Essentially, engineered cells are constructed as aerated composted piles. Typically used for treatment of surface contamination with petroleum hydrocarbons they are a refined version of landfarming that tend to control physical losses of the contaminants by leaching and volatilization. Biopiles provide a favorable environment for indigenous aerobic and anaerobic microorganisms (Vidali, 2001).

Bioreactors, slurry reactors or aqueous reactors are used for *ex situ* treatment of contaminated soil and water pumped up from a contaminated plume. Bioremediation in reactors involves the processing of contaminated solid material (soil, sediment, sludge) or water through an engineered containment system. A slurry bioreactor may be defined as a containment vessel and apparatus used to create a three-phase (solid, liquid, and gas) mixing condition to increase the bioremediation rate of soil-bound and water-soluble pollutants as a water slurry of the contaminated soil and biomass (usually indigenous microorganisms) capable of degrading target contaminants. In general, the rate and extent of biodegradation are greater in a bioreactor system than *in situ* or in solid-phase systems because the contained environment is more manageable and hence more controllable and predictable. Despite the advantages of reactor systems, there are some disadvantages. The contaminated soil requires pre treatment (e.g., excavation) or alternatively the contaminant can be stripped from the soil via soil washing or physical extraction (e.g., vacuum extraction) before being placed in a bioreactor (Vidali, 2001).

Advantages and disadvantages of bioremediation are listed in Table 12.

Table 12. Advantages and disadvantages of bioremediation (Vidali, 2001).

Technology	Examples	Benefits	Limitations	Factors to consider
In situ	*In situ* bioremediation Biosparging Bioventing Bioaugmentation	Most cost efficient Noninvasive Relatively passive Natural attenuation processes Treats soil and water	Environmental constraints Extended treatment time Monitoring difficulties	Biodegradative abilities of indigenous microorganisms Presence of metals and other inorganics Environmental parameters Biodegradability of pollutants Chemical solubility Geological factors Distribution of pollutants
Ex situ	Landfarming Composting Biopiles	Cost efficient Low cost Can be done on site	Space requirements Extended treatment time Need to control abiotic loss Mass transfer problem Bioavailability limitation	See above
Bioreactors	Slurry reactors Aqueous reactors	Rapid degradation kinetic Optimized environmental parameters Enhances mass transfer Effective use of inoculants and surfactants	Soil requires excavation Relatively high cost capital Relatively high operating cost	See above Bioaugmentation Toxicity of amendments Toxic concentrations of contaminants

2.6.3. Microorganisms in Bioremediation

Microorganisms can be isolated from almost any environmental conditions. Microbes will adapt and grow at subzero temperatures, as well as extreme heat, desert conditions, in water, with an excess of oxygen, and in anaerobic conditions, with the presence of hazardous compounds or on any waste stream. The main requirements are an energy source and a carbon source. Because of the adaptability of microbes and other biological systems, these can be used to degrade or remediate environmental hazards. We can subdivide these microorganisms into the following groups (Vidali, 2001):

Aerobic - In the presence of oxygen

Examples of aerobic bacteria recognized for their degradative abilities are Pseudomonas, Alcaligenes, Sphingomonas, Rhodococcus, and Mycobacterium. These microbes have often been reported to degrade pesticides and hydrocarbons, both alkanes and polyaromatic compounds. Many of these bacteria use the contaminant as the sole source of carbon and energy (Vidali, 2001).

Anaerobic - In the absence of oxygen

Anaerobic bacteria are not as frequently used as aerobic bacteria. There is an increasing interest in anaerobic bacteria used for bioremediation of polychlorinated biphenyls (PCBs) in river sediments, dechlorination of the solvent trichloroethylene (TCE), and chloroform (Vidali, 2001).

Ligninolytic fungi

Fungi such as the white rot fungus Phanaerochaete chrysosporium have the ability to degrade an extremely diverse range of persistent or toxic environmental pollutants. Common substrates used include straw, saw dust, or corn cobs (Vidali, 2001).

Methylotrophs

Methylotrophs are aerobic bacteria that grow utilizing methane as a source of carbon and energy. The initial enzyme in the pathway for aerobic degradation, methane monooxygenase, has a broad substrate range and is active against a wide range of compounds, including the chlorinated aliphatics trichloroethylene and 1,2-dichloroethane (Vidali, 2001). In addition, it is well known that the best hydrocarbon degraders are bacteria from the genera *Nocardia*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Micrococcus*, *Arthrobacter*, *Corynebacterium*, *Achromobacter*, *Rhodococcus*, *Alcaligenes*, *Mycobacterium* and *Bacillus* and the fungi *Rhodotorulla*, *Fusarium*, *Aspergillus*, *Mucor*, *Penicillium*, *Candida* and *Sporobolomyces* (Balba *et al*., 1998; Vidali, 2001).

2.6.4. Environmental conditions in soil bioremediation

Microbial growth and activity are readily affected by nutrients, pH, available oxygen, temperature, and moisture. Although microorganisms have been also isolated in extreme conditions, most of them grow optimally over a narrow range, so that it is important to achieve optimal bioremediation conditions (Vidali, 2001).

Optimum environmental conditions for the degradation of oil pollutants in soil are shown in Table 13.

Table 13. Environmental conditions affecting degradation (Vidali, 2001).

Parameters	Condition required for microbial activity in soil	Optimum value for oil degradation
Soil moisture	25 – 28 % of water holding capacity	30 – 90 %
Soil pH	5.5 – 8.8	6.5 – 8.0
Oxygen content	Aerobic, minimum air-filled pore space of 10 %	10 – 40 %
Nutrient content	N and P for microbial growth	C:N:P = 100:10:1
Temperature (°C)	15 – 45	20 – 30
Contaminants	Not too toxic	Hydrocarbon 5–10% of dry weight of soil
Heavy metals	Total content < 2000 ppm	< 700 ppm
Type of soil	Low clay or silt content	

2.6.4.1. Nutrients

Although the microorganisms are present in contaminated soil, they cannot necessarily be there in the numbers required for bioremediation of the site. Their growth and activity must be stimulated. Biostimulation usually involves the addition of nutrients and oxygen to help indigenous microorganisms. These nutrients are the basic building blocks of life and allow microbes to create the necessary enzymes to break down the contaminants (Vidali, 2001).

Carbon is the most basic element of living forms and is needed in greater quantities than other elements. With hydrogen, oxygen, and nitrogen it constitutes about 95 % of the weight of cells (Table 14; Vidali, 2001).

In addition to carbon, hydrogen and oxygen, there is at least another 11 macro- and micronutrients that must be present in the soil in adequate amounts, forms and ratios to ensure microbial growth. These nutrients are: nitrogen, phosphorus, potassium, sodium, sulfur, calcium , magnesium, iron, manganese, zinc and copper. Among them, after carbon, nitrogen is a major limiting factor for growth, but small amounts of phosphorus are necessary to stimulate biodegradation (ATSDR, 1999; Vidali, 2001).

It was found that the atomic ratios of elements that are optimal for microbial activity are: 10:1 for ratio of carbon to nitrogen and 30:1 for ratio of carbon to phosphorus (Vidali, 2001).

Table 14. Composition of a microbial cell (Stainer *et al*. 1986).

Element	Percentage	Element	Percentage
Carbon	50	Potassium	1
Nitrogen	14	Sodium	1
Oxygen	20	Calcium	0.5
Hydrogen	8	Magnesium	0.5
Phosphorous	3	Chloride	0.5
Sulfur	1	Iron	0.2

2.6.4.2. Available oxygen

In biochemical redox reactions microorganisms oxidize hydrocarbons consuming available electron acceptor. Complete conversion of organic compounds to CO_2 and H_2O takes place through the mediation of aerobic microorganisms with consumption of molecular oxygen, and this process is called "mineralization" (NRC, 2000; Bossert, Bartha, 1984). Aerobic biodegradation occurs much faster than anaerobic (Vidali, 2001) and aerobic microorganisms degrade wider range of hydrocarbon compounds than anaerobic (Margesin, 2000). However, the amount of molecular oxygen in the soils is often limited and the efficiency of biodegradation is often determined by the activity of other microorganisms. In the absence of molecular oxygen biodegradation is performed by anaerobic microorganisms which use as electron acceptors NO_3^-, SO_4^-, Mn (IV) or Fe (III) ions (Langwaldt, Puhakka, 2000; Widdel, Rabus, 2001). These processes are slower than aerobic and often can give products of incomplete degradation that can be more toxic than the starting molecules (ATSDR, 1999).

The amount of oxygen in the soil can be increased by tilling or sparging air. In some cases, hydrogen peroxide or magnesium peroxide can be introduced in the environment.

2.6.4.3. Temperature

Biodegradation of hydrocarbons in soil and sediments occurs in a wide temperature range (Bossert, Bartha, 1984).

The influence of temperature on biodegradation is primarily reflected through the influence on the enzymatic activity of microorganisms. With increase in the temperature the activity of enzymes increases (it doubles for each 10 ° C). On the other hand, the lowering of the temperature decreases the activity of enzymes till the temperature of 0 ° C when it is stopped by freezing water in the soil solution (ATSDR; Vidali, 2001). The optimal temperature range for biodegradation in soils is 30 to 40 °C (Bossert, Bartha, 1984).

2.6.4.4. Soil moisture

The presence of water in the pores of soil is essential for normal life processes of microorganisms (Bossert, Bartha, 1984). The water content in the soil affects the movement of microorganisms, the influx of nutrients and removal of metabolic products (ATSDR, 1999). However, excessive moisture content limits the flow of molecular oxygen and contributes to the development of anoxic conditions. The moisture content in the soil which is considered optimal for biodegradation, is in the range of 50 to 80% of the total available pore volume in soil (ATSDR, 1999; Bossert, Bartha, 1984).

2.6.4.5. Soil pH

The soil pH regulates the solubility, mobility, and the availability of the contaminants. Additionally, the growth and activity of soil microorganisms are very much dependant on the soil pH. For example, fungi predominate under acidic conditions (pH < 7) while bacteria and actinomycetes predominate near pH = 7.0. A soil pH of 7.8 has been shown to be the optimum pH for microorganisms' growth (Dibble, Bartha, 1979).

3. Our work

3.1. Introduction

Biotic degradation of petroleum pollutants is a very important transformation process that, actually, represents degradation of organic substances by microorganisms. Considering the facts that during these process microorganisms consume some compounds from oil pollutants as a source of energy, and in that way remove them permanently from the environment, biodegradation is in fact a process of self-purification of the environment.

Bioremediation is a modern method in which the natural biodegradation ability of microorganisms is employed for the reduction of the concentration and/or toxicity of various chemical substances (Singh, Ward, 2004). It has been proven to be very efficient in the removal of crude oil and some oil derivatives (Ollivier, Magot, 2005), especially when biostimulation and bioaugmentation are applied (Beškoski *et al*., 2011).

The aim of this thesis was investigation and definition of the influence of biostimulation factors on microbiological degradation of main components in petroleum-type pollutants.

The most significant biostimulation factors chosen to be investigated in this research were: addition of biomass of microbial consortia, addition of biosurfactants and addition of nutritive substances. All results obtained from this experiment were planned to be compared with results from a parallel non-biostimulated biodegradation experiment.

Bearing in mind that degradation intensity of some aromatic compounds is still not completely resolved, the main components in petroleum-type pollutants selected to be investigated during the stimulated biodegradation were phenanthrene and its methyl-, dimethyl- and trimethyl isomers. We supposed that these results could give significant conclusions especially if the degradability of these aromatic compounds is compared with the degradability of *n*-alkanes which are the most abundant and the most degradable compounds in crude oils and oil pollutants.

Accordingly, a soil polluted with heavy fuel oil was selected as a most appropriate medium for all investigation defined in the aim of this thesis.

Considering the fact that the biodegradation of petroleum pollutants is a process that takes place in the environment over time, we thought that the analysis of samples collected at regular intervals over a period of 6 months could lead to important conclusions about the influence of biostimulation factors on microbiological degradation of main components in petroleum-type pollutants.

For soil samples following basic physical, chemical and microbiological analyses were chosen: analyses of minerals, bulk density, moisture, water holding capacity, pH, loss on ignition, total petroleum hydrocarbons, organic carbon, inorganic carbon, total nitrogen, available phosphorus, available potassium, total chemoorganoheterotrophs, and hydrocarbon degraders.

A wide range of instrumental and non-instrumental techniques is available for isolation and identification of petroleum pollutants in the environment. Selection of appropriate compounds to be analyzed, and selection of methods to be used during these analyses depend primarily on the type of oil pollution, type of compounds to be analyzed, the compartment of the environment to be analyzed and, finally, expected need for comparison of results.

We decided to employ classical extraction methods to isolate oil pollutants from the samples of the investigated soil. Separation by liquid chromatography on the column of the adsorbents was chosen to separate isolated extracts into the fractions of saturated hydrocarbons, aromatic hydrocarbons, alcohols and fatty acid methyl esters.

For analyzes of the main components in the petroleum-type pollutant expected to be analyzed in this thesis, as the most appropriate and the most sensitive, instrumental analytical methods were chosen: gas chromatographic method for analysis of *n*-alkanes and gas chromatographic - mass spectrometric method for analyses of the total saturated fractions, *n*-alkanes, isoprenoids, sterane and terpane biomarkers, phenanthrene and its methyl-, dimethyl- and trimethyl isomers.

3.2. Investigated soil

As already stated, a soil polluted with heavy fuel oil was considered the most appropriate medium for all investigation defined in the aim of this thesis. Accordingly, the soil from surroundings of an energy power plant in New Belgrade (Serbia) was chosen as the most suitable for these criteria. Due to a break-down of the energy power plant facilities, this soil had been polluted with mazut and sediment from a mazut reservoir for a year. Because of that, we considered that this soil might contain high concentration of oil pollutant as well as a bioremediation potential high enough to satisfy the goals set by the aim of this thesis.

3.3. Research plan

Bearing in mind the objective of this thesis and the specific characteristics of the samples investigated, the following research plan was made:

1. Basic chemical, physico-chemical and microbiological analyses of the polluted soil.

2. Formation of the biremediation pile (biopile) using heavy fuel oil polluted soil.

3. Basic chemical, physico-chemical and microbiological analyses of the biopile.

4. Formation of the control pile.

5. Analysis of total petroleum hydrocarbons in the composite samples from the biopile.

6. Identification of microorganisms in cultures isolated from the polluted soil.

7. Determination of the number of microorganisms in the polluted soil and in the substrate for bioremediation.

8. Preparation and production of the microbial consortium to be used for reinoculation.

6. Biostimulation, bioaugmentation and reinoculation of the biopile during the period of 6 months.

10. Sampling from the biopile during the period of 6 months.

11. Isolation of total extractible organic substance from the samples collected from the biopile during the period of 6 months.

12. Isolation of alkane, aromatic, alcohol and fraction of fatty acid methyl esters from the isolated extracts using column chromatography.

13. Gas chromatographic - mass spectrometric analyses of the total saturated fractions.

18. Gas chromatographic - mass spectrometric analyses of phenanthrene and its methyl-, dimethyl- and trimethyl isomers.

19. Separation of *n*-alkanes from branched and cyclic alkanes by karbamide adduct.

20. Gas chromatographic analysis of *n*-alkanes separated by karbamide adduct.

21. Interpretation of results.

4. Experimental and results

Heavy fuel oil (mazut; Figure 22) is a low quality, heavy (chain length 12 – 70 C atoms) residual fuel oil (ASTM D396-09a; ISO 8217). In the United States and Western Europe mazut is blended or broken down with the end product being diesel. In Eastern Europe, however, mazut is used as a source of heating fuel. The long-term storage and use of mazut can leave hydrocarbon residues in the reservoir itself, with a high content of different mechanically-derived contaminants and water in the reservoir; this can potentially lead to dangerous pollution of the living environment (particularly soil) during cleaning, when there is a serious threat to underground water.

Figure 22. Storage of a heavy fuel oil.

4.1. The investigated soil

The heavy fuel oil polluted soil was excavated contaminated soil from an energy power plant (New Belgrade, Serbia). Due to a breakdown of the energy power plant facilities, the soil had been polluted with heavy fuel oil (mazut) and sediment from a heavy fuel oil reservoir for a year.

The level of contamination of soil investigated in this research, expressed through a set of parameters, including the content of total petroleum hydrocarbons, as well as basic chemical, physico-chemical and microbiological characteristics of the polluted soil are shown in Table 15.

Table 15. Characteristics of the mazut-polluted soil.

Characteristics	Results
Minerals	Quartz > clay minerals (clay mica-illite, kaolinite, montmorillonite) > calcite ≈ feldspar > dolomite > chlorites
Content of sand + clay [%]	61 + 35
Bulk density [kg m^{-3}]	1538 ± 100[a]
Moisture [%]	17.8 ± 0.3
WHC[b] [%]	18.3 ± 1.6
pH	7.3 - 7.5
Loss on ignition [%]	8.5 ± 1.2
TPH[c] [g kg^{-1}]	12.4 ± 0.5
Organic carbon [%]	1.79 ± 0.06
Inorganic carbon[d] [%]	1.45 ± 0.05
Total nitrogen [%]	0.16 ± 0.03
Available phosphorus [mg kg^{-1}]	47 ± 3
Available potassium [mg kg^{-1}]	18 ± 3
TC[e] [CFU[f] g^{-1}]	1.2×10^6
HD[g] [CFU g^{-1}]	2.7×10^5

[a]Error of determination. In all other results: ± standard deviation for five measurements; [b]Water Holding Capacity; [c]Total Petroleum Hydrocarbons; [d]Calculated on the basis of measured carbonates; [e]Total Chemoorganoheterotrophs; [f]Colony Forming Unit; [g]Hydrocarbon Degraders

4.2. Formation of the pile

The biopile (EPA, 2004) for bioremediation was prepared on a waterproof asphalt surface of approximately 1500 m^2 and with a 1 % sloping gradient

The mazut polluted soil (approximately 150 t; 210 m^3) was uniformly distributed over 300 m^3 of not rinsed ungraded sand from the Sava River (settlement Ostružnica, Serbia). River sand was added as a bulking and porosity increasing material.

The sawdust from poplar, beech, and oak (approximately 60 m^3) was added in order to increase the retention water capacity, but as alternative additional carbon source as well. To ensure homogeneity, the components were mixed three times with a front-

end loader, and finally, raked using a tractor fitted with a harrow. The entire homogenized material (volume of approx. 600 m^3), defined as a *substrate for bioremediation*, was then formed into a biopile shape with dimensions of 75 × 20 × 0.4 m (length, width, height), with bulldozers.

A perimeter drain enclosed the entire treatment area and directed all leachate and runoff to a joint vessel, from which they were pumped back onto the biopile.

The basic chemical, physico-chemical and microbiological characteristics of the substrate for bioremediation immediately after initial mixing are shown in Table 16.

Table 16. Characteristics of the substrate for bioremediation.

Characteristics	Results
Minerals	Quartz >> feldspar ≈ calcite ≈ clay minerals (clay mica-illite, kaolinite, montmorillonite) > chlorites > dolomite
Content of sand + clay [%]	77 + 17
Bulk density [kg m^{-3}]	1612 ± 100[a]
Moisture [%]	13.9 ± 0.6
WHC[b] [%]	26.1 ± 1.4
pH	7.4 - 7.6
Loss on ignition [%]	9.7 ± 1.1
TPH[c] [g kg^{-1}]	5.2 ± 0.2
Organic carbon [%]	2.44 ± 0.08
Inorganic carbon[d] [%]	0.67 ± 0.06
Total nitrogen [%]	0.12 ± 0,01
Available phosphorus [mg kg^{-1}]	25 ± 3
Available potassium [mg kg^{-1}]	10 ± 2
TC[e] [CFU[f] g^{-1}]	9.7 × 10^5
HD[g] [CFU g^{-1}]	5.6 × 10^4

[a]Error of determination. In all other results: ± standard deviation for five measurements; [b]Water Holding Capacity; [c]Total Petroleum Hydrocarbons; [d]Calculated on the basis of measured carbonates; [e]Total Chemoorganoheterotrophs; [f]Colony Forming Unit; [g]Hydrocarbon Degraders

4.3. Preparation and production of the microbial consortium used for reinoculation

A consortium of microorganisms was isolated from the polluted soil by enrichment in 200 mL volumes of mineral medium (10 vol. %; Loser *et al*., A consortium of microorganisms was isolated from the polluted soil 1998) containing mazut (2 g L^{-1}) as the only energy and carbon source in Erlenmeyer flasks (1 L).

Suspensions of the microbial consortium were used to seed four Erlenmeyer flasks (5 L), each with 2000 mL of the medium containing 23 g of nutrient broth (Torlak, Belgrade, Serbia); 100mL of soil extract (http://www.ccap.ac.uk/media/recipes/SE.htm); and 20 g of mazut. Commercial non-toxic and readily biodegradable surfactants, BioSolve CLEAR supplied by The Westford Chemical Corporation (Westford, MA, USA) were used as surface active agents to solubilize mazut. The original solution supplied by the manufacturer was used at a concentration of 1 mL L^{-1}.

The growth conditions were as follows: temperature 28 °C; 120 rpm; pH 7.0 (adjusted with 1 M HCl or NaOH). Duration of growth was 96 h.

The microbial population from all four flasks was used to inoculate (approx 1 % vol.) a bioreactor (total volume 1000 L) with a working volume of 800 L, producing the microbial consortium. The medium used was: 12 g L^{-1} meat peptone (Torlak, Belgrade, Serbia); 0.2 g L^{-1} $(NH_4)_2HPO_4$; 25 g L^{-1} of autoclave-sterilized soil sampled from undisturbed deciduous woodland; Bio-Solve CLEAR original solution (1 mL L^{-1}); and 10 g L^{-1} mazut.

The growth conditions were: non sterile, 25 °C, aeration and agitation 0.70 volume of air/volume of medium min^{-1}, pH 7.0 (adjusted with 10 M HCl or NaOH), duration 48 h and sunflower oil (1 mL L^{-1}) as antifoam.

4.4. Identification of microorganisms

Analytical profile index (API-Biomerieux) tests were used for identification of microorganisms.

Api 20NE tests were used for identification of gram-negative non-*Enterobacteriaceae.*

Api Rapid 20E tests were used for identification of *Enterobacteriaceae.*

API Coryne tests were used for identification of *Corynebacteria* and coryne-like organisms.

Api Bacillus 50 CHB/E were used for identification of *Bacillus*

All tests were used according to the manufacturer's instructions.

Analytical profile index (API-Biomerieux) tests conducted with isolated cultures of microorganisms identified: *Pseudomonas aeruginos, Rhodococcus sp., Pseudomonas sp., Pseudomonas Fluorescens, Sphingomonas Paucimobilis, Pseudomonas luteola, Achromobacter denitrificans, Stenotrophomonas maltoplilia* and *Aeromonas hydrophila.*

4.5. Determination of the number of microorganisms

The medium used for determination of total chemoorganoheterotrophs was nutrient agar (Torlak, Belgrade, Serbia).

The medium used for determination of hydrocarbon degraders was the mineral base medium (Löser *et al*., 1998) containing 2 g of standard D2 diesel fuel in 1 L of medium (Bossert *et al*., 2002).

The medium used for determination of yeast and molds was malt agar (Torlak, Belgrade, Serbia).

The number of microorganisms was determined by plating appropriate serial dilutions on agar plates incubated at 28 °C.

4.6. Applied conditions of biostimulation

After formation the biopile was sprayed once a month with the biomass of microbial consortia isolated from the heavy fuel oil – contaminated soil (re-inoculation) and nutritive substances (biostimulation).

Biomass and nutritive substances were sprayed over the biopile using an agricultural sprayer fitted to a tractor with a trailer unit. Biomass concentration was 1.44×10^7 cells mL^{-1}.

In order to achieve an optimal ratio of C/N/P/K the biopile was sprayed with a solution of dissolved ammonium nitrate (as a source of N), diammonium hydrogen phosphate (as a source of P and N) and potassium chloride (as a source of K). Spraying was also expected to result in the required moisture level in the biopile.

Biosurfactant of biosolve type was applied on the biopile at a concentration of 70 mL of the original solution per cubic meter of soil.

During bioremediation, the biopile was watered, turned and mixed each 2 weeks to maintain the required moisture and aeration levels.

The values of basic parameters at the beginning bioremediation (after mixing, watering, biostimulation and re-inoculation) are given in Table 17.

Table 17. Values of basic parameters at the beginning bioremediation (after mixing, watering, biostimulation and re-inoculation)

Characteristics	Results
Moisture [%]	15.4 ± 0.5
WHC [%][a]	26.4 ± 1.7
pH	7.3 - 7.5
Loss on ignition [%]	9.9 ± 1.1
Organic carbon [%]	2.46 ± 0.04
Inorganic carbon [%][b]	0.65 ± 0.03
Total nitrogen [%]	0.25 ± 0.03
Available phosphorus [mg kg^{-1}]	241 ± 5
Available potassium [mg kg^{-1}]	24 ± 3
Time of bioremediation [day]	0
TC[c] [CFU[d] g^{-1}]	2.0×10^6
HD[e] [CFU g^{-1}]	7.2×10^4
HD [%][f]	4

[a]Water Holding Capacity; [b]Calculated on the basis of measured carbonates; [c]Total Chemoorganoheterotrophs; [d]Colony Forming Unit; [e]Hydrocarbon Degraders; [f] Share of HD within the TC.

4.7. Control pile

At the beginning of the study, immediately after mixing, but before the addition of sawdust, biomass, nutrient substances, and biosurfactant, approximately 10 m^3 of the biopile mixture was set aside on the same waterproof asphalt surface, to be used as a control pile.

The complete analytical procedure that was applied to the samples was also applied to the control samples during an independent parallel non-biostimulated biodegradation experiment (Novaković *et al*., 2012; Ramadan *et al*., 2012).

4.8. Environmental conditions during the experiment

The average daily temperature during the six month experiment was 7.6 ± 6.3 °C (in the range from -2.3 to 23.5 °C).

Although the experiment was conducted in autumn and winter, due to the intensive microbiological activity, the temperature of the soil was stable, above 25 °C.

After each treatment the biopiles were covered with polyethylene foil to prevent reduction of the temperature and direct influence of precipitations and low temperatures (weather conditions) on the bioremediation substrate.

4.9. Analysis of minerals

The minerals in the samples were detected and determined semiquantitatively by roentgen diffraction analysis powder technique. X-ray diffractometer Philips (Netherlands) type PW 1050/ 00 was used with Cu Ka1 Ni-filtered radiation.

Preparation of samples: native, with glycerol and ignited.

Results were interpreted using Material Phases Data System program and ASTM Joint Committed on Powder Diffraction Standards card files.

The concentrations of heavy metals in all samples were below the reference values (Dutch, Standards, 2000) and because of that are not stated.

4.10. Analysis of total petroleum hydrocarbons

The content of TPH in the soil was determined using extraction as per method ISO 16703 and gravimetrical determination in accordance with DIN EN 14345.

Gas chromatographic analyses, as stated in the ISO standard, were used for quality analysis and comparison of results. The instrument used was: Agilent 4890D gas chromatograph with flame ionization detector. The column was HP-1MS 30 m x 0.25 mm, with hydrogen as a carrier gas. Injector temperature was 250 °C. The temperature program used was: initial temperature 40 °C, then heating till the temperature 285 °C at the rate 9 °C min-1 and finally isothermal at 285 °C for 12 min.

The results were controlled using certified European Reference Materials and a standard mixture of *n*-alkanes, pristane and phytane to compare the retention times.

Mass of samples, dilution and conditions for GC were identical for all samples.

4.11. Basic chemical, physico-chemical analyses

The basic chemical, physico-chemical characteristics analyzed in mazut polluted soil and substrate for bioremediation were: content of clay and sand, bulk density, WHC, moisture (for original material), pH, organic and inorganic carbon, nitrogen, and available phosphorus and potassium using standard methods (Wilke, 2005; Pansu, Gautheyrou, 2006).

4.12. Sampling

During the 6-month interval, the samples were taken five times (07/09/2009, 06/10/2009, 09/11/2009, 12/01/2010, and 18/03/2010).

Composite samples for analyses were taken from the biopile and control pile by ''zig-zag'' sampling with an Eijkelkamp auger soil sampler from 30 random places on the substrate for bioremediation and from 5 random places on the control pile. The composite samples (approximately 20 L from the biopile, and 10 L from the control pile) were sieved (1 mm grid), collected in stopped glass jars, and stored at 4 °C. Analyses were conducted within 12–24 h after sampling (Paetz, Wilke, 2005).

Samples taken from the substrate for bioremediation (the biopile that was treated with sawdust, biomass, nutrient, and biosurfactants) were marked M1 – M5:

M1 – 07/09/2009,
M2 – 06/10/2009,
M3 – 09/11/2009,
M4 – 12/01/2010 and
M5 – 18/03/2010.

4.13. Isolation of extracts

Organic substance from in total 5 soil samples was extracted with chloroform (HPLC, J.T., USA) using a Soxhlet apparatus (Figure 23).

From these extracts the most part of the solvent was removed using rotary vacuum evaporator, and the residue was quantitatively transferred to the glass weighing bottles using Pasteur pipettes. The rest of the solvent was evaporated in the flow of air without heating.

Analyses of extracts are schematically showed in Figure 24.
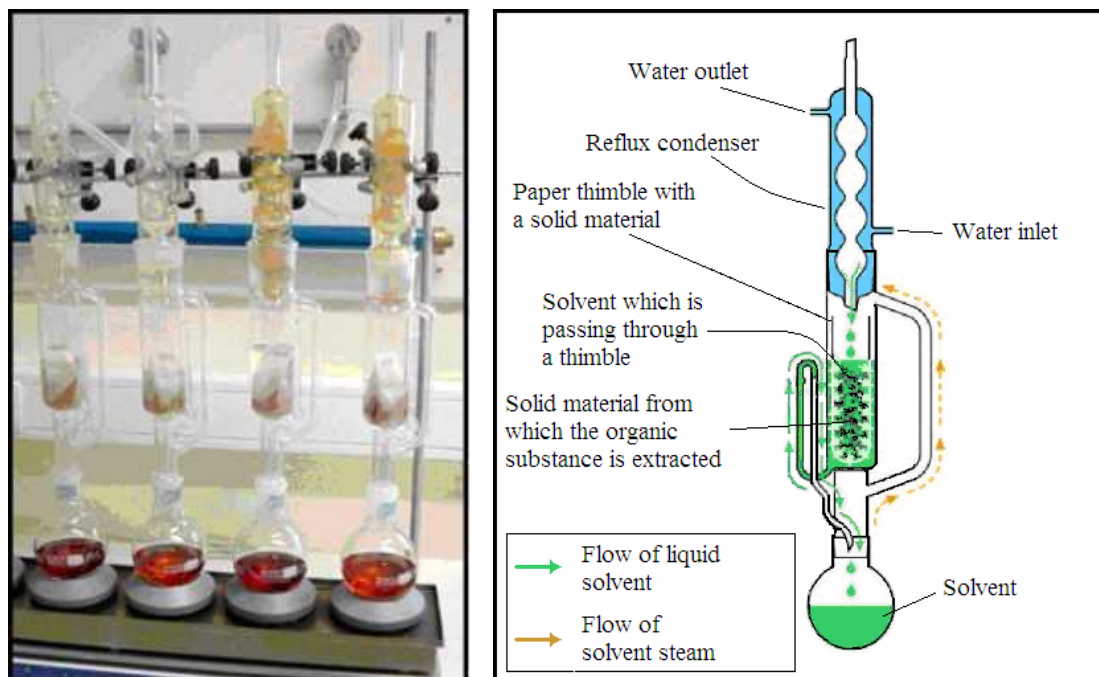
Figure 23. Soxhlet apparatus.

4.14. Isolation of alkane, aromatic, alcohol and fraction of fatty acid methyl esters

Fraction of saturated hydrocarbons, aromatic hydrocarbons, alcohols and fatty acid methyl esters were isolated from chloroform extracts of all samples. The extracts were first saponified, then neutralized and finally separated into four fractions (saturated hydrocarbons, aromatic hydrocarbons, alcohols and fatty acid methyl esters) by column chromatography.

1. **Saponification:**

Approximately 5 mg of a chloroform extract sample was measured into the appropriate glass container. 500 µl of 5 % solution of KOH in methanol (with 1 % of water) was added to the organic extract sample and the solution was left overnight at room temperature.
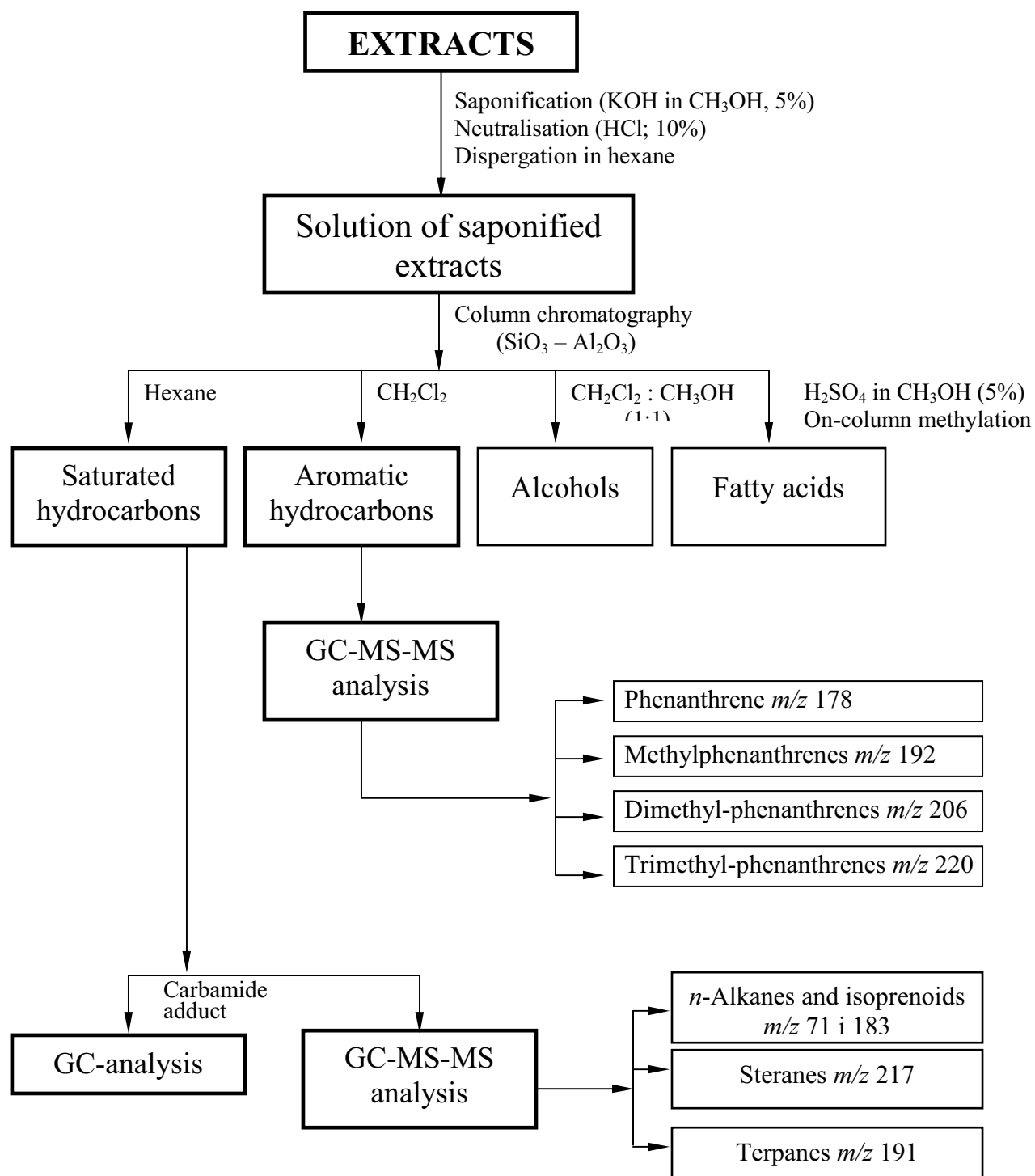
Figure 24. Scheme of the analyses of organic extracts.

2. Neutralization

After left over night, 130 microliters of HCl solution (10 %) were added into the saponified solution. Thereafter, the entire mixture was transferred in a glass container of approximately 40 ml, using the mixture of solvents methylene chloride – methanol (1:1).

Solvent and water were removed in a flow of nitrogen, with slight heating. Finally, 500 µl of CH_2Cl_2 with 1 % of CH_3OH, and 20 cm^3 of *iso*-hexane were added.

The solution was dried with anhydrous Na_2SO_4.

3. Chromatographic separation

- *Preparation of the column*

At the bottom of the glass column with diameter of approximately 1 cm and height of approximately 15 cm, a small Teflon filter was set. Above the filter, 13 mm high layer of aluminum oxide adsorbent ("basic", previously activated at 220 °C and deactivated with 4 % water) was formed. Above the aluminum oxide layer, 33 mm high layer of silicon dioxide (previously activated at 220 °C and deactivated with 10 % of water) was formed. Finally, on the top of the adsorbent column, 4 mm of Florisil (previously activated at 675 °C) was placed.

- *Chromatographic separation*

The entire amount of previously saponified and neutralized sample was transferred quantitatively on the top of the adsorbing column.

First fraction (saturated hydrocarbons) was eluted with 10 ml of hexane.

Second fraction (aromatic hydrocarbons, "keto" fraction) was eluted with 10 ml of methylene-chloride.

Third fraction (polar, "alcohol" fraction) was eluted with 10 ml of a mixture methylene-chloride – methanol (1:1).

Fourth fraction ("fatty acid" fraction) was eluted using 5 % solution of sulfuric acid in methanol four times in portions of 2 ml with intermissions of 20 minutes. Fatty acids were eluted in the form of their methyl esters.

From the first three fractions the solvent was removed in the flow of nitrogen.

Into the solution of the forth fraction approximately 30 ml of aqueous solution of NaCl (20 %) and 2 ml of hexane were added. The organic layer at the top was separated using pipette after energetic stirring. Procedure of addition/separation of hexane layer was repeated several times. From combined hexane extracts the excess of the solvent was removed in the flow of nitrogen.

4.15. Gas chromatographic - mass spectrometric (GC-MS) analyses

Hydrocarbons were analyzed by the gas chromatography – mass spectrometry (GC–MS) techniques. An Agilent 7890N gas chromatograph fitted with a HP5-MS capillary column (30 x 0.25 mm, 0.25 µm film; temperature range: 80 °C for 0 min; then 2 °C min-1 to 300 °C and held for 20 min) with helium as the carrier gas (flow rate 1 cm3 min-1) was used. The GC was coupled to a Hewlett-Packard 5972 MSD (Figure 23) operated at 70 eV in the 45–550 scan range. Preliminary analyses of the investigated samples were conducted in the full-scan mode. Detailed analyses of the target compounds were conducted in the single-ion monitoring mode (SIM), comprising the following ion chromatograms: 178 (phenanthrene), 192 (methylphenanthrenes), 206 (dimethyl-phenanthrenes), and 220 (trimethyl-phenanthrenes). Peaks of the phenanthrene, methyl-phenanthrenes, and dimethyl-phenanthrenes were identified according to organic geochemical literature data (e.g., Peters *et al*., 2005), or based on the total mass spectra, using mass spectra databases (NIST/EPA/NIH mass spectral library NIST2000, Wiley/NBS registry of mass spectral data, 7th ed., electronic versions). The peaks of trimethyl-phenanthrenes were labeled using the hypothesized elution order according to Stojanović *et al*., (2007). Phenanthrene and alkyl phenanthrene parameters were calculated from GC – MS chromatogram peak areas (software: MSD ChemStation E.02.00493; Copyright 1989 – 2000 Agilent Technologies, INC.).

Figure 24. Gas chromatograph Agilent 7890N and a Hewlett-Packard 5972 MSD.

Total ion chromatograms (TIC) of saturated fractions are shown in figure 25. Mass fragmentograms of phenanthrene (m/z 178), methyl-phenanthrenes (m/z 192), dimethyl-phenanthrenes (m/z 206) and trimethyl-phenanthrenes (m/z 220) are shown in figure 26.
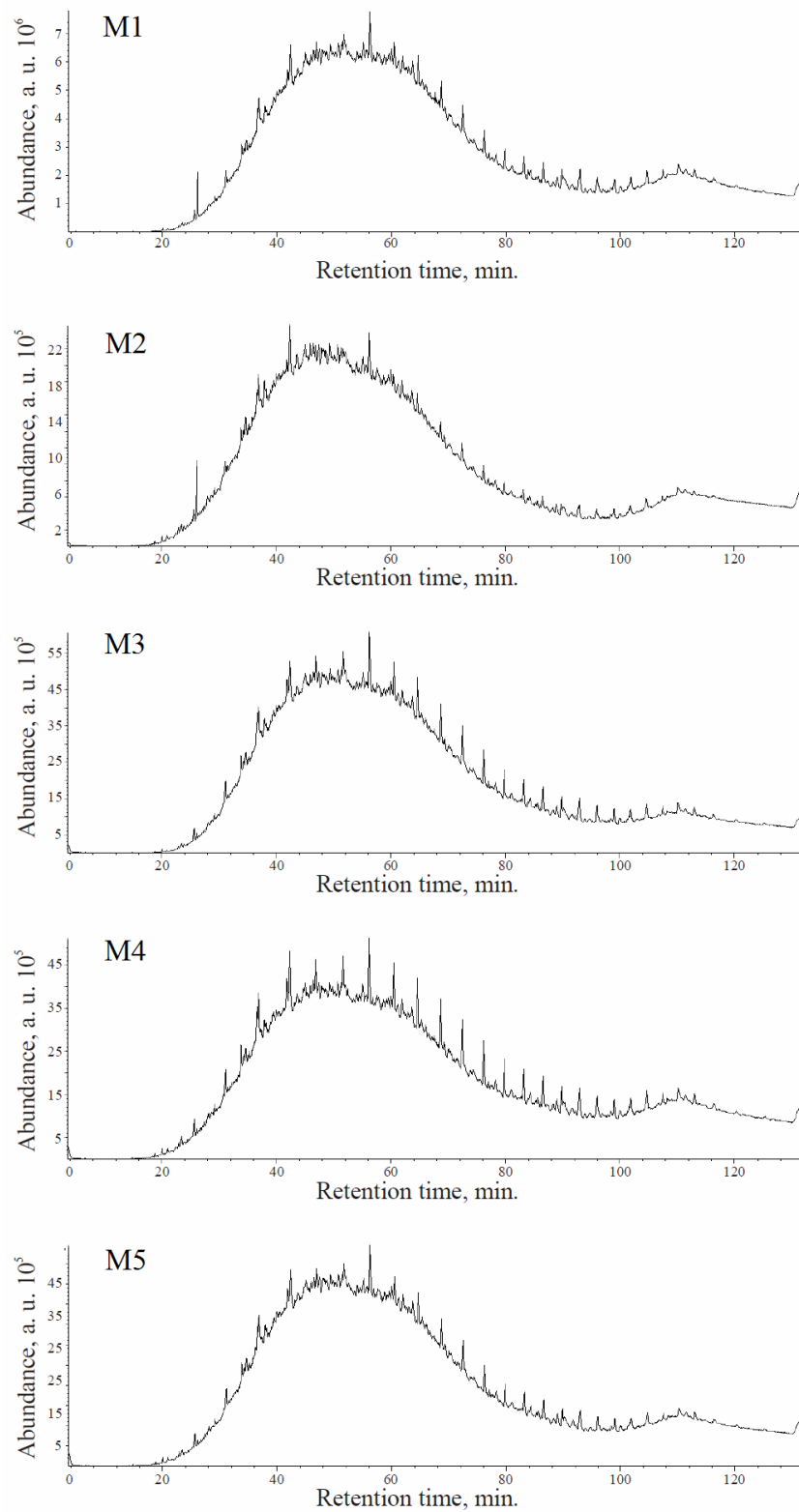
Figure 25. Total ion chromatograms (TIC) of saturated fractions isolated from extracts from soil samples M1 – M5.
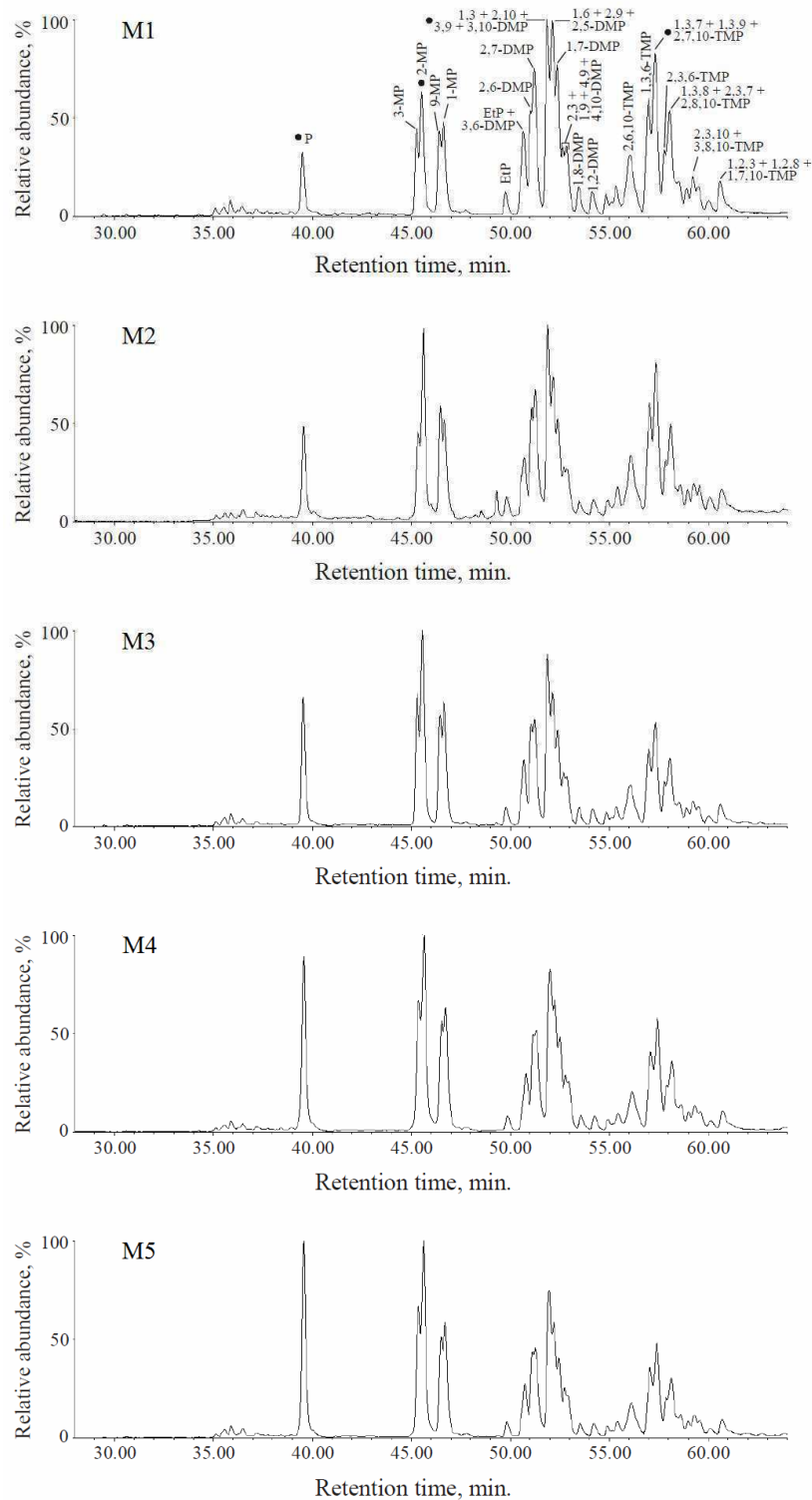
Figure 26. Mass fragmentograms of phenanthrene (m/z 178), methyl-phenanthrenes (m/z 192), dimethyl-phenanthrenes (m/z 206) and trimethyl-phenanthrenes (m/z 220) of aromatic fractions isolated from extracts from soil samples M1 – M5.

4.16. Separation of *n*-alkanes from branched and cyclic alkanes by karbamide adduct

Fraction of saturated hydrocarbons isolated by column chromatography is a mixture of cyclic and branched alkanes and *n*-alkanes. Since in this fraction (usually in very small quantities) are the most important analytical biomarkers, steranes and triterpanes, it is necessary to isolate them, that is, in a way, concentrated, and then analyze the instrumental techniques.

Due to the fact that *n*-alkanes can form inclusion adduct with urea, it is possible to isolate them from the mixture of saturated hydrocarbons. Considering the fact that good separation can be achieved only for higher *n*-alkanes, this procedure is not used for quantitative determination of content of *n*-alkanes in the fraction of saturated hydrocarbons.

In a glass centrifuge tube a sample of saturated hydrocarbons (weight no higher than 10 mg) was dissolved in 2 - 4 cm^3 of mixture petrol-ether/acetone (2:1, v/v).

To this solution a saturated solution of urea in methanol (approximately 0.5 cm^3) was added, dropwise, with stirring. The precipitate which was created consisted of carbamide adduct of n-alkanes, with some higher *iso-* and *anteiso*-alkanes.

The solvent was removed in the flow of nitrogen without heating.

This procedure was repeated four times.

The rest of Petroleum ether (3 - 5 cm^3) was added to the remainder and the nonadduct was dissolved. After centrifugation for 5 minutes at 300 rpm, the solution above the precipitate was carefully transferred to the glass weighing bottles using Pasteur pipettes.

This procedure of dissolution – separation of nonadduct was repeated in the same way four times.

From the combined solutions of nonadduct solvent was evaporated in the flow of nitrogen without heating.

Precipitate which remained in the centrifuge tubes was first dried in the flow of nitrogen and then dissolved in approximately 3 cm^3 of water. From the resulting aqueous emulsion *n*-alkanes were extracted with petroleum ether. Approximately 3 cm^3 of petroleum ether was added to the water emulsion. The mixture was stirred well using

vibromixer. After differentiation of water and petroleum ether layers, petroleum ether solution of *n*-alkanes was carefully transferred using Pasteur pipettes to the glass weighing bottles.

Extraction of *n*-alkanes was repeated three times. From combined petroleum ether solutions of *n*-alkanes the solvent was removed in the flow of nitrogen without heating.

4.17. Chromatographic analysis of *n*-alkanes

The *n*-alkanes in urea adducts were analyzed by gas chromatography. Gas chromatographic analyses were conducted on a GC8000 gas chromatograph (Fisons Instruments, Italy) equipped with a 30 m x 0.25 mm i.d. X 0.25 μm film ZB1 fused silica capillary column (Phenomenex, Germany). Chromatographic conditions were as follows: 1 μL split/splitless injection at 80 °C oven temperature (injector temperature 270 °C, splitless time 60 s), 3 min hold, then programmed at 5 °C/min to 300 °C. Hydrogen carrier gas velocity was 40 cm s^{-1}.

Gas-chromatograms of carbamide adducts from saturated fractions are shown in figure 31.

4.18. Preparation of laboratory glassware and chemicals

In order to avoid any possible contamination of samples during experimental work a special attention was given to the preparation of laboratory glassware and chemicals. Glassware was carefully washed before use, then rinsed with tap water and distilled water and dried in an oven at 105 °C.

All reagents used (except from the solvents used for instrumental techniques) were analytical (*p.a.*) grade. All solvents were distilled prior to use and used without any further purification.

The solvents used for instrumental techniques were the highest purity solvents available, suitable for gas - chromatographic applications.
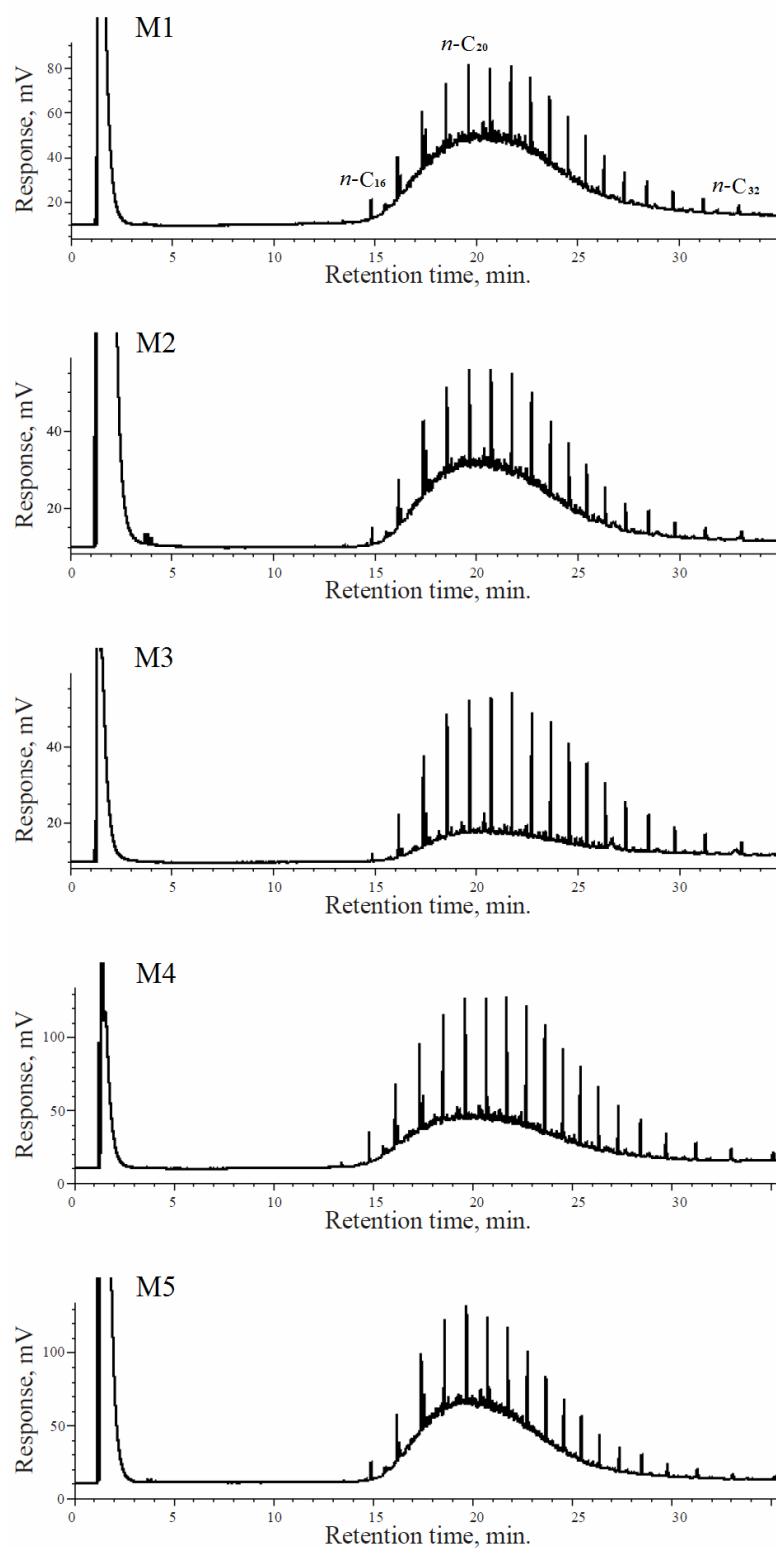
Figure 27. Gas-chromatograms of carbamide adducts from saturated fractions isolated from extracts from soil samples M1 – M5.

5. Discussion about results

5.1 Basic characteristics of the mazut-contaminated soil

As already explained in the section 2.6.4., microbial growth and activity in soils are readily affected by nutrients, pH, available oxygen, temperature, and moisture and most microorganisms grow optimally over a narrow range. Other factors important for proper growth and activity of microorganisms include presence of heavy metals and other contaminants which might act as growth and activity inhibitors, structure of the soil (porosity and bulk density) and content of sand, clay and minerals in the soil. In order to investigate if the selected soil polluted with heavy fuel oil is the appropriate medium for all investigation defined in the aim of this thesis, numerous physical, chemical and microbiological analyses were conducted (Chapter 4).

From a mineralogical point of view, the condition required for microbial activity in soil is a low clay or silt content (Vidali, 2001). Based on the concentration of sand and clay ((61 + 35) %; Table 15), this soil is classified as clayified sand (Wilke, 2005; Pansu, Gautheyrou, 2006). According to these results it can be concluded that the conditions for microbial activity in this soil would be better if the content of clay in it was lower.

Soil moisture required for microbial activity in soil is 25 – 28 % of water holding capacity, while the optimum value for oil degradation is 30 – 90 % (Vidali, 2001). According to the value for moisture content ((17.8 ± 0.3) % of water holding capacity; Table 15) it can be concluded that the moisture of the soil investigated is below the threshold value required for microbial activity in soil. Accordingly, it can be concluded that additional spraying is needed to achieve the optimum moisture level for oil degradation in this soil.

The growth and activity of soil microorganisms are very much dependant on the soil pH. Soil pH value required for microbial activity is within the 5.5 – 8.8 range with the optimum value for oil degradation in the 6.5 – 8.0 range (Vidali, 2001). According

to the results in the Table 15, it can be concluded that the pH value of the investigated soil (7.3 - 7.5) is within the optimum range for oil degradation.

The term "total petroleum hydrocarbons" (TPH) is generally used to describe the measurable amount of petroleum-based hydrocarbons in the environment. In this thesis the content of THP is determined using standard methods ISO 16703 and DIN EN 14345. The measured value for TPH in the investigated soil is (12.4 ± 0.5) g kg^{-1} (Table 15). According to the Dutch standard, which is one of the most often cited standards with soil criteria for evaluating the severity of contamination, based on the TPH value this soil is categorized as a soil that require immediate cleanup. According to the same Dutch standard, the background concentration in soil or detection limit is defined as 100 mg kg^{-1}. Based on this result it can be concluded that this soil has a content of TPH high enough for monitoring the bioremediation of soil polluted with heavy fuel oil.

Concentration of total chemoorganoheterotrophs in the soil investigated was 1.2×10^6 CFU g^{-1} (Table 15). The proportion of hydrocarbons degrading bacteria comprised approximately 20 % of the total number of bacteria in the mazut polluted soil (Table 15). This result indicated presence of an intensive biodegradative processes and, additionally, large bioremediation potential of the investigated soil.

The basic nutrients for microbial growth and activity are carbon, nitrogen, phosphorus and potassium and an optimum ratio of C/N/P/K is 100:10:1:0.1 (Vidali, 2001). According to the results given in Table 15, it can be concluded that the ratio of C/N/P/K in this soil is far from optimum and that for the effective bioremediation process the addition of nutrients is needed to help the growth and development of indigenous microorganisms.

The concentration of heavy metals in the mazut polluted soil was below that of reference values (Dutch Standards, 2000), which indicated that this soil did not contain these microorganism growth and activity inhibitors (data not shown).

All these results indicated that the chosen mazut polluted soil was potentially a good substrate for monitoring the bioremediation of petroleum pollutant: the soil pH was in the optimum range, the TPH content was high enough with presence of an intensive biodegradative processes and, accordingly, large bioremediation potential.

Furthermore, the content of heavy metals which are microorganism growth and activity inhibitors, in this mazut polluted soil was below that of reference values.

On the other hand, some parameters of this soil were not satisfactory for bioremediation purposes: the conditions for microbial activity in this soil would be better if the content of clay in it was lower, the moisture of the soil investigated was below the threshold value required for microbial activity in soil and the ratio of C/N/P/K in this soil was far from optimum.

According to these results it can be concluded that with an improvement of the soil structure and with biostimulation by addition of water and nutrients this soil could become an appropriate medium for all investigation defined in the aim of this thesis.

5.2. Basic characteristics of the substrate for bioremediation

The basic chemical, physico-chemical and microbiological characteristics of the substrate for bioremediation immediately after initial mixing are shown in Table 16.

Comparing with the content of sand and clay in the mazut polluted soil ((61 + 35) %; Table 15), a higher content of sand and a lower content of clay in the substrate for bioremediation are noticeable ((77 + 17) %; Table 16). It can be concluded that mixing of the mazut polluted soil with the river sand significantly lowered the content of clay. Accordingly, it can be concluded that the substrate for bioremediation has a good mineralogical composition for the microbial activity in soil regarding oil biodegradation (low clay or silt content; Vidali, 2001).

The water holding capacity, porosity and ability to be mixed increased during formation of the substrate for bioremediation (Tables 15 and 16) due to the addition of sawdust and raw river sand.

pH value of the substrate for bioremediation (7.4 - 7.6; Table 16) remained in the optimum range for oil degradation (6.5 – 8.0; Vidali, 2001).

Decrease in the value of TPH comparing the mazut polluted soil (12.4 ± 0.5 g kg^{-1}; Table 15) with the substrate for bioremediation (5.2 ± 0.2 g kg^{-1}; Table 16) can be explained as a consequence of dilution.

The ratio of C/N/P/K in this soil remained far from the optimum (Table 16) leading to the conclusion that for the effective bioremediation process the addition of nutrients is needed to help the growth and development of indigenous microorganisms. The difference between the mazut polluted soil and the substrate for bioremediation in the concentration of bacteria is noticeable. The concentration of total chemoorganoheterotrophs decreased from 1.2×10^6 CFU g^{-1} in the mazut polluted soil (Table 15) to 9.7×10^5 CFU g^{-1} in the substrate for bioremediation. As a consequence, the concentration of hydrocarbon degraders decreased from 2.7×10^5 CFU g^{-1} in the mazut polluted soil (Table 15) to 5.6×10^4 CFU g^{-1} in the substrate for bioremediation. Contrary to the mazut polluted soil which contained high proportion of hydrocarbon degrading bacteria (approximately 20 % of the total number of bacteria; Table 15), the substrate for bioremediation contained only approximately 6 % of hydrocarbon degrading bacteria (Table 16). All these differences in the proportion of hydrocarbon degrading bacteria between the mazut polluted soil and the substrate for bioremediation were a consequence of dilution.

The initial concentration of hydrocarbons degrading bacteria in the substrate for bioremediation before inoculation was 5.6×10^4 CFU g^{-1} (Table 16). In previous studies, it was confirmed that when the population of hydrocarbons degrading bacteria is less than 10^5 CFU g^{-1} of soil-substrate, no significant degree of bioremediation will occur, and, therefore, it is necessary to increase the number of these microorganisms (Forsyth *et al*., 1995). Based on these results it was concluded that the initial concentration of hydrocarbons degrading bacteria in the substrate for bioremediation before inoculation was not sufficient for significant degree of bioremediation. Because of that the biopile was re-inoculated each 30 d during the experiment.

The concentration of heavy metals in the substrate for bioremediation was also below that of reference values (Dutch Standards, 2000), which indicated that during the process of preparation of biopile the substrate for bioremediation was not contaminated with these microorganism growth and activity inhibitors.

According to all these results it can be concluded that due to the addition of sawdust and raw river sand to the mazut polluted soil some parameters improved as a consequence of dilution: the water holding capacity, porosity and ability to be mixed increased while the clay content became lower. On the other hand, the pH value remained within the optimum range for oil degradation. However, some parameters (C/N/P/K ratio and proportion of the hydrocarbon degrading bacteria) were not within the optimum range for oil degradation leading to the conclusion that for the effective bioremediation process of the substrate for bioremediation an increase in the number of hydrocarbons degrading microorganisms and addition of nutritive substances were needed.

5.3. Changes in the basic indicators after mixing, watering, biostimulation and re-inoculation

The addition of ammonium nitrate, diamonium phosphate and potassium chloride achieved the required balance of C:N:P:K = 102:10:1:0.1, as shown in Table 17.

In an independent parallel research where the changes in the basic indicators during the bioremediation were monitored (Beškoski *et al*., 2011) the results showed that during bioremediation, the WHC increased from 26% to 32%, which was a direct result of the reduction in the concentration of hydrophobic compounds and the increased polarity of the substrate for bioremediation. In the biopile remediation, the pH reduced somewhat due to the appearance of low molecular weight organic acids during degradation of the hydrocarbon compounds.

Changes in basic microbiological parameters during bioremediation showed that the level of the active bacterial consortium, particularly active hydrocarbon degrading microorganisms, was maintained and increased. It was shown that the inoculation and re-inoculation, followed by biostimulation and aeration, affected the microbial profile variation and maintained the necessary level (number) of hydrocarbon degrading microorganisms.

In all samples from control pile, a small number of hydrocarbon degrading microorganisms was detected and a small proportion, less than 10 % of hydrocarbon degrading in the number of total chemoorganoheterotrophs, which indicated a bioremediation potential which is not sufficient for self cleaning of this soil (Gojgić-Cvijović *et al*., 2006). Moreover, this share decreased over time. On the other hand, the technology applied to the biopile ensured that the number of hydrocarbon degrading and total chemoorganoheterotroph microorganisms increased in the substrate for bioremediation (Beškoski *et al*., 2011). This shows that re-inoculation together with aeration achieved by mixing and biostimulation will enable success of the applied bioremediation process.

5.4. Reliability of the sampling method

In order to assess whether the analysis of the composite sample was sufficiently representative and if it accurately represented the actual state in the biopile, a parameter for bioremediation monitoring, TPH, was analyzed. The TPH was determined for the composite sample taken from the substrate for bioremediation at the beginning of the experiment in five replications (five independent determinations), and in each of the 30 individual samples from which the composite sample was created. Statistical analysis of these results is shown in Table 18.

Coefficients of variation, or relative standard deviation for the composite sample and for individual samples were 10 % and 23 %, respectively (Table 18). Since the coefficient of variation indicates the homogeneity of properties of units in the set, large coefficients of variation (> 30 %) are often associated with increased experimental variability. Otherwise the property or the sample can be considered relatively homogenous (Miller, Miller, 2010). Therefore, it can be concluded that both, the composite sample and the individual samples were homogenous. Additionally, it can be concluded that the composite sample satisfactory reflected the properties of the whole biopile. Finally, it can be concluded that the analysis of the composite samples, which were sampled and prepared using the aforementioned procedures, was a valid technique

to be used in the current study where the biopile is applied for remediation of a soil contaminated with heavy fuel oil.

Table 18. Statistical analysis of single and composite samples
from the substrate for bioremediation at zero time.

Parameter	Composite sample[a]	Single samples[b]
	TPH (g kg^{-1})	
Number of samples	1	30
Number of determinations per sample	5	1
Mean	5.18	5.21
Std. error of mean	0.23	0.22
Median	5.42	5.12
Std. deviation	0.52	1.19
Coefficient of variation (%)	10	23
Minimum	4.38	2.11
Maximum	5.61	7.54
Range	1.23	5.43

[a] Five independent determinations of one composite sample.
[b] Single determinations of each 30 individual samples.

5.5. Influence of biostimulation factors on degradation of the main components of the petroleum pollutant

The primary mechanism for the elimination of hydrocarbons from contaminated sites is biodegradation by indigenous microorganisms. Bioremediation is a technology which exploits the biodegradative abilities of live organisms to restore polluted systems and can be efficiently used to clean the soils contaminated with petroleum hydrocarbons. Biostimulation involves the modification of the environment to stimulate existing bacteria capable of bioremediation (Gojgić-Cvijović *et al*., 2012).

The main biostimulation agents used in this research were: addition of biomass, addition of biosurfactants and addition of nutrients (Chapter 3). The influence of these

biostimulation factors on microbial degradation of the main components of the petroleum pollutant in soil was investigated.

5.5.1. Influence of biostimulation factors on degradation of the fraction of total saturated hydrocarbons

According to previous organic geochemical studies (Volkman *et al*. 1983; Peters *et al*. 2005) and research related to the fate of petroleum pollutants in the environment as well (for example: Jovančićević *et al*. 2003; Antić *et al*. 2006; Ilić *et al*. 2011; Šolević *et al*. 2011), it was shown that in the fraction of saturated hydrocarbons, the most susceptible to microbial degradation are *n*-alkanes and isoprenoid aliphatic hydrocarbons.

Total Ion Chromatograms (TICs) of saturated hydrocarbon fractions isolated from the biopile during the six month bioremediation period are shown in Figure 25.

In the sample M1, the fraction of total saturated hydrocarbons is characterized by a broad and prominent "hump" of an unresolved complex mixture (UCM; Figure 25). This shape of chromatogram is typical of oils altered by biodegradation. The peaks originating from *n*-alkanes are very low in intensity. Based on this total ion chromatogram, a precise conclusion about the intensity of microbial degradation of oil pollutant investigated can not be made.

However, considering the fact that *n*-alkanes are completely removed at the biodegradation level 4 (Head *et al*., 2003) and that they are still present but in a very low abundance in the sample M1, it can be concluded that according to these results the investigated oil pollutant from the biopile at the beginning of the experiment can be classified to be at the boundary between the third and the fourth Head's biodegradation level (Head *et al*., 2003).

In the samples M2 – M5, TICs of the fractions of total saturated hydrocarbons are very similar to the TIC of the sample M1. All of them are dominated by a broad and prominent "hump" of the UCM, with very low peaks originating from *n*-alkanes (Figure 25). However, based on these TICs, a precise conclusion about the intensity of microbial degradation of oil pollutant investigated can not be made.

Comparing with the results from the parallel non-biostimulated biodegradation experiment (Novaković *et al*., 2012; Ramadan *et al*., 2012), a similarity can be noticed. The TICs of saturated hydrocarbon fractions isolated from the oil pollutant at the beginning of the biodegradation and after 6 months, at the end of the experiment are quite similar to TICs corresponding to the samples analyzed in the present bioremediation experiment: all of them are dominated by UCM while the peaks originating from *n*-alkanes are very low in intensity. However, based on these TICs only, a precise comparison between the microbial degradation intensity of oil pollutant investigated in these two experiments (biostimulated and non-biostimulated) can not be made. Accordingly, a precise conclusion about the influence of biostimulation factors on degradation of the fraction of total saturated hydrocarbons can not be drawn.

5.5.2. Influence of biostimulation factors on degradation of phenanthrene and its methyl isomers

Biodegradation of the aromatic fraction of oil pollutant was examined together with saturated hydrocarbons in many organic geochemical studies (Peters *et al*. 2005). For this fraction can be said that it is generally more resistant to biodegradation than the fraction of saturated hydrocarbons (Volkman *et al*. 1983; Peters *et al*. 2005).

Mass fragmentograms of phenanthrene, methyl-, dimethyl- and trimethyl-phenanthrenes obtained by GC–MS analysis of aromatic fractions isolated from the extracts of samples M1 – M5 are shown in Figure 26. In the initial sample (M1), distributions of these aromatic hydrocarbons are typical for oil. Considering the fact that most of *n*-alkanes in the initial sample have already been degraded (Figure 25), and that phenanthrene and its methyl-isomers are still preserved, it can be drawn a conclusion that the investigated sample of oil pollutant at the beginning of the experiment was at the third, moderate level of Head's scale of oil biodegradation (Head *et al*., 2003).

In order to investigate the distribution of phenanthrene and its methyl isomers (mono-, di- and tri-), ratios of the relative concentrations of phenanthrene and the most abundant methyl, dimethyl and trimethyl isomers were calculated. Additionally, ratios

of the relative concentrations of methyl and trimethyl isomers were calculated. The values of these parameters for samples M1–M5 are shown in Figure 28.

Based on fragmentograms in Figure 26, as well as on the values of numerous parameters shown in Figure 28, it can easily be observed that during the process of bioremediation of soil contaminated by heavy residual fuel oil, there was a uniform increase in the relative abundance of phenanthrene compared to its methyl isomers. This increase was the most pronounced in the case of trimethyl-phenanthrenes (P/TMP = 0.42–2.45, Figure 28) and the least in the case of methyl-phenanthrenes (P/MP = 0.50–1.02, Figure 28). The ratio of MP/TMP was also uniformly increased from 0.84 to 2.40 (Figure 28). Based on these results, it can be drawn a general conclusion that the bioremediation process under the conditions described generally results in an increase in the concentrations of phenanthrene, but also its lower methyl homologue compared to the higher homologues.
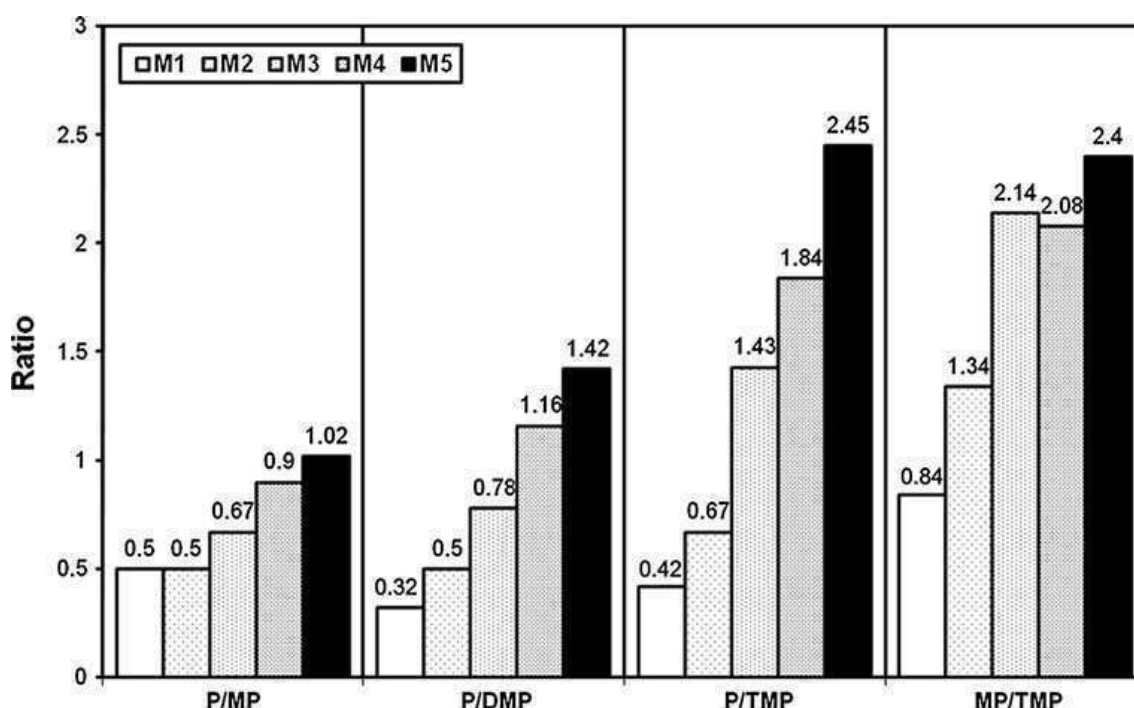
Figure 28. Ratios of phenanthrene (P) and most intense methylphenanthrene (MP), dimethylphenanthrene (DMP), and trimethyl-phenanthrene (TMP), as well as MP and TMP isomers for M1–M5 samples taken from soils that were treated with sawdust, biomass, nutrient, and biosurfactants during the 6-month bioremediation. The uniform increase in all ratios shows decrease in the relative abundance of methyl isomers compared to phenanthrene and also its higher methyl homologues compared to the lower homologues (See Figure 26).

From a total of 9 types of microorganisms identified in the zymogen consortium, 6 of them belong to the group of efficient petroleum hydrocarbons' degraders. These are *P. aeruginosa*, *Rhodococcus sp.*, *Pseudomonas sp.*, *P. fluorescens*, *P. luteola* and *A. denitrificans*, *S. maltophilia*, and *A. hydrophila* (Bossert, Bartha 1984, Singh, Ward 2004). As already noted, the process of re-inoculation, that is, addition of biomass of microbial consortia isolated from crude oil-contaminated soil, was performed once a month after the biopile was mixed and turned around. At the same time, the nutrients (N, P, and K) necessary for cellular metabolism and successful development of microorganisms were added (Alexander, 1994; Atagana *et al*. 2003).

There is a possibility that the observed changes in the distribution of phenanthrene and its methyl isomers occurred as a result of demethylation (Huang *et al*. 2004) through the process of oxidative decarboxylation. In this way, an increase in absolute concentration of phenanthrene could have happened at the expense of degradation of methyl-, dimethyl-, and trimethyl-phenanthrenes. However, demethylation is a thermodynamically less favorable process. Therefore, it is more likely that the biostimulation process favored those bacteria strains in consortium that decompose methyl isomers (first of all trimethyl-phenanthrenes). This biodegradation pattern can be a consequence of better interaction of reactive methyl groups with the active centers on the surface of bacterial cells and, in this way, promoted decomposition of methyl-phenanthrene derivatives (Lamberts *et al*. 2008). The presence of biosurfactant that increases solubility and thus the availability of products with a higher degree of alkylation could also contribute this process. In this way, it could have happened a regular increase in ratios of P/MP, P/DMP, and P/TMP during the process of bioremediation of contaminated soils.

On the other hand, during the bioremediation, a decrease in the relative concentration of trimethyl-phenanthrenes relative to the methyl-phenanthrenes was observed. This is reflected through an increase in the ratio MP/TMP. This result could indicate that the interaction of phenanthrene isomers with bacterial cells is increased if phenanthrene contains a larger number of methyl substituents.

Monitoring of changes in the distribution of phenanthrene and its methyl isomers in 5 samples belonging to the control trials (Novaković *et al*., 2012) actually gives an estimate of the transformations that occur during the natural microbial degradation of oil pollutant. Contrary to the bioremediation process (samples M1–M5), sawdust, biomass, nutrient substances, and biosurfactant were not added to the control samples. Under such conditions, comparing with the process of stimulated biodegradation, an opposite trend can be noticed (Novaković *et al*., 2012): the relative concentration of phenanthrene is reduced relative to methyl-phenanthrenes, dimethyl-phenanthrenes, and trimethyl-phenanthrenes. This decrease is the most pronounced in comparison with the trimethyl isomers (P/TMP = 0.85–0.11; Novaković *et al*., 2012) and the least pronounced in comparison with the methyl isomers (P/MP = 0.91–0.61; Novaković *et al*., 2012). MP/TMP ratio was also uniformly decreased from 0.93 to 0.17 and 1.15 (Novaković *et al*., 2012). It can be concluded that, generally, in this process, a decrease in the relative concentration of phenanthrene and lower methyl homologue comparing to the higher homologues occurred. These changes in the distribution of phenanthrene and its methyl isomers during biodegradation can be characterized as typical (Šolević *et al*. 2011).

According to these results, a general conclusion can be drawn that this process of applied bioremediation generally resulted in an increase in the relative concentrations of phenanthrene, but also its lower methyl homologue compared to the higher homologues. During the process of natural microbial degradation of oil pollutants (control samples, Novaković *et al*., 2012), a different trend was observed: the relative concentration of phenanthrene is reduced relative to methyl-phenanthrenes and dimethyl-phenanthrenes and especially relative to trimethyl- phenanthrenes. Similarly, the concentration of methyl-phenanthrenes is reduced relative to the trimethyl derivatives (Novaković *et al*., 2012).

Demethylation is a process that could have led to the degradation of methyl derivatives of phenanthrene and an increase in the absolute concentration of phenanthrene. However, this process is thermodynamically less favorable. Accordingly, it was assumed that re-inoculation, biostimulation, and the addition of sawdust and biosurfactant promoted the process of decomposition of methyl-phenanthrene

derivatives, by favoring the bacterial strains in the consortium (*P. aeruginosa*, *Rhodococcus sp.*, *Pseudomonas sp.*, *P. fluorescens*, *S. paucimobilis*, *P. luteola*, *A. denitrificans*, *S. maltophilia*, and *A. hydrophila*) that break down methyl isomers (first of all trimethyl-phenanthrenes). This biodegradation pattern is explained as a result of better interaction between more reactive methyl groups with active centers on the surface of bacterial cells. Direct laboratory experiments would be useful for the confirmation of this assumption.

On the other hand, changes in the distribution of phenanthrene and its methyl isomers during the "unstimulated" biodegradation (Novaković *et al*., 2012) can be characterized as typical, and they are described in earlier papers.

On the basis of this research, a general conclusion can be drawn that an increase in the availability of phenanthrene and its methyl derivatives to microorganisms can increase degradability of methyl-phenanthrenes compared to phenanthrene. In this study, an increased availability of phenanthrene and its methyl derivatives to microorganisms was accomplished by re-inoculation, biostimulation, as well as by the addition of sawdust and biosurfactants. Additionally, it can be concluded that the level of degradability in these conditions depends on the number of methyl groups, that is, on the level of alkylation.

5.5.3. Influence of biostimulation factors on degradation of *n*-alkanes

Considering the fact that in the initial sample *n*-alkanes within the fraction of saturated hydrocarbons were presents in a very low amount (Figure 25), in order to facilitate the analysis of these compounds, a concentration by urea adduction technique was used (Section 4.16). Due to their ability to build up channel inclusion compounds with urea molecules, using urea adduction technique *n*-alkanes can be successfully concentrated i.e. separated from branched, cyclic and polycyclic alkanes in the mixture of saturated hydrocarbons.

Gas chromatograms of carbamide adduct from samples M1 - M5 are given in Figure 27. Although the amount of *n*-alkanes within the total mixture of saturated hydrocarbons, both at the beginning and at the end of the experiment was very low (Figure 25), they were successfully concentrated by urea adduction technique. Accordingly, in gas chromatograms of all samples investigated a homologous series of alkanes, ranging from C16 to C32 and maximizing at *n*-C20 were identified. It is noticeable that the abundance and the distribution of *n*-alkanes during biodegradation of oil pollutant did not change. The abundance of *n*-alkanes remained at the initial low level, even at end of the experiment, after six months of the intensive stimulated bioremediation.

As it was shown in the Section 5.5.2., a specific biodegradation pattern of methyl-phenanthrene homologues was observed during the process of stimulated biodegradation, investigated in this research. This process of applied bioremediation resulted in an increase in the relative concentrations of phenanthrene, but also its lower methyl homologue compared to the higher homologues as a result of more intense degradation of higher homologues.

Considering the fact that phenanthrene and its methyl-, dimethyl- and trimethyl-homologues were seriously affected by biodegradation during the investigated six month period of applied bioremediation, the fact that the abundance and the distribution of *n*-alkanes during the same process did not change is surprising. According to the generally accepted principles of oil biodegradation (Head *et al*., 2003; Huang *et al*., 2004; Volkman *et al*., 1983), *n*-alkanes should have already been completely removed before or simultaneously with the major alternation of phenanthrene and its methyl isomers.

According to these results it can be concluded that even in conditions of intensive stimulated bioremediation, biodegradation of individual components of oil pollutants will not proceed (not even in the case of   *n*-alkanes which are the most biodegradable hydrocarbons in oils) unless they are present in some minimum "threshold" amount.

Comparison with the distribution of *n*-alkanes in 5 samples belonging to the control trials (Ramadan *et al*., 2012) can give an estimate of the transformations that

occur during the natural microbial degradation of oil pollutant. Similarly to the stimulated bioremediation process, during the six month experiment of natural oil pollutant biodegradation, the abundance and the distribution of n-alkanes did not change (Ramadan *et al*., 2012). In all samples they were identified in the C_{16} to C_{31} range, maximizing at n-C_{20}. Considering the fact that in these control samples phenanthrene and its methyl-isomers were degraded up to > 6 biodegradation level, this similarity in abundance and the distribution of n-alkanes during these two experiments (stimulated and non-stimulated biodegradation) is even more pronounced.

Interpreting the results obtained during the "unstimulated" biodegradation experiment (Ramadan *et al*., 2012), it was concluded that in conditions of reduced availability of certain class of compounds microorganisms opt for those which are, although less biodegradable, more accessible, i.e. those which are found in a substrate in higher amount. Consequently, the reason for lower biodegradability might be the lower amount of n-alkanes in the pollutant.

Another explanation was that n-alkanes could have been captured by incorporation, i.e. formation of inclusions with nonbiodegradable components such as humic substances or fulvic acids, present in recen sediments as native organic compounds. It should be stressed that results about biodegradation of n-alkanes during the experiment of stimulated biodegradation presented in this thesis support this explanation.

6. Conclusions

According to the results presented in this thesis, following general conclusions can be drawn:

1. The mazut polluted soil chosen for this research was potentially a good substrate for monitoring the bioremediation of petroleum pollutant: the soil pH was in the optimum range, the TPH content was high enough with presence of an intensive biodegradative processes and, accordingly, large bioremediation potential. With an improvement of the soil structure and with biostimulation by addition of water and nutrients this soil could become an appropriate medium for monitoring the bioremediation of petroleum pollutant.

2. Due to the addition of sawdust and raw river sand to the mazut polluted soil some parameters improved as a consequence of dilution: the water holding capacity, porosity and ability to be mixed increased while the clay content became lower. On the other hand, the pH value remained within the optimum range for oil degradation. However, some parameters were not within the optimum range for oil degradation leading to the conclusion that for the effective bioremediation process of the substrate for bioremediation an increase in the number of hydrocarbons degrading microorganisms and addition of nutritive substances were needed.

3. Changes in basic microbiological parameters during bioremediation showed that the level of the active bacterial consortium, particularly active hydrocarbon degrading microorganisms, was maintained and increased. The inoculation and re-inoculation, followed by biostimulation and aeration, affected the microbial profile variation and maintained the necessary number of hydrocarbon degrading microorganisms.

4. Re-inoculation together with aeration by mixing and biostimulation can enable success of the applied bioremediation process.

5. Thanks to a special sampling method, the composite sample and the individual samples were homogenous and satisfactory reflected the properties of the whole biopile. That made the analysis of the composite samples a valid technique to be used in this study where the biopile is applied for remediation of a soil contaminated with heavy fuel oil.

6. According to the results of the analyses of the total saturated fraction, the investigated oil pollutant from the biopile at the beginning of the experiment was classified to be at the boundary between the third and the fourth Head's biodegradation level (Head *et al*., 2003).

7. In the bioremediation experiment under the conditions applied in this research, monitoring the changes in the fraction of total saturated hydrocarbons cannot lead to any precise conclusions about the intensity of microbial degradation of oil pollutant investigated.

8. Based on the results of the analysis of the fraction of total saturated hydrocarbons, a precise comparison between the microbial degradation intensity of oil pollutant investigated in the present biostimulated experiment and a paralel non-biostimulated, cannot be made. Accordingly, a precise conclusion about the influence of biostimulation factors on degradation of the fraction of total saturated hydrocarbons cannot be drawn.

9. The stimulated biodegradation process under the conditions described (re-inoculation, biostimulation, aeration and the addition of sawdust and biosurfactant) generally results in a preferable biodegradation of higher homologues of methyl-phenanthrenes comparing to the lower ones. This effect is the most pronounced in the case of trimethyl-phenanthrenes and the least in the case of methyl-phenanthrenes.

10. As a result of specific biodegradation pattern during the stimulated biodegradation process described in this research, an increase in the relative abundance of

phenanthrene compared to its methyl isomers occured. This increase is the most pronounced in comparison of phenanthrene with trimethyl-phenanthrenes and the least comparing phenanthrene with methyl-phenanthrenes.

11. The biodegradation pattern observed in this research can be a consequence of better interaction of reactive methyl groups with the active centers on the surface of bacterial cells and, in this way, promoted decomposition of methyl-phenanthrene derivatives. The presence of biosurfactant that increases solubility and thus the availability of products with a higher degree of alkylation could also contribute this process.

12. Re-inoculation, biostimulation, aeration and the addition of sawdust and biosurfactant promoted the process of decomposition of methyl-phenanthrene derivatives, by favoring the bacterial strains in the consortium (*P. aeruginosa*, *Rhodococcus sp.*, *Pseudomonas sp.*, *P. fluorescens*, *S. paucimobilis*, *P. luteola*, *A. denitrificans*, *S. maltophilia*, and *A. hydrophila*) that break down methyl isomers (first of all trimethyl-phenanthrenes). This biodegradation pattern is explained as a result of better interaction between more reactive methyl groups with active centers on the surface of bacterial cells. Direct laboratory experiments would be useful for the confirmation of this assumption.

13. The biodegradation trend among phenanthrene and its methyl-, dimethyl- and trimethyl-homologues observed in this research is opposite to the typical biodegradation trend of phenanthrene and its methyl isomers during the natural "unstimulated" biodegradation.

14. An increase in the availability of phenanthrene and its methyl derivatives to microorganisms can increase degradability of methyl-phenanthrenes compared to phenanthrene. In this study, an increased availability of phenanthrene and its methyl derivatives to microorganisms was accomplished by re-inoculation, biostimulation, as well as by the addition of sawdust and biosurfactants.

Additionally, the level of degradability in these conditions depends on the number of methyl groups, that is, on the level of alkylation.

15. The distribution of *n*-alkanes during this experiment did not change. The abundance of *n*-alkanes remained at the initial low level, even at end of the experiment, after six months of the intensive stimulated bioremediation.

16. Individual components of oil pollutants must be present in soil in some minimum "threshold" amount to be biodegraded. Otherwise, their biodegradation will not proceed even in conditions of intensive stimulated bioremediation. This applies even on *n*-alkanes which are the most biodegradable hydrocarbons in oils.

17. Low biodegradability of *n*-alkanes during the investigated six month period of applied stimulated bioremediation was quite similar to the low biodegradability of *n*-alkanes during the parallel nonstimulated biodegradation experiment. These results support the explanation from the previous research (Ramadan *et al*., 2012) that low biodegradability of *n*-alkanes might be a consequence of their capture by incorporation with nonbiodegradable components in soil such as humic substances or fulvic acids.

7. References

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Прилог 1.

Изјава о ауторству

Потписани-а _Муфтан Мохамед Али Рамадан_

број индекса _2 /2009_

Изјављујем

да је докторска дисертација под насловом

The study of influence of biostimulation factors on microbiological degradation of main components in petroleum-type pollutant

- резултат сопственог истраживачког рада,

- да предложена дисертација у целини ни у деловима није била предложена за добијање било које дипломе према студијским програмима других високошколских установа,

- да су резултати коректно наведени и

- да нисам кршио/ла ауторска права и користио интелектуалну својину других лица.

Потпис докторанда

У Београду, _22. Маја 2013._

Прилог 2.

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора ___MUFTAH MOHAMED ALi RAMADAN___

Број индекса _____2/2009_____

Студијски програм ___DOKTOR HEMIJSKIH NAUKA___

Наслов рада ___THE STUDY OF INFLUENCE OF BIOSTIMULATION FACTORS ON MICROBIOLOGICAL DEGRADATION OF MAIN COMPONENTS IN PETROLEUM -TYPE POLLUTANT___

Ментор ___DR BRANIMIR JOVANČIĆEVIĆ, RsoPROFESOR HEMIJSKOG FAKULTETA UNIVERZITETA u BEOGRADU___

Потписани/а ___MUFTAH MOHAMED ALi RAMADAN___

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис докторанда

У Београду, ___22. MAJA 2013.___

Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић" да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

THE STUDY OF INFLUENCE OF BIOSTIMULATION FACTORS ON MICROBIOLOGICAL DEGRADATION OF MAIN COMPONENTS IN PETROLEUM-TYPE POLLUTANT

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

1. Ауторство

2. Ауторство - некомерцијално

3. Ауторство – некомерцијално – без прераде

4. Ауторство – некомерцијално – делити под истим условима

5. Ауторство – без прераде

6. Ауторство – делити под истим условима

(Молимо да заокружите само једну од шест понуђених лиценци, кратак опис лиценци дат је на полеђини листа).

Потпис докторанда

У Београду, 22. MAJA 2013.

1. Ауторство - Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце, чак и у комерцијалне сврхе. Ово је најслободнија од свих лиценци.

2. Ауторство – некомерцијално. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца не дозвољава комерцијалну употребу дела.

3. Ауторство - некомерцијално – без прераде. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, без промена, преобликовања или употребе дела у свом делу, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца не дозвољава комерцијалну употребу дела. У односу на све остале лиценце, овом лиценцом се ограничава највећи обим права коришћења дела.

4. Ауторство - некомерцијално – делити под истим условима. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце и ако се прерада дистрибуира под истом или сличном лиценцом. Ова лиценца не дозвољава комерцијалну употребу дела и прерада.

5. Ауторство – без прераде. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, без промена, преобликовања или употребе дела у свом делу, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца дозвољава комерцијалну употребу дела.

6. Ауторство - делити под истим условима. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце и ако се прерада дистрибуира под истом или сличном лиценцом. Ова лиценца дозвољава комерцијалну употребу дела и прерада. Слична је софтверским лиценцама, односно лиценцама отвореног кода.