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VOLUME II

CONTENTS

Development of a procedure for bioremediation treatment of underground waters and soils polluted by petroleum

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ABSTRACT

Crude oil as a raw material, and all its refined products that are used as fuel, is a highly complex mixture of a large number of saturated and aromatic hydrocarbons and various nitrogen, sulphur and oxygen compounds (NSO compounds). On the other hand, there are some new inorganic and organic compounds formed during the usage of the refined products. It can be easily concluded that they are very serious environmental pollutants. The man has a duty and responsibility to clean the contaminated segments of the environment by petroleum and return them to the form in which they were before pollution. In this sense, remediation procedures are undertaken. Microbial bioremediation procedure (i.e. bioremediation), is justifiably considered the most efficient remediation procedure.

This paper will present research aimed at optimizing the conditions for bioremediation of various segments of the environment (in the first place of underground waters and soils) contaminated with oil-type pollutants. This research covers a ten-year long pathway starting from the laboratory experiments, through *ex situ* processes, then *in situ* conditions and finally to practical application.

Key words: Petroleum. Biomarkers. Environment pollutants. Bioremediation. Zymogenous microorganisms.

1. INTRODUCTION

The impression that petroleum (crude oil) is the most important fossil fuel, and the most important material of general importance in the last hundred years and more, has its reverse side. Crude oil as a raw material, and all its refined products that are used as fuel, is a highly complex mixture of a large number of saturated and aromatic hydrocarbons and various nitrogen, sulphur and oxygen compounds (NSO compounds). On the other hand, there are some new inorganic and organic compounds formed during the usage of the refined products. Some of these compounds have a harmful effect on human health, and on the living systems on Earth in general. It can be easily concluded that they are very serious environmental pollutants. In addition, the use of oil as fossil fuel contributes to the most prominent and most dangerous negative effect on the environment, the greenhouse effect, which, according to some of the most pessimistic forecasts may lead to fatal disorders of ecochemical balance on our planet.

The man has a duty and responsibility to clean the contaminated segments of the environment by petroleum and return them to the form in which they were before pollution. In this sense, remediation procedures are undertaken.

Remediation procedures can be classified into three groups: physical, chemical and biological. Physical methods of pollutant degradation processes are burning and sorption procedures. Chemical ones are e.g. dechlorination, reduction or oxidation procedures, and biological include bioremediation procedures. Bioremediation may be microbiological, phytoremediation and zooremediation. Microbial bioremediation procedure (i.e. bioremediation), is justifiably considered the most efficient remediation procedure.

However, bioremediation as a technique for removing petroleum pollutants has its limitations. For example, not all compounds are biodegradable. A very complex mixture of organic compounds, such as crude oil, contains also some of them that are not easily and completely degradable. Moreover, the success of the process depends on many factors, the most important of which are the appropriate selection of metabolically capable populations of microorganisms, defining favourable conditions for their growth in the environment, appropriate concentrations of nutrients and pollutants. Also, in some cases, microbial metabolism of a pollutant can produce toxic metabolites.

This paper will present research aimed at optimizing the conditions for bioremediation of various segments of the environment (in the first place of underground waters and soils) contaminated with oil-type pollutants. This research covers a ten-year long pathway starting from the laboratory experiments (1), through *ex situ* processes (2), then *in situ* conditions (3) and finally to practical application (4).

2. RESEARCH APPROACHES

Biodegradation of the oil pollutant in the laboratory (1)

From the very beginning, the research assumption was that the most efficient are the experiments using zymogenous microorganisms, i.e. those microorganisms that have been identified at the site where the pollutant is found. For example, in the waste water of Pancevo oil refinery (Serbia, alluvial formation of the Danube River) bacteria of the genus *Bacillus* and *Actynomycetes* and fungi of the genus *Penicillium* were identified [1]. Simulated biodegradation experiments under aerobic conditions using these microorganisms were conducted. Paraffinic type petroleum was used as substrate, and experiments lasted 15, 30, 45, 60 and 75 days.

Gas chromatographic-mass spectrometric analysis (GC-MS) gave total ion chromatograms (TIC) of saturated hydrocarbons in the initial sample paraffinic crude oil and the sample exposed to biodegradation. In this way it is possible to monitor changes in the abundance and

distribution of *n*-alkanes, isoprenoids and polycyclic alkanes.

Degradation of the lower members of homologous series of *n*-alkanes starts already after 15 days. After 60 days they are almost completely degraded. In these experiments, degradation of *n*-alkanes and isoprenoids runs in parallel. Ion chromatograms *m/z* 217 and *m/z* 191 confirmed that used microorganisms under the applied conditions did not decompose polycyclic alkanes (steranes and triterpanes).

GC-MS analysis of aromatic fraction (ion chromatograms of phenanthrene, *m/z* = 178; methylphenanthrenes, *m/z* = 192 and dimethylphenanthrenes, *m/z* = 206) confirmed that during the crude oil biodegradation, phenanthrene, and its methyl derivatives get almost completely degraded. According to some earlier theories, it was believed that during oil biodegradation, after the decomposition of *n*-alkanes, isoprenoid degradation begins followed by degradation of polycyclic alkanes [2]. However, later investigations of microbial degradability of some classes of organic compounds led to conclusions according to which aromatic hydrocarbons, in particular phenanthrene and its methyl isomers, are less resistant to biodegradation than polycyclic alkanes of sterane and terpane types [3].

Bioremediation – *ex situ* conditions (2)

During *ex situ* bioremediation, soil or a sediment layer contaminated with oil, or some of its refined products is dug out and then transported to designated places where the process is to be conducted. In this process biopile, or pile, is formed.

In preparation for the bioremediation process it is important to define a consortium of microorganisms that will be used for pollutant degradation. Zymogenous microorganisms are most commonly used for this purpose, too.

Preparation of biopile involves homogenization of the contaminated soil with wood chips. This will increase the water holding capacity. Aeration is carried out using a system of perforated pipes, and the growth of microorganisms, and their increase is achieved also by bio-stimulation processes. In this sense, during preparation, and later, biopile is added with fertilizer, as a source of nitrogen and phosphorus. Furthermore, biosurfactants are added in order to boost the effect of biodegradation.

For example, in the period from September 2009 to March 2010 the process of *ex situ* bioremediation was performed on soil contaminated with heavy oil [4], [5]. About 150 tons of soil was evenly distributed on 300 m^3 of unwashed sand. Then the mass was added chips from poplar, beech and oak. The whole mass was homogenized to form a 75 x 20 x 0.4 m biopile by means of excavator. It was continuously sprayed by biomass from the 5 m^3 reservoir. Biomass was made of consortium of zymogenous microorganisms: *Pseudomonas aeruginos, Rhodococcus sp., Pseudomonas sp., Pseudomonas fluorescens, Sphingomonas paucimobilis, Stenotrophomonas maltophilia, Pseudomonas luteola, Aeromonas hydrophila,* and *Achromobacter denitrificans*. Biopile was continuously being added fertilizer, as a source of nutrients (nitrogen and phosphorus) and biosurfactant ("Biosolve" type) at a concentration of 70 mL of the original solution per cubic meter of land. Within a six-month period, five control samples were taken in equitable time intervals.

During the process of biodegradation of a petroleum type pollutant, similarly to previous experiences, *n*-alkanes and isoprenoid aliphatic alkanes were degraded. Polycyclic alkanes remained unchanged, and aromatic hydrocarbons of phenanthrene type (with their methyl-, dimentil- and trimethyl-isomers) were partially degraded.

Bioremediation – *in situ* conditions (3)

Theoretically, carrying out of *in situ* bioremediation process for cleaning the oil-contaminated soil, recent formation or water, has advantages over the *ex situ* procedure. The cost of transportation of polluted material is thus eliminated. The theoretical approach is

then very similar to that applied in *ex situ* conditions.

Our research included ground waters which contained dissolved hydrocarbons and a floating layer of an oil pollutant. They were treated 60 days with filtration-adsorption remediation technique, using the columns filled with natural inorganic hydrophobic adsorbents, and *in situ* bioremediation based on the principle of "bipolar" model [6]; [7].

In situ bioremediation of ground waters and soil layers in contact with groundwater was accomplished by chemical and biological stimulation, augmentation and aeration in closed "bipolar" system (pumping out – pumping in), with adsorption in the "external unit". This combination of methods is original and applied for the first time.

Bioaugmentation was achieved by injection of biomass of zymogenous microorganisms isolated from treated polluted ground waters. Groundwater from this site contained hydrocarbons. A part of them was dissolved, and the other, the insoluble portion, floated on the water surface. These waters were first treated with the filtration/adsorption remediation technique. Natural inorganic hydrophobic adsorbents were used. Then the *in situ* bioremediation was applied on a "bipolar model" principle. Chemical and biological stimulations were applied, as well as augmentation and aeration in a closed "bipolar" model (pumping out – pumping in), with adsorption in the "outdoor unit". Microbiological processes in the treated groundwater were further stimulated by aeration processes. Biomass was increased by periodic injection of consortium of zymogenous microorganisms.

Comprehensive GCxGC-MS analysis (*m/z* 71, 191, 217, 178, 192, 206 and 220) showed that at the beginning of the process, *n*-alkanes, isoprenoids, triterpanes, steranes and phenathrene and its methyl-, dimethyl- and trimethyl- isomers had typical oil distributions in the isolated extracts [7].

After 60 days of carrying out the bioremediation process all five classes of organic compounds were almost completely degraded. In this way, it could be concluded that the bioremediation process under *in situ* conditions was the most efficient.

All the described biodegradation processes of oil as a contaminant (biodegradation in the laboratory, *ex situ* bioremediation, *in situ* bioremediation) generally confirmed earlier findings about the sequence of degradation of some organic compounds that was previously defined: 1) *n*-alkanes, 2) isoprenoids pristane and phytane, 3) phenathrene, 4) methylphenathrenes, 5) dimethyilphenathrenes, 6) trimethyilphenathrenes, 7) steranes, 8) terpanes [3].

Instead of conclusions - practical application (4)

The investigations of the microbial degradation of the petroleum pollutant carried out in the laboratory, in *ex situ* and *in situ* conditions with zymogenic microorganisms, led to the definition of the procedure for practical application, that is, for defining a bioremediation method for water and soil cleaning in real conditions. For example, the "New Belgrade" Thermal Plant located in Belgrade, Serbia, is the oldest heat generation plant in Belgrade. Annual consumption of oil is about 45000 tons (13% of total energy). The thermal plant is one of the major sources of environmental pollution in a densely populated area on the left bank of the Sava River. The presence of various petroleum pollutants in sediment and soil has been previously confirmed [8]. For the purpose of remediation of contaminated sediment and soil at this site, the process of combined *in situ* and *ex situ* bioremediation is in progress. Microorganisms (degraders of hydrocarbons) used for this bioremediation process are isolated as zymogenic consortium in laboratory conditions and then multiplied in a large number in a mobile bioreactor. The obtained preliminary results (decomposition of total petroleum

hydrocarbons) proved the justification of applied bioremediation techniques.

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