CZECH UNIVERSITY OF LIFE SCIENCES - PRAGUE
Faculty of Environmental Sciences

BACHELOR THESIS

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CZECH UNIVERSITY OF LIFE SCIENCES, PRAGUE
Faculty of Environmental Sciences

Bachelor academic studies
Study programme: Environmental Engineering

BACHELOR THESIS'S ASSIGNMENT

Evaluation of the *ex-situ* bioremediation of the petroleum hydrocarbons contaminated soil

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CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

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BACHELOR THESIS ASSIGNMENT

Andjela Stanojevic

Environmental Engineering

Thesis title

Evaluation of the ex-situ bioremediation of the petroleum hydrocarbons contaminated soil

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Objectives of thesis

Bioremediation of the soils contaminated with various organic pollutants belong to the effective measures for environmentally friendly removal of these pollutants from the soil. Ex-situ bioremediation by using biopiles represents a possible measure for enhancement of the bioremediation procedure. Soil contaminated with oil or oil derivatives (TPH), which contains TPH higher than 5 g/kg can be purified with the help of a consortium of microorganisms (MO)
examine the degree of environmental pollution.
The objectives of the thesis will be:
1) to determine the content of total petroleum hydrocarbons (TPH) at the beginning and at the end of bioremediation.
2) to isolate microorganisms that participate in the process of bioremediation, then I would scale them up from laboratory level to industrial level
Hypothesis: the identification of the microbial consortium responsible for effective bioremediation of the TPH in soil will be helpful for the establishment of the further bioremediation projects.

Methodology

The M0 consortium would be isolated directly from the polluted environment, which would be multiply and then return to the environment. At the beginning, during and at the end of the experiment, the parameters would be monitored:

1) total number of MOs, number of yeasts and molds, number of anaerobic MOs and number of MOs of hydrocarbon degraders originating from pollutants;

2) TPH and n-Hexane Extractable Substances (HES) would be determined;

3) Gravimetry and gas chromatography (GC) will be used for the quantification of the contaminant levels;

4) statistical and graphical programs will be used for an evaluation of the data

The proposed extent of the thesis

30 pages

Keywords

bioremediation, petroleum hydrocarbons, biopile, soil

Recommended information sources

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Zhang C. (2020): Soil and Groundwater Remediation, Fundamentals, Practices, and Sustainability. John Wiley & Sons, Inc., USA.

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Dean

Prague on 28. 02. 2022

BT author's statement

I hereby declare that I have independently elaborated the bachelor/final thesis with the topic of: **"Evaluation of the *ex-situ* bioremediation of the petroleum hydrocarbons contaminated soil"** and that I have cited all of the information sources that I used in the thesis as listed at the end of the thesis in the list of used information sources.

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Acknowledgements

And where is the beginning?
Who is in the first place and who is in the last place on this list? All of you who helped me during my studies and in drafting this thesis are in my heart.

Thank you, Prof. Ing. Jiřina Száková, CSc. Czech University of Life Sciences, Prague, who had faith in me and agreed to be my mentor, as well as a great help in drafting this thesis.

This bachelor's thesis was done in cooperation the Czech University of Life Sciences, Prague with the Institute of Chemistry, Technology and Metallurgy, University of Belgrade and BREM Group Company, Belgrade, Serbia. That is why I owe a great deal of gratitude to Prof. Dr. Miroslav Vrvić, a retired full professor at the University of Belgrade - Faculty of Chemistry, and owner of BREM GROUP doo Company, Belgrade, and director of Department for research and development this Company, who selflessly supported me and showed me what it is like to be a "great professor". Professor Vrvić showed me in a wonderful way that the rule "once a professor - always a professor" is possible.

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Thanks to my parents Slađana and Boban who constantly give me great love and inspiration that makes my head spin, which is why I feel a responsibility to be better and better all the time.

Anđela Stanojević

Abstract

The amount of waste entering the environment at the present time is too large to be removed naturally - by self-purification. One of the technologies that are increasingly used in the world for the remediation of polluted environments, primarily soil, is bioremediation. One of the most effective types of bioremediation is the use of non-pathogenic microorganisms (bioaugmentation) for which organic pollutants are an important source of nutrients.

The main objective of this thesis was to answer the question: Is it possible in the applied conditions of bioremediation to successfully purify the soil contaminated with fuel oil? In this study, *ex-situ* bioremediation of soil contaminated with waste oils from petroleum products consisted from different areas in Serbia was performed. Contaminated soil was delivered to Dobanovci in Serbia. Chemical and microbiological indicators of bioremediation were monitored immediately after the preparation of the bio-pile and at the end of the bioremediation procedure, as well as in a control sample (which was prepared without microorganisms). Standard analytical methods were applied.

After bioremediation the tested samples showed a slightly increased alkalinity (pH = 7.0-7.5). The loss of organic substances for all the samples after bioremediation was about 34% and the loss of carbonate was about 36%. The value of n-hexane extractable substance (HES) and total petroleum hydrocarbons (TPH) content in the soil after bioremediation decreased by ~90%. The soil sample at the initial state of bioremediation had a significant presence of hydrocarbons with 17C and 18C atoms (pristane and phytane), while after the completion of bioremediation, a significant decrease in the presence of this petroleum hydrocarbons in the soil was observed. After bioremediation, a significant decrease in the content of total (from ~17% to 1.29%) and organic (from ~17% to 0.53%) carbon, as well as total hydrogen (from ~3% to 0.13%) was registered. However, statistically significant difference in total sulfur content (0.07-0.08%) was not registered. The presence of a large number of microorganisms showed that intense aerobic and anaerobic processes take place in soil samples.

Microorganisms had a significant role on the decrease of organic substances, HES and TPH, which indicates a very high efficiency of the applied microorganisms in the process of bioremediation with a very favorable bioremediation potential. The general conclusion and answer to the question posed in this thesis is that the applied bioremediation procedure has successfully cleaned the soil contaminated with fuel oil.

Key words: bioaugmentation, fuel oil, pristane, phytane, organic substances, microorganisms

Abstrakt

Množství odpadu, které se dostává do životního prostředí, je příliš veliké, takže při jeho likvidaci již selhává přirozená samočistící schopnost přírodních systémů. Jednou z technologií, které se stále více používají pro remediaci znečištěného prostředí, zejména půdy, je bioremediace. Jednou z nejúčinnějších metod bioremediace je pak aplikace nepatogenních mikroorganismů (bioaugmentace), pro které jsou organické polutanty významným zdrojem živin.

Hlavním cílem této bakalářské práce je odpovědět na otázku: Je možno za daných podmínek bioremediace dosáhnout úspěšného vyčištění půdy kontaminované topným olejem? Byl proveden *ex-situ* bioremediační experiment, který byl aplikován v případě půdy kontaminované odpadními oleji. Tato půda pocházela z různých oblastí Srbska a byla shromážděna v lokalitě Dobanovci v Srbsku. Byly sledovány vybrané chemické a mikrobiologické indikátory v hromadě půdy bezprostředně po založení pokusu a po ukončení bioremediace. Pro srovnání byla založena stejným způsobem kontrolní hromada (bez aplikace mikroorganismů). Byly využity standardní analytické metody.

Po skončení bioremediace byla zaznamenána zvýšená alkalita vzorků půdy (pH = 7.0-7.5). Ztráta organického materiálu po skončení bioremediace byla přibližně 34% a úbytek obsahu karbonátů byl 36%. Obsahy sloučenin extrahovatelných n-hexanem (HES) a celkový obsah ropných uhlovodíků (TPH) v půdě po skončení bioremediace poklesl o ~90%. Půdní vzorek odebraný na začátku bioremediace obsahoval ve významném zastoupení uhlovodíky s počtem atomů 17C a 18C (pristan a fytan), zatímco po skončení bioremediace byl pozorován významný pokles obsahu těchto ropných uhlovodíků v půdě. Po skončení bioremediace byl také zaznamenán úbytek celkového obsahu uhlíku (ze ~17% na 1,29%) obsahu organického uhlíku (ze ~17% na 0.53%) a celkového obsahu vodíku (ze ~3% na 0.13%). Naproti tomu významné rozdíly v obsahu síry (0.07-0.08%) zaznamenány nebyly. Přítomnost velkého množství mikroorganismů pak dokládá, že v půdních vzorcích probíhaly intenzivní aerobní i anaerobní procesy.

Mikroorganismy prokázaly významnou roli ve snížení obsahu organických látek, HES a TPH, což naznačuje vysokou účinnost aplikovaných mikroorganismů v procesu bioremediace a slibný bioremediační potenciál. Výsledky tedy můžeme shrnout tak, že na otázku vytyčenou na začátku výzkumu lze odpovědět ano, použitý bioremediační postup vedl k úspěšnému vyčištění půdy kontaminované topnými oleji.

Klíčová slova: bioaugmentace, topný olej, pristan, fytan, organické sloučeniny, mikroorganismy

Contents

1. INTRODUCTION

In the last hundred years, huge amounts of waste material have been dumped into the environment. Until the beginning of the Second World War, this waste material and its negative effects were not significant, but after its end, due to the irresponsible disposal of all types of waste, serious problems began to appear. Nowadays, the public is very interested in solving these problems. The amount of waste that is in the environment today is too large to be removed naturally - by self-purification. Therefore, it is necessary to clean the polluted areas and return to the state before the pollution, i.e., to remediate the contaminated environment. For most of the methods currently used in the world, it is characteristic that as a consequence of their use, new waste is generated that requires controlled disposal.

One of the technologies that are increasingly used in the world for the remediation of polluted environments, primarily soil, is bioremediation. Bioremediation is especially effective in remediation of environments contaminated with oil and oil derivatives, but it is also used for the treatment of waste that has not yet reached the environment, and it is increasingly used for environments contaminated with toxic elements. One of the most effective types of bioremediation is the use of non-pathogenic microorganisms for which the organic pollutants are a source of nutrients. Microorganisms that are naturally present at the site of pollution, translate toxic substances into products that are non-toxic to humans and the environment. Organic pollutants can be transformed to both carbon dioxide and water, and toxic elements can be converted to their non-toxic form.

Although microorganisms that have been transferred from another contaminated or even unpolluted environment can also be used in bioremediation, the best effect is shown by those that are isolated at the site of contamination. Many components of pollutants can be broken down only by the joint action of several strains of microorganisms - a consortium.

Environmental pollution with petroleum-type contaminants is an ever-present problem and challenge for researchers. Microorganisms are constantly relevant as "biological agents" in cleaning, especially historical pollution, protection and preservation of the environment. Biochemical pathways and mechanisms of degradation and inactivation of pollutants are challenges, both for researchers and those involved in the application.

2. OBJECTIVES OF THE THESIS

The main objective of this thesis was to answer the following question: Is it possible in the applied conditions of bioremediation to successfully purify the soil contaminated with fuel oil?

In this study, *ex-situ* bioremediation of contaminated soil consisted from different areas in Serbia that were contaminated with waste oils from petroleum products was performed. Contaminated soil was delivered to Dobanovci in Serbia. Chemical and microbiological indicators of bioremediation were monitored immediately after the preparation of the bio-pile and at the end of the bioremediation procedure (after 6 months). At the beginning of the experiment (before the addition of microorganisms), a control sample of bio-pile was separated, which was also chemically and microbiologically analyzed.

Basic chemical analysis of samples before and after bioremediation, as well as control sample, was performed. An elementary organic analysis of all samples was also performed, as well as the determination of hexane-soluble substances and gravimetric and gas chromatography analysis of total petroleum hydrocarbons. In addition, a microbiological analysis was performed to determine the number of microorganisms in the samples before and after bioremediation, as well as the control sample. All the obtained results were statistically analyzed.

3. THEORETICAL BACKGROUND

3.1 ECOSYSTEMS

An ecosystem is a community of biotic and abiotic factors in a particular environment. Biotic factors include living organisms - plants, animals and microorganisms, and abiotic factors include sunlight, air, water, mineral salts, temperature, humidity, chemical composition, etc. These biotic and abiotic factors are interrelated through the biogeochemical cycles of the elements as well as through the flow of energy (Miller & Spoolman, 2012). Ecosystems ecology is studied as the main processes of energy transformation and biogeochemical cycles. An ecosystem can be defined as a network of interactions between organisms, but also as relationships between organisms and their environment (Schulze et al. 2005).

Energy, water, carbon, nitrogen, phosphorus and minerals from the soil are essential abiotic components of the ecosystem (Supplementary figures 9.1-9.4). The energy that flows through the ecosystem is obtained from solar energy. It enters the ecosystem through photosynthesis, a process that also introduces carbon from the atmosphere. Plants, animals and microorganisms play an important role in the movement of compounds and energy through the system. The decomposition of extinct organisms by microorganisms' discharges carbon and other nutrients back into the atmosphere and soil, where they can be reused (Chapin et al. 2011).

3.1.1 Processes in ecosystems

There are two basic ideas about the functioning of ecosystems:

- the circulation of energy and
- the circulation of matter (biogeochemical cycles).

These two processes are related but not exactly the same (Figure 3.1.).

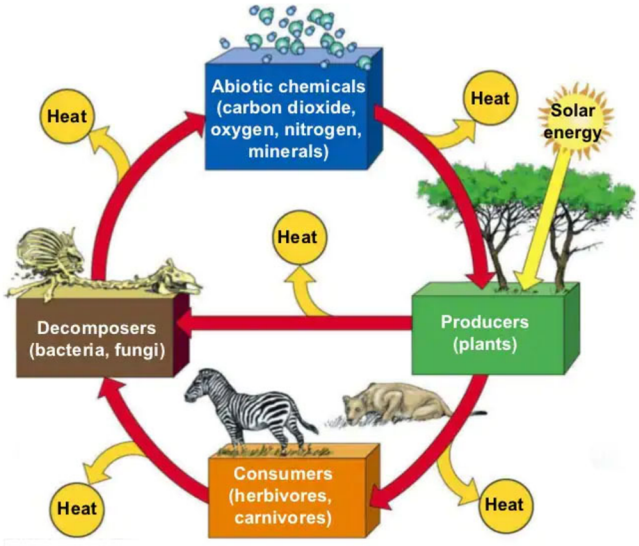

Figure 3.1: Circulation of energy and matter in nature (Miller & Spoolman, 2012).

The energy enters the biological system from the energy of the Sun and is converted into the chemical energy of organic molecules in cellular processes that include photosynthesis and respiration, and is eventually converted into thermal energy. This energy is wasted because it is released from the systems in the form of heat and as such can no longer be renewed (Chapin et al. 2011).

Elements such as carbon, nitrogen and phosphorus enter living systems in different ways. Plants get elements from the environment: the atmosphere, water and earth. Animals can also ingest these elements directly from the environment, but they mostly get them by consuming other organisms. These elements then enter the composition of many molecules in the body, but sooner or later due to the excretion or death of the organism, they return to their inorganic form. Microorganisms usually complete this cycle of elements in the process of decomposition or mineralization. Since the elements are not destroyed or lost within the system itself, we believe that the Earth is a closed system in relation to microelements (Chapin et al. 2011).

Ecosystems provide a large number of various material goods and various "services" that are necessary for man. Material goods include "tangible material products" produced by the ecosystem, such as food, building materials, medicinal plants (Brown et al. 2007), but also less tangible products such as tourism, recreation and the genes of wild animals and plants that can be used to improve domestic species (Christensen et al. 1996). The services provided by the ecosystem may include all those that improve living conditions, such as maintaining the water cycle, purifying the air, water and soil, maintaining the amount of oxygen in the atmosphere, pollinating crops (Christensen et al. 1996; Brown et al. 2007).

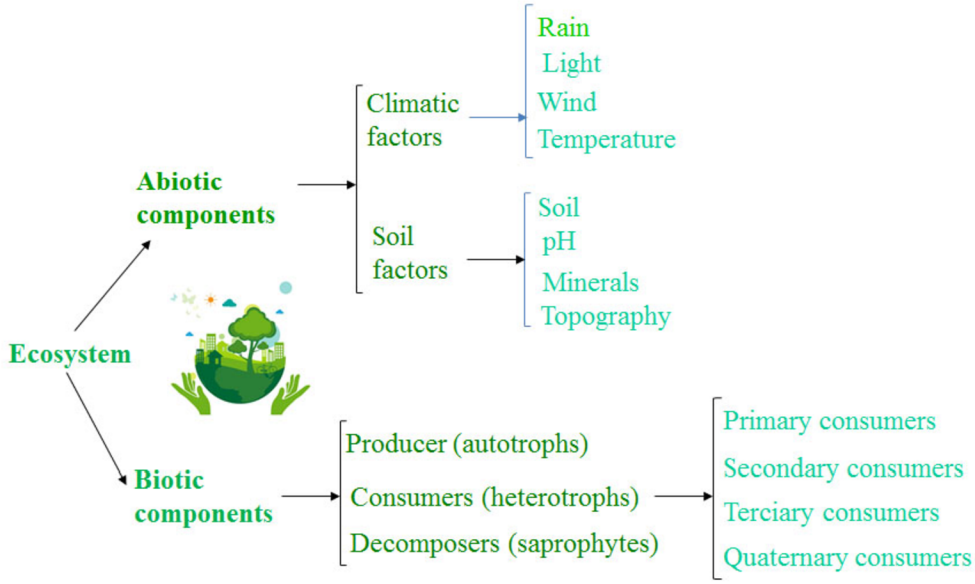

Figure 3.2: Ecosystem components.

3.1.2 Structural and functional components of ecosystems

The functional components of ecosystems are abiotic and biotic, which consist of a number of particular components. The structure and relationships between system components are shown in Figure 3.2.

3.2 SOIL AS A PART OF THE ECOSYSTEM

Soil is an important factor that affects the development and productivity of various ecosystems on our planet. Many life forms depend on the soil, such as vascular plants, and their development and growth further affect other organisms in the food chain. Although the importance of soil has always been known, only in the last few decades has soil been examined in a scientific sense (Jenny, 2005).

The term soil is not easy to define and there are several definitions **(**Hartemink, 2016). Hilgard (1914) defines soil as "the more or less loose and friable material in which, by means of their roots, plants may or do find a foothold and nourishment, as well as other conditions of growth". This definition of soil is quite outdated, but it is still used. It is one of many where the importance of soil stands out only in terms of plant growth and development. Raman (1928) defines soil as "the upper weathering layer of the solid earth crust." Joffe (1936), a representative of the Russian school, believes that Raman's formulation does not distinguish soil from rocky materials. According to him, the soil is naturally differentiated into layers of minerals and organic substances; it is usually loose depending on the depth. It differs from the parent layer in the lower layers in morphology, physical properties and constitution, chemical properties and composition, as well as in biological characteristics (Jenny, 2005).

Soil as an environment can be defined as a set of living organisms that inhabit it, meaning plants, animals and microorganisms, as well as their abiotic environment (Voroney, 2007).

3.2.1 Soil composition

Soil is a very complex structure and contains air, water, mineral and organic substances and various types of living organisms (Figure 3.3.); the soil formation is influenced by climate, living organisms, materials below the soil surface and weather.

Over time, a simple mixture of sand, silt, clay and organic matter will grow into a soil profile consisting of two or more layers called horizons. They differ from each other in texture, structure, density, porosity, temperature, color, thickness and reactivity. The horizons have no sharp boundaries with each other. Mature soil in

temperate regions has three main horizons (A~25cm; B~75cm; C~120cm) which are shown in Figures 3.4. and 10.5.

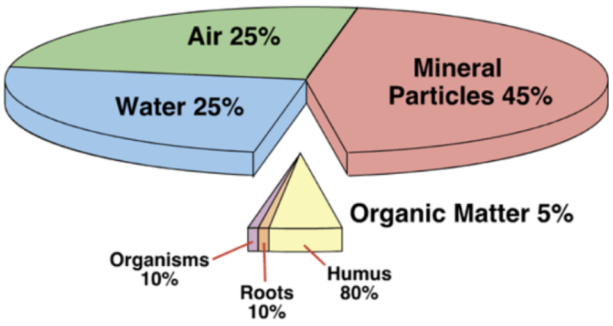

Figure 3.3: Soil composition.

Horizons A and B are also called "*solum*" or "*real soil*", since most chemical and biological activities take place in these layers (Sastre et al. 2002). In areas where the climate is tropical, the soil can have only one horizon.

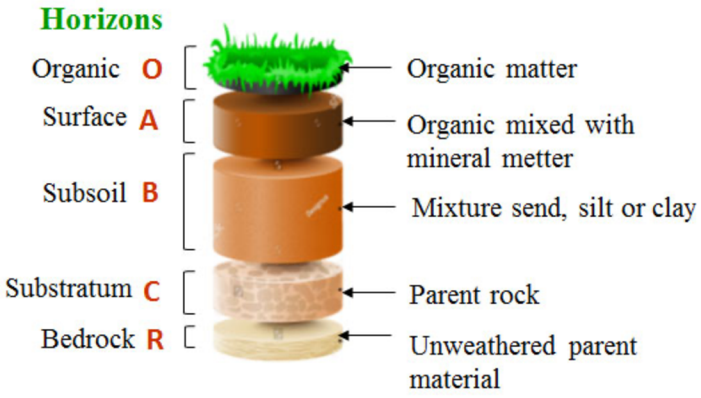

Figure 3.4: Soil profile.

3.2.2 Soil organic matter

The term organic matter of the soil means the total organic material in the soil, including macroorganic material that is scattered on the surface of the soil (litter) necessary for the circulation of nutrients in forests and meadows, then light fractions (plant residues in different stages of decomposition), microbial biomass (the most common are bacteria, *Actinomycetes*, fungi, algae and *Protozoa*), water-soluble organic substances and stabilized organic matter – humus (Stevenson, 1994).

Humus is the main factor that determines the character of the soil profile and which is mainly concentrated in the upper layers of the soil (at a depth of up to 15 cm). One of the many definitions of humus is that humus is a "complex and quite resistant

mixture of brown or dark brown amorphous and colloidal organic compounds that are formed during the microbiological decomposition of larger molecules, but also during microbiological synthesis. This mixture has very important chemical and physical properties for the soil and the organisms that live there" (Mirsal, 2008). Humus is a product of decomposition of various surface waste materials (litter) but also a product of root decomposition. In the average soil, humus contains about 4-6% of organic matter composed of dead matter (which makes up about 85% of organic matter), live roots and rhizomes (about 8.5%) and living organisms (about 6.5%) (Mirsal, 2008).

The role of soil organic matter is its importance in plant growth through its effect on the physical, chemical and biological properties of the soil (Magdoff & Van Es, 2021). The organic substances also have a nutritional effect in which it serves as a source of nitrogen, phosphorus and sulfur necessary for plant growth. The biological function of an organic substance is its significant effect on the activity of microflora and microfauna, but also its physical function in creating a good soil structure, whereby aeration and retention water capacity increase (Stevenson, 1994).

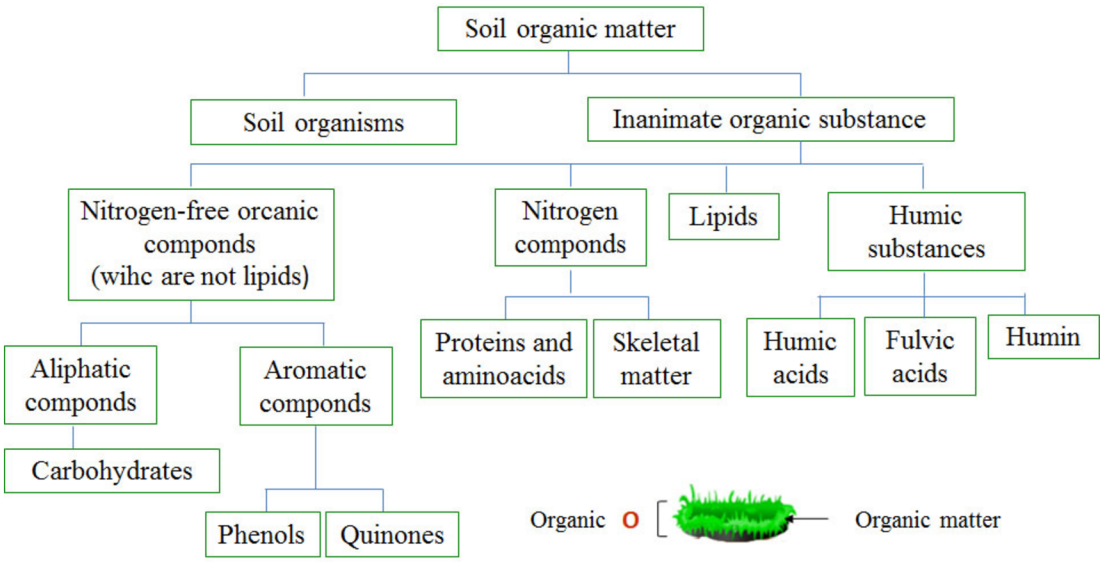

Figure 3.5: Classification of soil organic matter (Mirsal, 2008).

3.2.3 Soil organisms

Living organisms can be divided into macrofauna, mesofauna and microorganisms (microfauna and microflora) according to their size. Macrofauna includes mollusks, beetles, larvae of large insects, as well as some vertebrates that live underground and feed on smaller organisms (Mirsal, 2008).

The mesofauna consists of four basic groups:

1. Nematodes. Unsegmented worms that reach a size of 0.5-1 mm. In the soil of 1 m2 and depth of 30 cm there are between 106 - 2 x 107 of these organisms.
2. Arthropods. These include mites, hexapods (but not insects), centipedes, plant lice, beetles, insect larvae and termites
3. Anelide. Segmented worms, earthworms.
4. Molluscs. Lengths of 2-20 mm. They include snails (Mirsal, 2008).

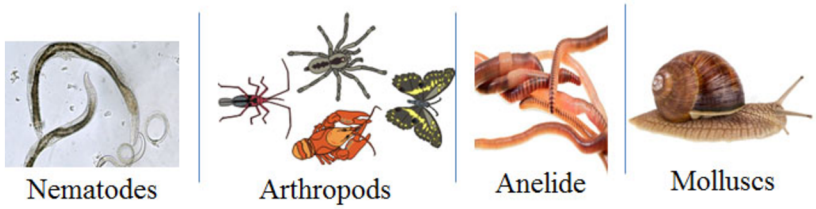

Figure 3.6: Some representatives of the mesofauna in the soil.

Microorganisms are usually divided into four groups:

1. Bacteria. These are single-celled organisms whose cell size is between 0.1 and 20 microns. Due to the high rate of reproduction, there are about 1012-1015 cells in one m^3 of soil. The effects of bacteria in the soil are great, because they decompose a large number of different materials under different conditions, e.g., oxidation of reduced sulfur compounds by *Acidithiobacillus ferrooxidans* (Zhang et al. 2018) or formation of nitrogen-fixing nodules on the roots of legumes by *Rhizobium sp*. (Lindström & Mousavi, 2020) Some bacteria are able to metabolize a wide range of chemicals (e.g., *Pseudomonas*, which metabolizes pesticides; Van Eerd et al. 2021).

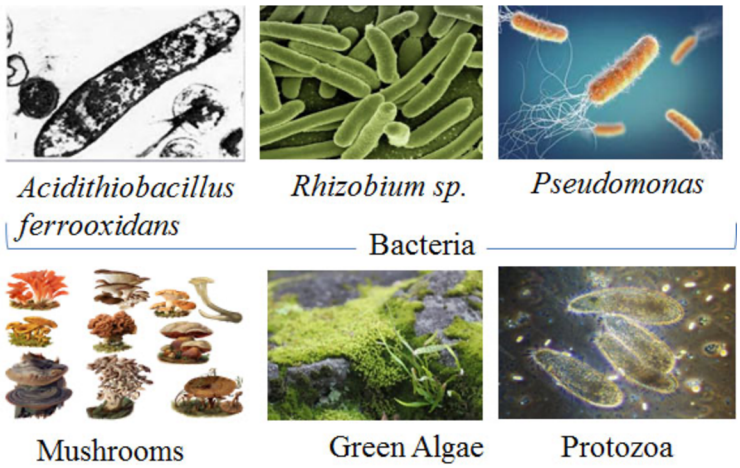

Figure 3.7: Some representatives of the soil microorganisms.

2. Mushrooms. Depending on whether they use dead organic material or a living organism, fungi can be saprophytic or parasitic. They have a filamentous structure (hyphae) whose diameter is about 0.5-10 μm and which build a dense

network - mycelium. They grow mainly on the surface of the soil, especially where the conditions are more acidic. Some fungi live as symbiosis on plant tissue. If the conditions are favorable (primarily acidity) they can be responsible for the decomposition of up to 80% of the organic matter of the soil (Becher et al. 2021).

3. Algae. Photosynthetic organisms limited to the upper soil layers (Paul, 2007).
4. Protozoa. They live on an aqueous film that encompasses soil particles. They control the number of bacteria and fungi (Paul, 2007).

3.2.4 Inanimate organic material

Soil organic material, which is formed by the metabolic action of soil organisms, but also by the decomposition of organic matter, can be divided into four classes (Figure 3.5.):
- organic compounds that do not contain nitrogen in their structure (and which are not lipids);
- nitrogen compounds;
- lipids;
- complex compounds, including humic substances (Mirsal, 2008).

The decomposition of organic material in the soil can be divided into several stages. Earthworms, worms, etc. play an important role in reducing the amount of plant residues. Microorganisms are responsible for further transformations. The initial phase of microbiological decomposition is characterized by the rapid loss of easily degradable organic substances, resulting in the release of CO_2, NH_3, H_2S, and by-products such as organic acids and other incompletely oxidized substances. In the next few phases, intermediates and newly formed microbial biomass are degraded by a large number of microorganisms. In the last phase, there is a gradual decomposition of more resistant parts of plants (such as lignin) in which actinomycetes and fungi play the most important role (Stevenson, 1994).

3.3 SOURCES OF SOIL POLLUTION

The soil pollution caused by various anthropogenic activities origins from main sources listed by Mirsal (2008); see Figure 3.8.

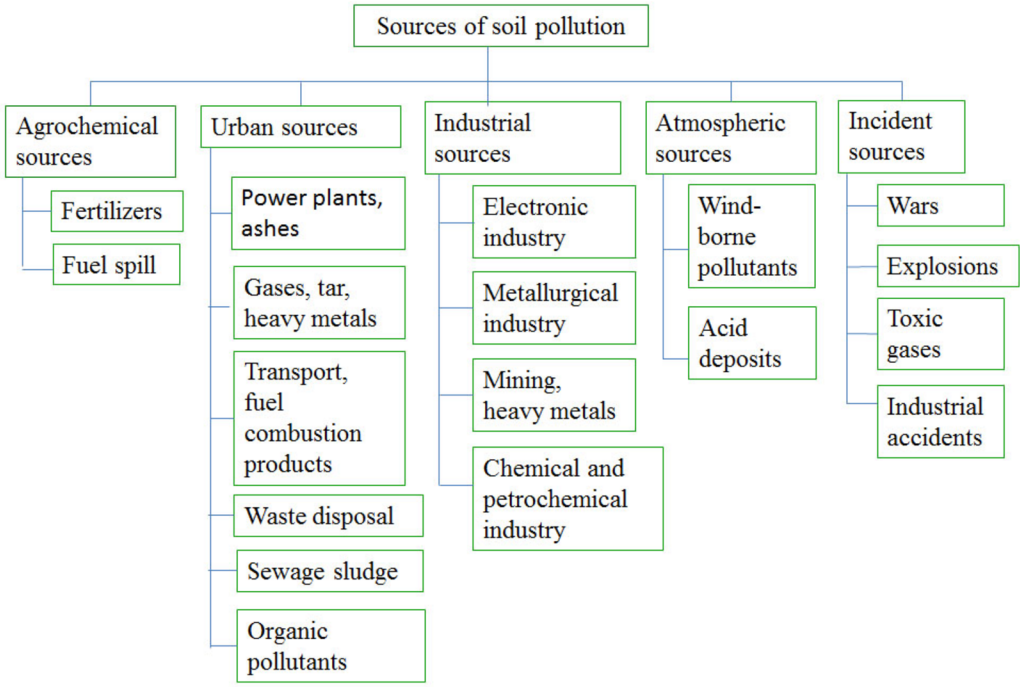

Figure 3.8: Sources of soil pollution (Mirsal, 2008).

3.3.1 Environment pollution by oil, petroleum products and other organic pollutants

Pollution of the environment with oil and its derivatives and disturbance of the natural balance is the result of accidental spills during exploitation, transport, processing, storage and use. Contaminants reach the soil mainly through their careless application, spillage and leakage, as well as atmospheric deposition. Only 10% of pollution comes from large incidental spills, which cause contamination of the sea, lakes or watercourses and attract significant media attention. It is estimated that about 5 million tons of crude oil and its derivatives reach the environment each year as a result of anthropogenic activities, of which 2.3 million tons reach the seas and oceans (Beškoski, 2011; Godleads et al. 2015).

Organic pollutants can be divided into following groups according to their chemical structure (Das et al., 2012):
- aliphatic hydrocarbons (e.g., alkanes released during oil spills or formed by petrochemical industry activity);
- alicyclic hydrocarbons;
- aromatic hydrocarbons (e.g., monoaromatic petrochemical solvents benzene and toluene or polyaromatic pyrene);
- chlorinated aliphatic hydrocarbons (chloroform);
- chlorinated flavors (polychlorophane biphenyls (PCBs), dichloro-diphenyl-trichloro-ethene (DDT) and dioxins);

- aromatics containing nitrogen (trinitro toluene - TNT).

The rate of biodegradation of oil and its derivatives in the soil depends on the nature and amount of hydrocarbons, the presence and degradation properties of microorganisms in the soil, as well as the characteristics of the soil itself (Beškoski et al., 2012).

3.4 REMEDIATION OF POLLUTED ENVIRONMENTS

The aim of remediation is to bring the polluted environment into a sustainable environment, while the concentration of pollutants is reduced below the legally allowed maximum (Mirsal, 2008). In the case of land, remediation can be reported at the place where the pollution is located (*in-situ*) or after being taken to a special plant (*ex-situ*). Remediation can be reported using physical and chemical methods or bioremediation (Mirsal, 2008).

3.4.1 Planning and implementation of soil remediation

A successful remediation plan is based on information obtained during preliminary research procedures that must be done before remediation can begin. These tests must answer the following questions:
- what are the types and what is the chemical nature of the pollutants at the test site;
- degree of pollution and dimensions of polluted space;
- what is the level of risk;
- which remediation techniques would be most optimal for the contaminated site;
- whether there are financial restrictions for selected remediation methods (Mirsal, 2008).

3.4.2 Remediation technologies

Depending on the degree of pollution, risk levels, financial and time constraints, soil treatment can start at the site of pollution (*in-situ*) or can be transferred to a plant specifically designed and adapted for bioremediation (*ex-situ*). Vidali (2001) defined the *in-situ* and *ex-situ* techniques as "*in-situ* those that are applied to soil and groundwater at the site with minimal disturbance" and "*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)".

There are four classes of remediation that can be abiotic and biotic (Beškoski et al. 2012):

1. chemical and physical methods,
2. biological methods,
3. fixation methods (storage and immobilization methods),
4. methods of thermal destruction.

Each of these methods can be performed by different procedures (Figure 3.9).

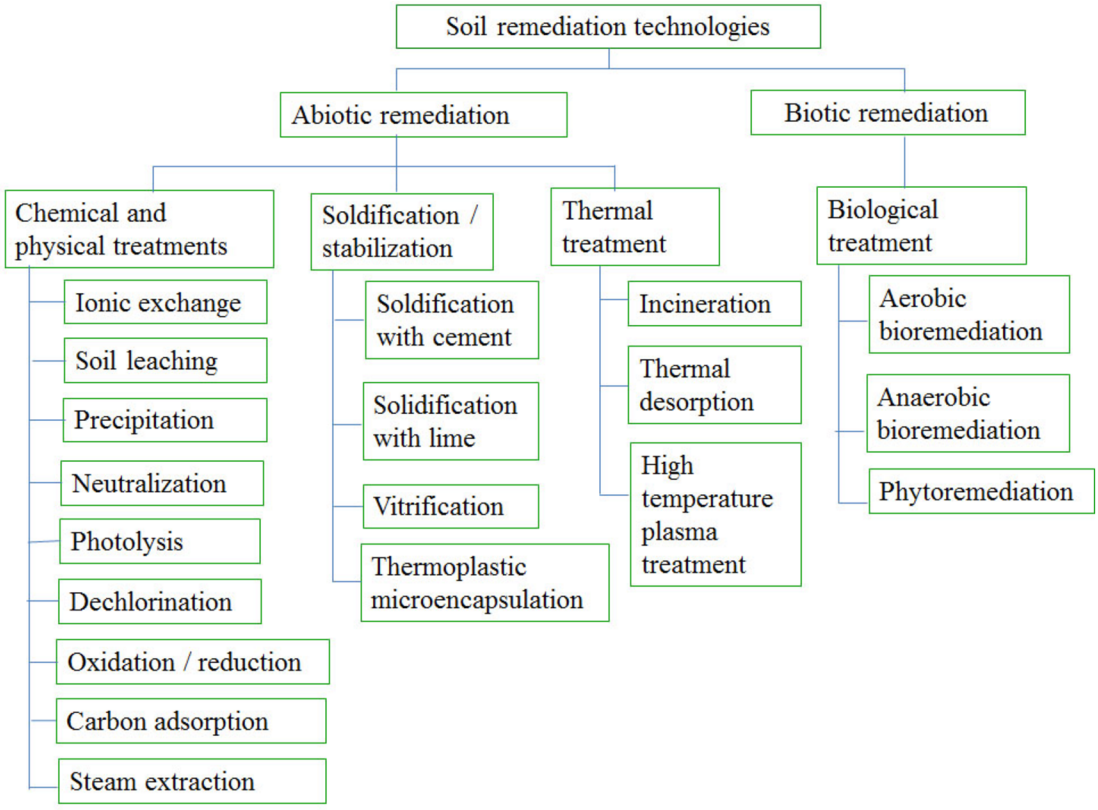

Figure 3.9: Remediation technologies (Mirsal, 2008).

Nowadays there are many technologies that are available for the treatment of contaminated sites, the choosing depends on site and contaminant characteristics, regulatory requirements, expenses, and time constraints (Khan et al. 2004). Combination of treatment processes may offer the most effective remediation. As noted in Table 3.9, various remediation technologies can be divided, according to the underlying mechanisms, into physical/chemical, biological, and thermal technologies. Technologies based on physical/chemical principles include, but are not limited to, solidification/stabilization, physical separation/chemical extraction, soil vapor extraction, soil washing, air stripping and pump-and-treat with granular activated carbon. Thermal treatments are unique in environmental remediation, from incineration to thermally enhanced soil vapor extraction and thermal desorption using steam, as well as high-temperature vitrification and pyrolysis. There are many varieties of biologically based remediation for contaminated soil, including bioremediation, slurry-phase bioremediation, bioventing, solid-phase bioremediation, composting and phytoremediation (Zhang, 2020).

Bioremediation is a more eco-friendly (least damaging to the environment, especially when applied *in-situ* and corresponds to the strategy of sustainable development), cost-effective and efficient environmental clean-up technique when compared to chemical and thermal treatments or physical removal of hazardous compounds from

contaminated soil (Beškoski et al. 2012; Tyagi, et al. 2011). **Enhanced bioremediation**, is an application biostimulation and/or bioaugmentation in a contaminated area in order to accelerate natural biodegradation processes (Philp & Atlas, 2005). The introduction of living microbial biomass into the soil for the purpose of soil purification is called **bioaugmentation**. The addition of nutrients needed by indigenous hydrocarbon degrading microorganisms is called **biostimulation** (Parra-Guevara & Skiba, 2014). Enhanced bioremediation (involving bioaugmentation and/or biostimulation), being an economical and eco-friendly approach, has emerged as the most advantageous soil clean-up technique for contaminated sites containing toxic elements and/or organic pollutants (Tyagi, et al. 2011).

3.4.3 Bioremediation - biological treatment of soil remediation

Biological treatment of contaminated soil is a remediation technique that uses microorganisms that are naturally present in the soil, and which are able to degrade toxic substances (Supplementary table 9.1.). These organisms include bacteria, fungi and yeasts. Some bacteria are able to digest a wide range of organic contaminants that are otherwise difficult to separate or degrade with previously known methods (Mirsal, 2008).

Bioremediation is an easy and effective method for removing organic contaminants (such as oil and other products derived from oil and its derivatives). For microorganisms, pollutants are a substrate for growth, by converting them into carbon-dioxide and water. The bioremediation process accelerates and optimizes the natural processes of biodegradation (so-called - stimulated bioremediation) by:

- aeration (oxygen addition below the soil surface, which stimulates aerobic decomposition processes);
- biostimulation (nutrient addition - nitrogen, phosphorus, oxygen); and
- bioaugmentation (microorganism addition - primarily bacteria, but also yeasts and other microbes) (Beškoski et al. 2012).

The time required to complete remediation depends on whether it is performed *in-situ* or *ex-situ* (Table 3.2). *In-situ* bioremediation refers to the bioremediation process which is carried out at the original site of the contamination. *In-situ* bioremediation process is primarily used to treat contaminations in soil and ground water. The remediation rate and the effectiveness of the process depend on various factors, such as:

- the type of the contaminant,
- contaminant distribution and concentration site-specific characteristics,
- microbial community of the site,
- temperature of the site,
- pH of the medium,
- moisture content,

- nutrient supply, (Đukić, et al 2013).

The manipulation of these factors is not highly feasible in *in-situ* bioremediation. Because of that, e*x-situ* technologies are usually faster and more efficient than *in situ* technologies (Mirsal, 2008). *Ex-situ* bioremediation is a process which treats the contaminants away from the site where they were found. Contaminants are excavated or pumped out from the original location and treated within the controlled environments. For this purpose, remediation of bio-piles (Figure 3.10.), special reactors or tanks can be used (Mirsal, 2008). A broad range of hydrocarbons is purified by ex situ bioremediation. Contaminated soils are excavated and placed on the surface of the ground and treated using indigenous microorganisms. *Ex-situ* bioremediation can be controlled and managed which ensures needed conditions (Mirsal, 2008).

Table 3.2. What is the difference between *in-situ* and *ex-situ* bioremediation?

***In-situ* bioremediation**	***Ex-situ* bioremediation**
process is performed at the original site of the contaminant	process is performed out of the location where the contaminant is found
this process is less expensive	this process is expensive
this process is less thorough	this is a more thorough remediation method
this process is less manageable	this process is manageable
this process is less effective	this process is more effective

Figure 3.10: Bioremediation bio-pile.

In their research Tomei and Daugulis (2013) ask and answer two important questions:

1) Why use *ex-situ* methods? The answer lays in the predictability and high efficiency of *ex-situ* bioremediation methods.

2) When to use *ex-situ* methods? Primary, when a safe and highly effective intervention is required (in the presence of severe contamination of extremely hazardous compounds). Second, when a high degree of treatment is required when the contaminated area is easily approachable for excavation, and when the contamination is concentrated in the superficial soil layer.

Biological treatment can be performed under aerobic and anaerobic conditions. When there is presence of sufficient oxygen (aerobic conditions), and other nutrient elements, microorganisms will eventually transform various organic contaminants to carbon-dioxide, water, and microbial cell mass (FRTR, 2020).

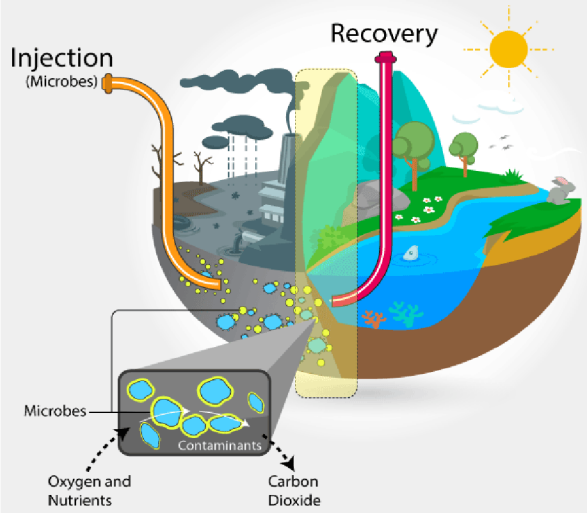

Figure 3.11: Stimulated bioremediation.

Stimulated (improved) aerobic bioremediation is often used, when in addition to the adding of oxygen, groundwater or unpolluted water is injected (to which nutrients have been added that are saturated with dissolved oxygen). Sometimes other sources of oxygen (e.g., hydrogen-peroxide) as well as acclimatized microorganisms (bioaugmentation) are added (FRTR, 2020).

When there is absence of oxygen (anaerobic conditions) - the organic pollutants will be metabolized to methane, limited amounts of carbon-dioxide, and trace amounts of hydrogen gas. Anaerobic degradation is much slower than aerobic, but is significant in conditions of reduced oxygen concentration and very often allows the decomposition of polychlorinated biphenyls (PCBs) (Beškoski, 2011).

Aerobic bioremediation is effective for remediation of soil contaminated with oil hydrocarbons, pesticides and nitrotoluenes, especially when the degree of contamination is low or when the contaminant leaks from the underground reservoir in small quantities. The advantages of this method are minimal disturbance of the topography of the terrain, no waste generation, easy to operate and control and low energy demand. However, high concentrations of pollutants can be toxic to

microorganisms, to can lead to increased mobility of the contaminant and its passage into groundwater, and under anaerobic conditions the contaminant can be transformed into an even more toxic compounds (Beškoski, 2011).

Table 3.3. Environmental conditions affecting degradation (Vidali 2001).

Parameters	Condition required for microbial activity	Optimum value for an oil degradation
Soil moisture	25-28% WHC[1]	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	15-45°C	20-30°C
Contaminant	Not too toxic	Hydrocarbon 5-10% of dry weight of soil
Toxic elements	Total content 2000 ppm	700 ppm
Type of soil	Low clay or silt content	-

[1]WHC - of water holding capacity (WHC is the ability of soil to hold its own or added water during the application of force, pressure, centrifugation, or heating).

In the process of bioremediation, environmental conditions have a great influence Table 3.3. (Dibble & Bartha, 1979). The most significant chemical and physical characteristics of soil which can influence the process of bioremediation are: temperature, pH, carbonate content, density and water retention capacity, moisture, presence of toxic elements, as well as the availability of oxygen and nutrients (Rogers et al., 1993). Bioremediation process is slowed down by low temperatures. When contaminated sites have low soil temperature, heat blankets could be used to cover the soil surface so that soil temperature is increased and with that the degradation rate (FRTR, 2020).

3.4.4 The role of microorganisms in the environment bioremediation

With the continuous development of industry and global dependence on fossil fuels, as well as the development of agrochemical measures, the natural environment is contaminated with a whole spectrum of organic pollutants and toxic elements. Two bioremediation processes can be used to degrade hydrocarbons: bioaugmentation and biostimulation. The introduction of living microbial biomass into the soil for the purpose of soil purification is called **bioaugmentation**. The addition of nutrients needed by indigenous hydrocarbon degrading microorganisms is called **biostimulation** (Parra-Guevara & Skiba, 2014).

The degradation of hydrocarbons by microorganisms begins by the conversion of the polycyclic aromatic hydrocarbon or alkaline chain into alcohol. Then by oxidation

alcohol converts to an aldehyde and then into an acid and at the end into water, carbon dioxide and microbial biomass (Parra-Guevara & Skiba, 2014). The main precondition for the application of bioaugmentation is that microorganisms can synthesize appropriate enzymes that participate in the decomposition of pollutants, as well as those microorganisms in biomass are resistant to metals in concentrations located at the site of bioremediation (Kothe & Varma, 2012). Namely, some pollutants are synthetic and microorganisms have not developed the ability to synthesize enzymes that are necessary to catalyze degradation processes. In addition, some organic pollutants that enter the soil are toxic to microorganisms (Paul, 2007).

Hydrocarbon decomposing microorganisms are widespread in soil, salt and fresh water. Of the various types of microorganisms, bacteria, yeasts and fungi are the main degraders of hydrocarbons. On the other hand, algae and protozoa have been found to play no significant role in hydrocarbon degradation (Bossert & Bartha, 1984; O'Brien & Dixon, 1976). Microorganisms that are capable of decomposing naturally occurring hydrocarbons in the environment can also decompose hydrocarbons originating from petroleum pollutants. Bacteria play the most important role in the degradation of petroleum pollutants and act as primary degraders of hydrocarbons derived from spilled oil (Brito et al. 2006). Also, molds have been observed as hydrocarbon degraders (Chaillana et al. 2004). Mixed microbial cultures have an advantage due to the wider degradation potential: synergism (Beškoski et al. 2012). The fastest and most complete degradation of most organic pollutants is done under aerobic conditions (Novaković, 2013).

In order for microorganisms to be used in the process of bioremediation, it is necessary to isolate, cultivate and determine the strains beforehand (Paul, 2007). Strains can also be isolated from unpolluted environments, but they are much less efficient (Kothe & Varma, 2012). Microorganisms need small amounts of essential elements (micronutrients such as Cu, Zn, Fe, etc.). These micronutrients are part of enzymes and growth hormones. The essential trace elements that are necessary for microorganisms are iron, zinc, manganese, copper, boron, molybdenum, nickel, cobalt, chromium, selenium and tin (Paul, 2007).

The important groups of microorganisms in the process of bioaugmentation are the following (Vidali, 2001):

- *aerobic bacteria* use the contaminant as the sole source of carbon and energy and they can to degrade hydrocarbons, pesticides as well as alkanes and polyaromatic compounds;

- *methylotrophs* are aerobic bacteria that grow utilizing methane by containing the enzyme methane-monooxygenase which has a wide range of substrates on which it can act (such as. 1,2-dichloroethane and chlorinated aliphatics trichloroethylene);

- *anaerobic bacteria* they can be used for decomposition of polychlorinated biphenyls in river sediments as well as for dichlorination of the solvent trichloroethylene, and chloroform; in practice they are used less frequently than aerobic bacteria;

- *ligninolytic fungi* have the ability to degrade an extremely diverse range of toxic environmental pollutants (e.g., polycyclic aromatic hydrocarbons; Bamforth & Singleton, 2005);

- *yeasts* can degrade a wide range of hydrocarbons (alkanes, aromatic, and polyaromatic hydrocarbons) as well as toxic elements (Bahafid et al. 2017.); their ability to decompose high amounts of organic pollutants at temperatures down to 1° C has been established, which is very important for their application in bioremediation processes in areas that permanently low temperatures (Margesin, 2014).

3.4.5 Benefits and limitations of applying the bioremediation process

The process of bioremediation has many benefits, however, a few boundaries can be noticed (Vidali, 2001.).

The benefits of bioremediation process:

1. Bioremediation is a natural process and because of that it is seen as an adequate waste treatment process for polluted material like soil. Microbes that are capable of degrading the pollutant rise in numbers when the pollutant is present; when the pollutant is degraded, the biodegradative population decreases. The remnants from the process are usually harmless products such as carbon dioxide, water, and cell biomass.

2. In theory, bioremediation is effective in the complete destruction of a broad variety of pollutants. Numerous pollutants that are legally considered to be hazardous can be altered to harmless products. This eliminates the risk of future responsibility related to the treatment and disposal of contaminated material.

3. Rather than relocating pollutants from one environmental medium to another, for instance, from soil to air or water, total destruction of intended pollutants is possible. Bioremediation can be conducted on site, usually without causing a significant disturbance of normal activities. *In-site* bioremediation excludes the need of transporting quantities of waste off site and the possible threats to human health and the environment that can emerge during transportation.

4. Bioremediation can be way cheaper compared to other techniques that are used for clean-up of hazardous waste.

5. Bioremediation is one of the most cost-effective methods of soil remediation. It often takes longer than other methods, but is significantly cheaper.

6. Bioremediation is a process that doesn't produce other types of pollution, unlike others, such as solidification, where solid material that remains needs to be disposed of. During bioremediation final products are CO_2, H_2O, and smaller molecules that are not harmful for the environment.

7. By applying the process of bioremediation the degree of purification is more than 90% (Parra-Guevara & Skiba, 2014).

The limitations of bioremediation (Boopathy, 2000; Vidali, 2001.):

1. Bioremediation is restricted to those compounds that can be biodegraded. Not all compounds can be liable to fast and complete degradation.

2. There are some concerns that the products of biodegradation could be more persistent or toxic compered to parent compound.

3. Biological processes are usually very specific. Influential site factors needed for success include the presence of metabolically capable microbial populations, appropriate environmental growth conditions, and appropriate levels of nutrients and contaminants.

4. It can be complicated to extrapolate from bench and pilot-scale studies to full-scale field operations.

5. Further research is necessary to develop and engineer bioremediation technologies that can be suitable for areas with complex mixtures of contaminants that aren't evenly spread in the environment. Contaminants can exist as solids, liquids, and gases.

6. Processes such as excavation, removal of soil or incineration are much faster than bioremediation processes.

7. Regulatory uncertainty remains concerning satisfactory performance criteria for bioremediation process. There isn't defined definition of "clean", evaluating performance of bioremediation is tricky, and there aren't acceptable endpoints for bioremediation treatments.

Supplementary table 9.2. summarizes the benefits and limitations of bioremediation.

4 METHODOLOGY

4.1 CHARACTERISTICS OF THE STUDY AREA

The sample of contaminated soil consisted of soil from different areas in Serbia that were contaminated with waste oils from petroleum products. Contaminated soil was delivered to Dobanovci in Serbia.

The *ex-situ* bioremediation process was performed at the BREM plant in Dobanovci (44°49′22″N 20°13′19″E /44.822833°N 20.222°E). Dobanovci is a city settlement in the city municipality of Surčin in the city of Belgrade. Belgrade is the capital of Serbia, lying at the confluence of the Sava and the Danube, where the Pannonian Plain joins the Balkan Peninsula (44 ° 49'14 "N 20 ° 27'44" E), Figure 4.1.

Figure 4.1: Geographical position of Dobanovci.

Chemical analysis was conducted at the Institute of Chemistry, Technology and Metallurgy (IHTM), University of Belgrade. Isolation of microorganisms was done in the field and multiplication of microorganisms was done at IHTM and BREM GROUP doo Company (Beograd-Kneževac).

4.2 PREPARATION THE BIO-PILE FOR *EX-SITU* BIOREMEDIATION OF SOIL CONTAMINATED WITH FUEL OIL

Bio-pile for bioremediation was made on a waterproof asphalt surface, dimensions 1000 m^3 and 1250 m^2 (mass approx. 15000 T; Figure 4.1.A) (Beškoski et al. 2011). Bio-pile consisted of a mixture containing soil contaminated with waste oil (e.g. expired oil, or oil used in engines and later discarded). So, the bio-pile had contained oil hydrocarbons. Due to the increase in porosity, river sand (300 m^3) was added.

To ensure homogeneity, the bio-pile was mixed with a bulldozer and finally leveled with a tractor. The final dimensions of the bio-pile were 75 x 20 x 0.5-0.8 m (length x width x height). The optimal ratio of nutrients - C: N:P: K (100: 10: 1: 0.1) was

achieved by biostimulation. Namely, dissolved ammonium nitrate, ammonium phosphate and potassium chloride were sprayed all over the bio-pile (Figure 4.2.B).

Figure 4.2: A: Preparation of bioremediation bio-pile. B: Bio-pile inoculation and biostimulation.

Bio-pile contained 1000 m^3 of substrate for bioremediation. During bioremediation (6 months - January-June, 2021), the bio-pile was soaked, rotated and mixed every 15 days to achieve the necessary humidity and aeration. Bio-pile was inoculated every 30 days with a prepared consortium of microorganisms (*Pseudomonas sp., Bacillus sp., Rhodococcus sp., Brevibacterium sp.*).

After mixing, the bio-pile was covered with plastic polyethylene foil to avoid the direct influence of weather conditions. BioSolve CLEAR was also added to the holder in a volume of 70 mL of the original solution per cubic meter (which has the role of making "non-polar" pollutant molecules available to microorganisms). Chemical and microbiological indicators of bioremediation were monitored immediately after the preparation of the bio-pile and at the end of the bioremediation procedure which lasted for 6 months from January until June 2021.

At the beginning of the experiment (before the addition of microorganisms), a control sample of 10 m^3 of bio-pile was separated, which was also chemically and microbiologically analyzed.

4.3 SOIL SAMPLING

Soil samples were sampled with a soil auger (Eijkelkamp, Netherlands) with a depth of 0-70 cm, (Figure 4.3.A.). Sampling was performed from several positions on the bio-piles before/and after bioremediation/control bio-pile, and then the samples from the same bio-pile were mixed and homogenized. The individual composite samples thus obtained were used for analysis.

Individual soil samples for chemical determinations were packed in glass jars and for microbiological determinations in sterile Petri dishes (Figure 4.3.B.). The samples were transported to the laboratory in a field refrigerator at a temperature of 4°C and then stored in a cold chamber at the same temperature. Microbiological analyzes of all samples were performed immediately upon delivery to the laboratory, and chemical and physico-chemical within 12-24 hours.

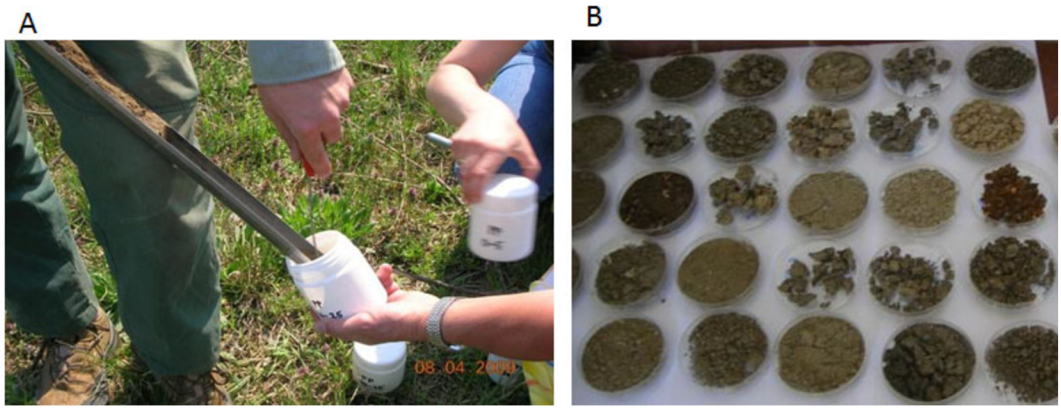

Figure 4.3: A: Soil sampling. B: Preparation of soil samples.

4.4 ANALYTICAL METHODS

4.4.1 Determination of the percentage of hygroscopic moisture

The moisture percentage was determined in a hygrometer (model MOC-120H; Shimadzu Co., Japan) with a sample drying program at 105°C, 1.5 h (ISO, 1993).

4.4.2 Potentiometric determination of pH value

The pH of the solid samples was measured in a suspension prepared from a homogenized sample and boiled demineralized water in a ratio of 1: 2.5 after 30 minutes of stirring on a magnetic stirrer according to the standard method (ISO, 2002). The measurement was performed on a digital pH meter (type pH 300i; WTW, Weilhem, Germany) with a combined electrode from the same manufacturer.

4.4.3 Determination of ash content

The ash content was determined in porcelain crucibles by burning the organic substance of the sample using a burner until the cessation of white vapor evolution, and then heating in an oven at 550°C and 800°C for 2 h. Determination of ash at 550°C was done in order to determine the loss of organic substance, while, determining the ash at 800°C showed the loss of carbonate (by subtracting the ash content (%) from 100%).

4.4.4 Determination of CO_2 content

The CO_2 and content of the samples was determined by the standard volumetric method (ISO, 1995).

4.4.5 Elementary analysis

The total content of: carbon, hydrogen, nitrogen and sulfur were determined from the dry powdered substance by automatic analyzer (Vario EL III, CHNS-O, Elemental, Germani). The inorganic carbon content was calculated on the basis of CO_2 content, while the organic carbon content was calculated as the difference between the total and inorganic carbon content (Wilke, 2005; Pansu & Gautheyrou, 2006).

4.4.6 Gravimetric determination of total petroleum hydrocarbons

Total petroleum hydrocarbons (TPH) were extracted according to ISO 16703 (2004) and gravimetrically determined according to DIN EN 14354 (2004) (Beškoski et al. 2011). Briefly, the hydrocarbons were extracted with a mixture of n-heptane and acetone (2: 1) and afterwards washed in a separatory funnel. The aqueous fraction, and with it everything that was dissolved in acetone, was discarded. The n-heptane fraction was further purified by passing through a column with previously inflicted florisil and anhydrous sodium sulfate (which actually acted as a specific filter). Sodium sulfate removed excess water, and florisil retained soil pigments. The heptane layer was collected in pre-weighed vegeglas, the substance was dried in a stream of nitrogen and then the mass was measured.

4.4.7 Gas chromatographic determination (GC) of petroleum hydrocarbons

Total petroleum hydrocarbons (TPH) were determined according to ISO 16703 (2004). This International Standard is applicable to the determination of hydrocarbons from $C_{10}H_{22}$ to $C_{40}H_{82}$ and is not applicable to the quantitative determination of hydrocarbons < C_{10}. The gas chromatographic analyses were conducted on a gas chromatograph - Agilent 7890A (United States) with a flame ionization detector (FID). A film chromatographic column HP-5 (30 m long, 0.32 mm in diameter, 0.25 μm thickness of stationary phase) was used. The carrier gas was hydrogen at a velocity of 30 cm/s. The chromatographic conditions were: the starting temperature was 60°C, the injector temperature was 250°C, and the detector temperature was 300°C; the temperature was ramped up at 4°C/min. For data processing ChemStation, Agilent Technologies software was used.

4.4.8 Determination of n-hexane extractable substances

n-Hexane extractable substances (HES) were determined by the modified EPA method (1998). Briefly, hexane-soluble substances were extracted with n-hexane

then the extract was evaporated on an evaporator and then (with gentle rinsing with n-hexane) passed through a column with previously inflicted florisil and anhydrous sodium sulfate. The mass of the n-hexane fraction was measured.

4.5 BIOLOGICAL PARAMETERS

In all microbiological procedures, the rules of work in microbiological chemistry were respected, and substances of purity *pro analysis* were used for the preparation of microbiological media (Gojgić-Cvijović & Vrvić, 2003).

4.5.1 Preparation of microbiological media

All microbiological media was prepared by suspending and/or dissolving of components in distilled water and autoclaved to 0.10 MPa for 20 minutes, unless otherwise noted. In all media the pH is adjusted by the addition of H_2SO_4 (1M) or NaOH (1M).

4.5.1.1 Media for determining the number of total hemoorganoheterotrophic aerobic and facultative anaerobic bacteria

Total hemoorganoheterotrophic mesophilic aerobic and facultative anaerobic bacteria (TB) were determined on nutrient agar. Nutrient agar was prepared by dissolving the dry medium according to the manufacturer's instructions (Torlak, 2010).

Media composition (g / L):

- pepton-1 "Torlak" 15 g
- meat extract "Torlak" 3 g
- sodium chloride 5 g
- potassium phosphate 0.3 g
- agar 18 g
- pH ≈ 7

4.5.1.2 Media for determining the number of total anaerobic mesophilic hemoorganoheterotrophic bacteria

Total anaerobic mesophilic hemoorganoheterotrophic bacteria (AN) were determined on nutrient agar with 0.5% glucose by seeding under anaerobic conditions.

4.5.1.3 Media for determining the number of yeasts and spores of molds

The total number of yeasts, and spores of molds (YM) was determined on malt agar which was prepared by dissolving the dry medium according to the manufacturer's instructions (Torlak, 2010).

Media composition (g/L):

- pepton-1 "Torlak" 5 g
- meat extract "Torlak" 30 g
- agar 18 g
- pH ≈ 5.7

4.5.1.4 Media for determining the total number of bacteria that decompose hydrocarbons

Hydrocarbon-degrading microorganisms (HD) were determined on a mineral hydrocarbon medium with diesel D2 (UG) as the sole carbon source (Miličić-Terzić et al. 2000, 2001).

Media composition (g / L):

- ammonium nitrate 1 g
- potassium hydrogen phosphate 0.25 g
- soil extract 50 mL
- agar 16 g
- demineralized water 1 L
- pH ≈ 7

After sterilization, diesel D2 in the amount of 2000 ppm was added to the microbiological media which is partially cooled (to the temperatures between 40 and 50 ° C) in the sterile zone (2 g or 2.35 mL).

4.5.2 Determination of the number of microorganisms

The number of microorganisms was determined by the method of serial dilution (Gojgić-Cvijović & Vrvić, 2003; Collins et al. 2004). Total grown colonies of hemoorganoheterotrophic mesophilic aerobic and facultative anaerobic bacteria (TB), as well as total anaerobic mesophilic hemoorganoheterotrophic bacteria (AN) were counted after 48 hours. Yeasts and spores of molds (YM) were counted after 72 hours, while colonies of microorganisms that decompose hydrocarbons (HD) were counted after 7 to 8 days. The seeded agar plates were incubated at 28 ° C.

4.6 STATISTICAL ANALYSIS

Data are expressed as mean and standard deviation of two replicates. Pearson¨s correlation coefficients ($p < 0.05$) among different parameters were computed to assess the relationships among these parameters. A one-way analysis of variance (ANOVA) at $p<0.05$, followed by Tukey's test, were applied to assess the effectivity of the bioremediation. Statistica software version 8.0 (StatSoft Co., Tulsa, Oklahoma, USA) was applied for statistical analysis.

5 RESULTS

Because of high adaptability to different environmental conditions and huge diversity there is a growing interest in microorganisms as sources of new biologically active substances and/or metabolic pathways.

5.1 Chemical characterization of the examined soil

Moisture content was first analyzed on soil samples before and after bioremediation, as well as on the control sample. The tested samples were found to contain 28.8-30.3 % moisture (Table 5.1). The ash content in the samples after incineration at 550°C was 65.2-96.3 %, and after incineration at 800°C it was 63.5-95.8 % (Table 5.1).

In this part of the research, the pH value of the examined soil before and after bioremediation was determined, as well as on the control sample. The pH values of the samples were from 7.0-7.5 (Table 5.1).

Table 5.1. Moisture and ash content (%) and pH value of the examined soil

Tested parameter	Initial state	After bioremediation	Control sample
Moisture	30.32 ± 0.68^{a}	$29.58 \pm 0.81^{a\,b}$	28.76 ± 0.25^{b}
Ash (550°C)	$65.64 \pm 1.10^{a}{}_{1}$	$96.34 \pm 0.64^{b}{}_{1}$	$65.18 \pm 1.68^{a}{}_{1}$
Ash (800°C)	$63.46 \pm 0.53^{a}{}_{2}$	$95.79 \pm 0.53^{b}{}_{1}$	$63.91 \pm 0.01^{a}{}_{2}$
pH	7.0 ± 0.06^{a}	7.9 ± 0.06^{b}	7.3 ± 0.06^{a}

Ash is expressed on dry matter.
Means in the same row with different roman letters in superscript are significantly different ($p < 0.05$).
Means in the same column with different number in subscript are significantly different ($p < 0.05$).

The loss of organic substance for the sample before bioremediation and the control sample was about 34% after incineration at 550°C and the loss of carbonate was about 36% after incineration at 800°C (Table 5.2). On the contrary, for the soil sample after bioremediation the registered loss of content organic substances was 3.66% after incineration of samples on 550°C and the loss of carbonate was 4.21% after incineration of samples on 800°C (Table 5.2).

The content of n-hexane extractable substances (HES) for the sample before bioremediation and the control sample was around 23-24%, while HES content for the soil sample after bioremediation was around 2% (Table 5.2).

Table 5.2. Content of organic substances, n-hexane extractable supstance and total petroleum hydrocarbons of the examined soil

Tested parameter	Initial state	After bioremediation	Control sample
%			
Organic substances (Loss of ignition at 550°C)	34.36 ±1.10 $^{a}{}_{1}$	3.66±0.64 $^{b}{}_{1}$	34.82 ±1.68 $^{a}{}_{1}$
Carbonate (Loss of ignition at 800°C)	36.54 ±0.68 $^{a}{}_{2}$	4.21 ±0.53 $^{b}{}_{1}$	36.09 ±0.25 $^{a}{}_{2}$
g/kg			
HES[1]	24.02 ±0.79 ^{a}	2.34 ±0.17 ^{b}	23.45 ±0.88 ^{a}
TPH[2]	22.82 ±0.66 ^{a}	1.62 ±0.14 ^{b}	22.13 ±0.83 ^{a}

The results are given on dry matter.
[1]HES - n-hexane extractable supstances;
[2]TPH - Total Petroleum Hydrocarbons;
Means in the same row with different roman letters in superscript are significantly different ($p < 0.05$).
Means in the same column with different number in subscript are significantly different ($p < 0.05$).

The content of total petroleum hydrocarbons (TPH), obtained by the gravimetric method, for the sample before bioremediation and the control sample was around 22%, while TPH content for the soil sample after bioremediation was 1.62% (Table 5.2).

5.2 Determination of total petroleum hydrocarbons by the gas chromatography (GC)

The GC spectrum of extracted total petroleum hydrocarbons (TPH) before bioremediation is shown on Figure 5.1., while the GC-spectrum after the performed bioremediation is shown in Figure 5.2.

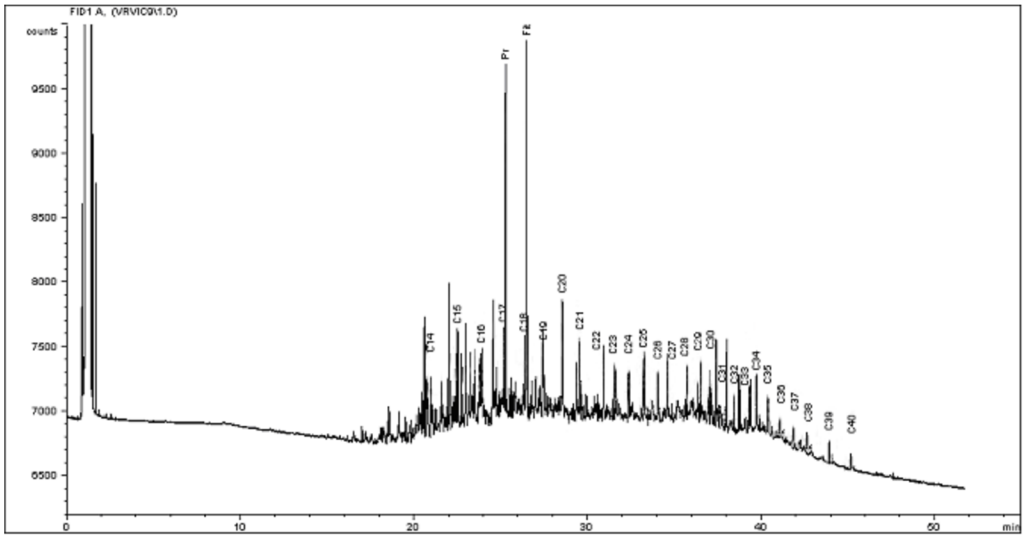

Figure 5.1. GC spectrum of extracted petroleum hydrocarbons before bioremediation.

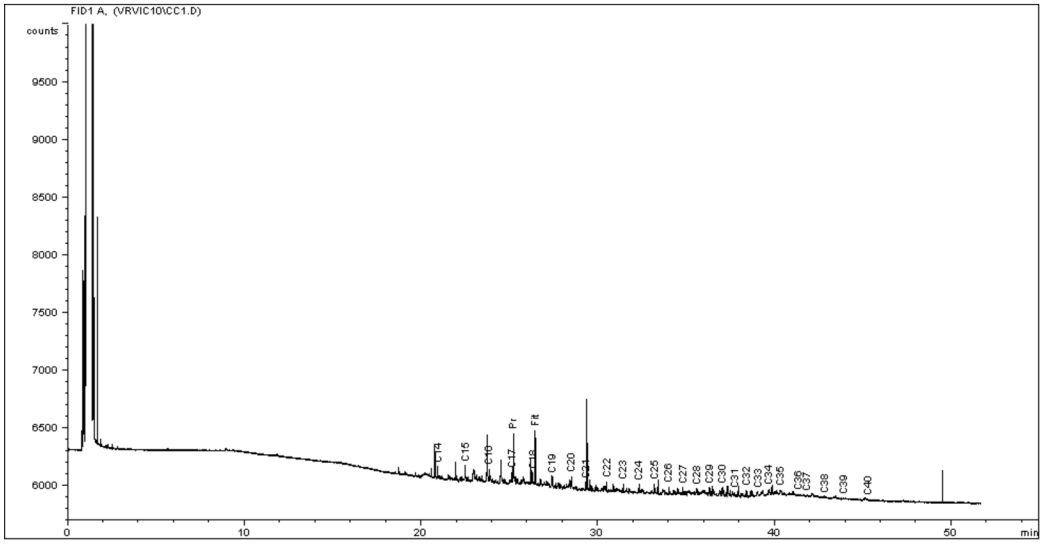

Figure 5.2. GC spectrum of extracted petroleum hydrocarbons after bioremediation.

5.3 Elemental analysis of the investigated soils

The total contents of carbon, hydrogen, nitrogen and sulfur and the results are presented in Table 5.3.

Table 5.3. Results of elemental organic analysis (%)

Test parameter	Initial state	After bioremediation	Control sample
C total	17.27 ±0.29 [a]	1.29 ±0.28 [b]	17.51 ±0.27 [a]
C organic	17.00 ±0.28 [a]	0.53 ±0.26 [b]	16.80 ±0.26 [a]
C inorganic	0.27 ±0.01 [a]	0.76 ±0.02 [a]	0.71 ±0.02 [a]
N total	1.05 ±0.07 [a]	1.15 ±0.11 [a]	1.11 ±0.04 [a]
H total	3.45 ±0.15 [a]	0.13 ±0.02 [b]	3.65 ±0.05 [a]
S total	0.07 ±0.01 [a]	0.08 ±0.01 [a]	0.07 ±0.01 [a]
CO_2	0.99 ±0.04 [a]	2.79 ±0.07 [b]	2.61 ±0.05 [c]

The results are on dry matter.
Means in the same row with different roman letters in superscript are significantly different ($p < 0.05$).

The total carbon content in the samples before bioremediation and in the control sample was around 17%. After bioremediation, the determined content of total carbon was 3.66% (Table 5.3). The inorganic carbon content was lower than 1% for all tested samples (Table 5.3). The organic carbon content was calculated from the difference between the total carbon content and the inorganic carbon and for the soil sample after bioremediation the registered content of organic carbon was lower than

1% (Table 5.3). However, the organic carbon content in the samples before bioremediation and in the control sample was around 17% (Table 5.3).

The contents of total nitrogen and sulfur were not significantly altered during the bioremediation. The hydrogen content in the samples before bioremediation and for the control sample was about 3%, while for the sample after bioremediation was lower than 1% (Table 5.3).

5.4 Microbiological analysis of the examined soil

The presence of total hemoorganoheterotrophic aerobic and facultative anaerobic bacteria in the soil before bioremediation was $2x10^9$ cfu/g, while after bioremediation it was $4x10^8$ cfu/g. In the control sample, the presence of these microorganisms was registered with $7x10^7$ cfu/g (Table 5.4).

The presence of yeasts, spores and molds in the soil before bioremediation was $2x10^6$ cfu/g, while after bioremediation it was $3x10^4$ cfu/g. In the control sample, the presence of these microorganisms was registered with $9x10^5$ cfu/g (Table 6.4).

Table 5.4. Results of microbiological analysis

Test parameter	Initial state	After bioremediation	Control sample
cfu[1]/g			
TB[2]	2×10^9	4×10^8	7×10^7
YM[3]	2×10^6	3×10^4	9×10^5
HD[4]	7×10^7	2×10^6	2×10^5
AN[5]	4×10^3	9×10^3	1×10^4
%			
Bioremediation potential (HD/TB · 100)	3.5	0.5	0.29

The results are on dry matter.
[1] cfu/g - colony forming unit;
[2]TB - total hemoorganoheterotrophic aerobic and facultative anaerobic bacteria
[3]YM - yeasts, spores and molds;
[4]HD - bacteria that degrade (decompose) hydrocarbons;
[5]AN - anaerobic bacteria.

The presence of bacteria that degrade/decompose hydrocarbons in the soil before bioremediation was $7x10^7$ cfu/g, while after bioremediation it was $2x10^6$ cfu/g. In the control sample, the presence of these microorganisms was registered with $2x10^5$ cfu/g (Table 5.4).

The presence of anaerobic bacteria in the soil before bioremediation was $4x10^3$ cfu/g, while after bioremediation it was $9x10^3$ cfu/g. In the control sample, the presence of these microorganisms was registered with $1x10^5$ cfu/g (Table 5.4).

6 DISCUSSION

High adaptability to different environmental conditions and huge diversity is the reason why there is interest in microorganisms as sources of new biologically active substances. In this study, a bioremediation procedure by microorganisms was applied in order to detoxify the soil consisted from different areas in Serbia that were contaminated with waste oils from petroleum products was performed. Contaminated soil was delivered to Dobanovci in Serbia, where the bioremediation measures were provided.

6.1 Basic chemical analyzes

Literature data indicate, that the moisture has a strong effect on biochemical processes in contaminated soils (Boopathy, 2000). The soil moisture content of all tested samples was increased compared to the results obtained by Beškoski et al. (2011) who registered a moisture content of 13.4% after bioremediation (in soil contaminated with fuel oil due to a leak from the power plant after a breakdown, Obrenovac, Serbia). The reason for this may be that the preparation of the bio-pile in this study was done in winter conditions, when moisture content in the soil is increased. The moisture content of the bio-pile during bioremediation was within the limits that are optimal for this technique (12%-30%; Beškoski et al. 2012). The range of values of optimal moisture for the bioremediation process arises from the need to provide an adequate environment for the growth of microorganisms, in order to avoid excessive moisture content which increases diffusion resistance to transport oxygen to microorganisms (Tomei & Daugulis, 2013). Thus, too much humidity reduces aeration in soil, while insufficient moisture limits and reduces the growth of microorganisms (Beškoski et al. 2012).

According to the obtained results, there was no significant difference in the content of ash between the samples before bioremediation and the control sample regardless of the incineration temperature (Table 5.1.), which was to be expected, since the control sample was not treated by addition of microorganisms. Compared to these samples, the soil sample differs significantly after the applied bioremediation. After bioremediation, the ash content in the samples was increased by about 30% (regardless of the incineration temperature). This change in ash content was consistent with a significant reduction in organic matter (after incineration at 550°C) and carbonate (after incineration at 800°C) in soil samples after bioremediation. Namely, after incineration at a temperature of 550°C, a significant loss of organic substances (~30%) as well as after incineration at 800°C, a significant loss of carbonate/inorganic substances (~30%) in the soil after bioremediation was registered. This result was confirmed a very high degree of success of the applied bioremediation, that is, efficient work of microorganisms, since the soil pollution was mainly caused by organic pollutants. Namely, the content of organic matter in

the soil after bioremediation is ~30% lower than in the control sample. The loss of organic substances and carbonate for the samples before bioremediation were close to the values for the control sample and do not differ statistically.

Statistical difference was found between the ash content in the soil samples without bioremediation and in the control sample depending on the applied incineration temperature. At a combustion temperature of 550°C, organic carbon burns, while at 800°C, inorganic carbon burns. Considering that the organic and inorganic carbon content is one of the parameters of bioremediation process success (besides, for example, TPH and HES content), it is expected that these values of ash content differ in samples after bioremediation and control sample. Correlation analysis showed that the ash content obtained by burning the samples at 550°C was in a very strong correlation with the ash content obtained by burning the sample at 800°C (r = 1.00), which indicates that the loss of organic and inorganic carbon has the same trend. The content of after application of both combustion temperatures of the samples is in a very strong inverse correlation with the moisture content (r = -1.00) in the samples (Table 6.1), which indicates that if the moisture content is higher, the ash content will be lower.

Soil pH influences biodegradation through its effect on microbial activity, on microbial enzymes that aid in the degradation processes and on microbial community and diversity (Myazin et al. 2021). pH values in soil samples before bioremediation and in the control sample are close and do not differ statistically. Compared to these samples, the soil after bioremediation has a slightly increased alkalinity, which may be the result of the activity of microorganisms, as well as consequence of the buffering effect of some cationic substances of some elements (potassium, calcium, magnesium, etc.; Myazin et al. 2021). However, all samples had a pH value in the range that is optimal for the growth of microorganisms (pH = 6-8; Tomei and Daugulis, 2013) and for oil degradation (pH = 6.5-8; Myazin et al. 2021). Statistical analysis showed that soil pH values are strongly correlated with ash content (r = 0.87) and moisture content (r = -0.87), however, these dependences are not statistically significant at the probability level p = 0.05 (Table 6.1).

The fractions eluted with the solvent n-hexane (HES) are referred to as alkanes (Lin et al. 2010). The content of HES and TPH were very strongly correlated (r = 1.00; Table 6.1). If we observe that the value of HES content in the soil before bioremediation of 24.0 g/kg is actually 100%, we can notice that the content of HES in the soil after bioremediation decreased by 90.3 %. Contrarily, the content of HES in the control sample compared to the soil sample before bioremediation decreased by only 2.4 %. Based on the results of Fan et al. (2014), it can be assumed that the presence of yeast in the inoculation medium contributed to such a successful removal of HES. Namely, these authors, after biostimulation–bioaugmentation with yeast, achieved an n-alkane removal effect of 71.5-100 % in petroleum-contaminated soils.

The obtained results of the analysis of TPH and HES contents indicated a significantly more efficient *ex-situ* procedure compared to the *in-situ* procedure. Namely, Lin et al. (2010), after 1 year of *in-situ* bioremediation, registered significantly higher contents of alkanes (30.3 g/kg) and TPH (100.6 g/kg).

Similar results, as for HES content, were obtained for the TPH content. Namely the TPH content decreased by 92.9 % in the bioremediation process, while in the control sample it decreased by only 3.02%. These results indicate a very high efficiency of the applied microorganisms in the process of bioremediation. This significant reduction in TPH content in the soil after bioremediation is in accordance with the results obtained after treatment of soil (lasting 6 months) from the region of the Pančevo refinery (Serbia), where the TPH content after bioremediation (lasting 6 months) was reduced by 89% (from 29.8 g/kg to 3.3 g/kg; Milić et al. 2009). Similar results were obtained by Bešoski et al. (2010) after applied bioremediation (lasting 6 months) of soil contaminated with transformer oil in the Bor, Serbia, when the TPH content was reduced from 19.4 g/kg to 1.6 g/ kg. A very successful process of bioremediation using multiple inoculations of microorganisms was registered by Łebrovska et al. (2011) by reducing TPH content by 80% in soil contaminated with diesel oil and 98% in soil contaminated with aviation fuel.

6.2 Gas chromatographic analysis of petroleum hydrocarbons

Petroleum hydrocarbons were detected by GC-analysis. The obtained results were compared with the presentation of a gas chromatogram illustrating the ranges of the most abundant hydrocarbons in individual petroleum derivatives (Figure 6.1.).

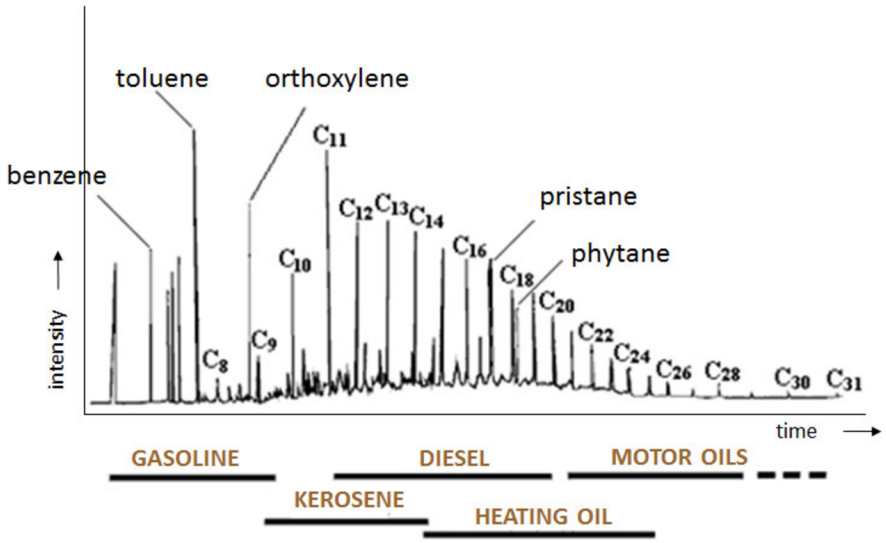

Figure 6.1: The model gas chromatogram of the most abundant hydrocarbons in some petroleum derivatives (Novaković, 2013).

Gas chromatograms indicate the presence of TPH with 14-40 C-atoms with peaks of different intensity before and after bioremediation (Figure 6.2). The TPH with C_{14}-

C_{28} peaks refer to low-molecular-weight hydrocarbons and the $TPH_{C28-C40}$ peaks refer to high-molecular-weight hydrocarbons (Lin et al. 2010). By comparing the size of the peaks on GC chromatograms (Figure 6.3), a significant decrease in the presence of both light and heavy TPH fractions can be observed after bioremediation, which is in line with the study by Bento et al. (2005), who registered a significant percentage of degradation of both light and heavy TPH fractions after 12 weeks of diesel oil in soil by natural attenuation, by biostimulation and by bioaugmentation.

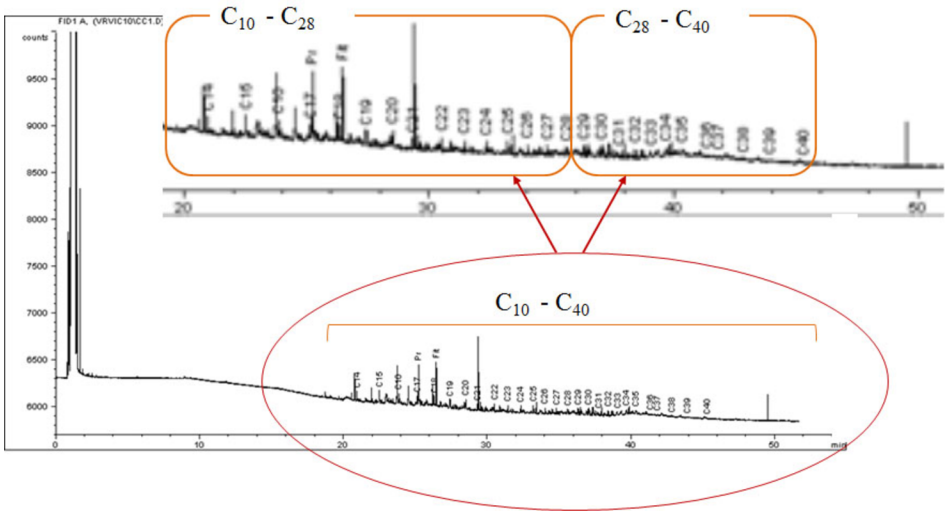

Figure 6.2: Comparison of the TPH chromatogram showing $TPH_{C14–C40}$ portions in bio-pile after bioremediation.

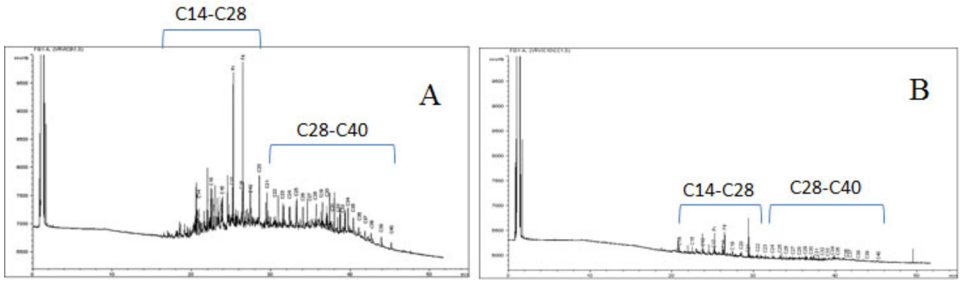

Figure 6.3. Comparative view of GC chromatograms before (A) and after bioremediation.

Based on the obtained results, it can be concluded that the soil sample at the initial state of bioremediation had a significant presence of hydrocarbons with 17 and 18 C atoms. This corresponds to presence of pristane (C17) and phytane (C18; Figure 6.4.).

Based on GC-analysis, after the completion of bioremediation, a significant decrease in the share of petroleum hydrocarbons - pristane and phytane in the soil was observed (Figure 6.5).

These results indicate a very successful bioremediation of soil with a very favorable bioremediation potential, where obviously the number of bacteria that successfully degrade hydrocarbons is large in relation to the total number of bacteria. In this process, hydrocarbons are used as a source of energy for growth of microorganisms (Novaković, 2013).

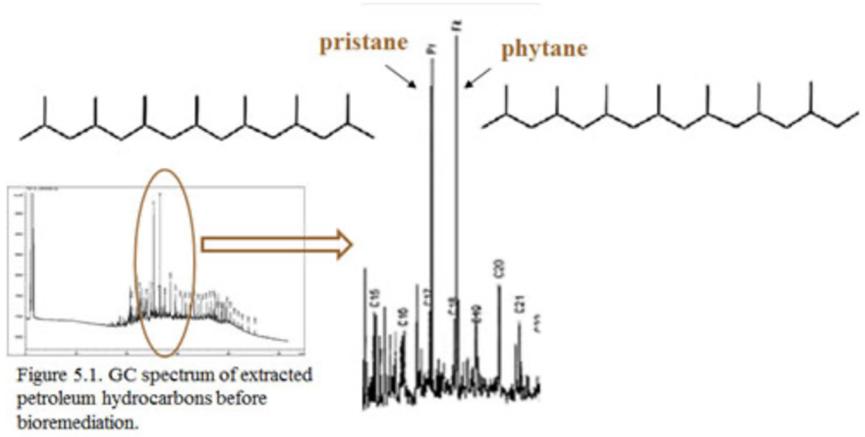

Figure 6.4: A review of the dominant participation of pristane and phytane in soil before bioremediation.

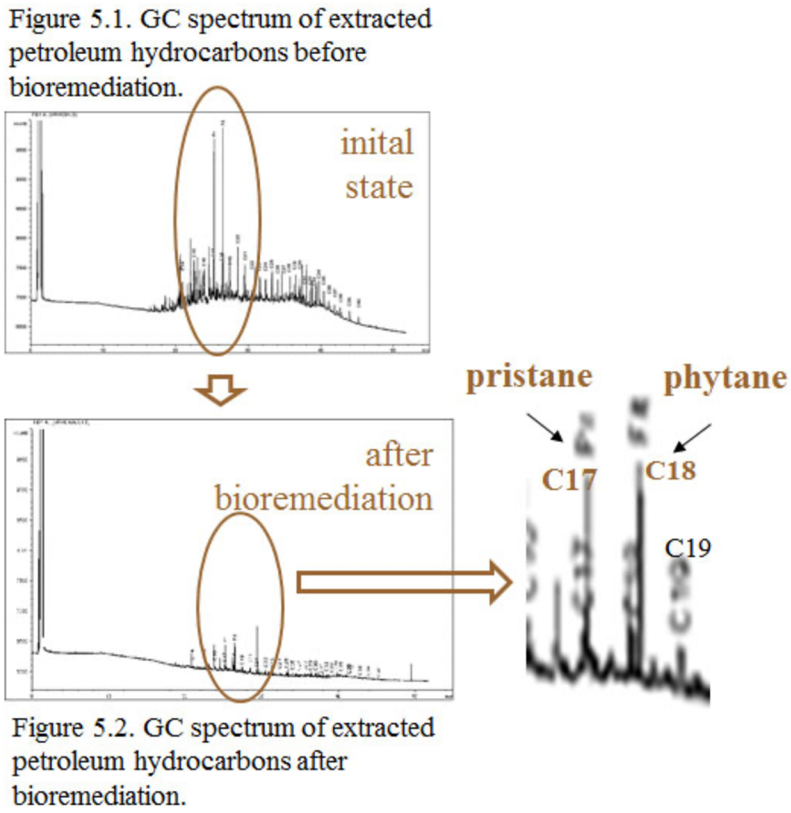

Figure 6.5: Overview of a significant reduction in the share of pristane and phytane in the soil after bioremediation.

6.3 Elementary analysis

Statistical and elementary organic analysis showed that there were no significant differences in the total carbon content of the soil before the start of bioremediation and in the control sample. However, after bioremediation, the total carbon content in the soil was by 92.5% lower than in the soil before the start of bioremediation and by 92.6% lower than in the control sample. Similar relations have been observed for the content of organic carbon (Table 5.3). Namely, the organic carbon content was 96.88% lower in the soil after bioremediation than in the soil before bioremediation and 96.85 % lower than in the control sample. The results of this study showed a more significant reduction in organic and inorganic carbon contents after bioremediation of contaminated soil than in the study by Beškoski et al (2011), where the reduction in organic and inorganic carbon contents was 56.1% and 13.85% respectively, after 5 months of soil bioremediation. For inorganic carbon content, no statistical difference was observed before and after bioremediation, as well as in relation to the control sample (Table 5.3). Correlation analysis indicates a very strong dependence of total carbon content with organic (r = 1.00) and inorganic (r = 1.00) carbon content, as well as between organic and inorganic carbon content (r = 1.00; (Table 6.1).

Table 6.1. Correlation coefficients between some properties[a*]

Relationship	*r*
Ash (550°C) - ash (800°C)	1.00
Ash (550°C) - moisture	-1.00
Ash (800°C) - moisture	-1.00
Ash (550°C) - pH value	0.87[b]
Ash (800°C) - pH value	0.87[b]
Moisture - pH value	-0.87[b]
TPH[1] – HES[2]	1.00
C_{total} - $C_{organic}$	1.00
C_{total} - $C_{inorganic}$	1.00
N_{total} - C_{total}	1.00
N_{total} - $C_{organic}$	1.00
N_{total} - $C_{inorganic}$	1.00
N_{total} - H_{total}	-1.00
S_{total} - C_{total}	-1.00
S_{total} - $C_{organic}$	-1.00
S_{total} - - $C_{inorganic}$	-1.00
S_{total} - N_{total}	-1.00

[a]Significant et $p < 0.05$. [b]Not significant et $p < 0.05$.
[1]HES - n-hexane extractable supstance.
[2]TPH - Total Petroleum Hydrocarbons.
*See Supplementary tables 9.3.-9.11.

Elementary organic analysis indicates significant soil contamination before bioremediation because the total nitrogen content is high. Namely, the average nitrogen content in the soil is ~0.2%, (Marty et al. 2017.) while in the examined samples (even the soil after bioremediation) a significantly higher content of total nitrogen of 0.2% was registered. However, the ratio of total carbon to nitrogen differed significantly among the samples. A very high C_{total}/N_{total} ratio could be observed for bioremediation soil (16.54) as well as for the control sample (15.77), while the soil after bioremediation showed a low ratio C_{total}/N_{total} (1.16; Table 5.3.). Soil contaminated with organic contaminants is known to have a high C/N ratio (Marty et al. 2017.) Bioremediation did not significantly affect the change in total nitrogen content (namely, the total nitrogen content before and after bioremediation was similar), which is in accordance with the results by Beškoski et al (2011). A very strong correlation was registered between the content of total nitrogen with the content of total, organic and inorganic carbon, as well as with the content of total hydrogen (r = 1.00; Table 6.1).

The content of total hydrogen in the soil samples before bioremediation and the control sample differ significantly from the hydrogen content in the soil after bioremediation. However, no statistical difference in total sulfur content was registered between these samples Table 5.3.). Correlation analysis showed a very strong positive relationship (r = 1.00) between the content of total hydrogen with all the examined parameters in the elementary organic analysis, except with the content of total sulfur. Namely, a very strong negative correlation was registered with the total sulfur content with all parameters examined in the elementary organic analysis of the soil (r = -1.00; Table 6.1). Such strong correlations of hydrogen content with the content of other elements are expected, because, the activities of H_2-producing and consuming microbes shape the global H_2 cycle and may have vital relationships with the global cycling of other elements. There are numerous pathways of microbial H_2 emission and consumption that could have an effect on the structure and function of microbial communities. A wide range of microbial groups employ H_2 as an electron donor to catalyze the reduction of pollutants such as organohalides, azo-compounds, and trace metals (Teng et al. 2019). Probably for this reason, there was a very significant decrease (96.2%) in the total hydrogen content in the tested sample after bioremediation.

Considering the practically unchanged content of total sulfur in the soil samples before and after bioremediation, it can be concluded that microorganisms that reduce sulfates were not present in the applied consortium of microorganisms. Namely, a consortium of microorganisms that reduce sulfates are efficient in optimizing the acid waste of mining tailings in industrial processes, and are most often used in this area (Ayangbenro et al. 2018).

6.4 Microbiological analysis

The presence of a large number of microorganisms in the range of 10^3-10^9 cfu/g, showed that intense aerobic and anaerobic processes take place in soil samples. The results showed that the number of total hemoorganoheterotrophic aerobic and facultative anaerobic bacteria (TB) is maintained very high both before and after bioremediation (Table 5.4.). Similarly, a high number of bacteria have been registered that degrade (decompose) hydrocarbons (HD) in soil samples before and after bioremediation; a slightly lower number was registered in the control sample.

The number of microorganisms before and after bioremediation presented in the literature is different and significantly depends on the applied conditions and duration of bioremediation. Then, the number of microorganisms significantly depends on whether and how the biostimulation procedure was applied. Today, the use of various additives in biostimulation is being investigated, such as ammonium sulfate, K-nitrate or urea (Kahraman et al. 2017) or by-products of the food industry such as: potato skin, soycake and tea leaf (Dadrasia & Agamuthu, 2014). All this can significantly affect the degree of soil purification, as well as on the number of microorganisms. As expect, the addition of nutrients significantly increases the number of microorganisms (Fan et al. 2014). Additionally, research under controlled/defined conditions in bio-reactors indicates that it is very important for efficient bioremediation to maintain a high density of bacteria, not just their number (Balsero-Romero et al. 2019).

The HD/TB ratio is very important for the bioremediation process and shows so-called "bioremediation potential". The bioremediation potential shows the possibility of bioremediation and the decomposition of more contaminants. This number depends on the number of bacteria that degrade hydrocarbons in relation to the total number of bacteria (Gojgić-Cvijović et al 2006). High bioremediation potential was registered in the soil prepared for bioremediation (in the initial state). That is why there was a significant decomposition of hydrocarbons during bioremediation. Lowest bioremediation potential is noted in control sample where microorganisms weren't added (Table 5.4.).

6.5 What future research could focus on?

The problems that belong to the field of ecology are numerous today and represent a global problem. The bioremediation process is one of the possibilities for solving environmental problems with the aim of improving the quality of contaminated soil. The results presented in this study showed a successfully performed process of *ex-situ* bioremediation (bioaugmentation) of soil in the area of Dobanovci (Serbia). In order to obtain a more complete picture, in this study, the content of toxic elements toxic elements before and after the bioremediation procedure could be examined in the future, as well as the determination of humic acids.

Why is it important to examine the content of toxic elements?

The importance of determining the presence and content of toxic elements in the soil, as a consequence of its pollution by spills of petroleum products, is multiple. Based on available literature (Adams et al, 2014), oil contamination increases the presence of toxic elements in soil, which can bio-accumulate causing adverse effects on health. It is well known that toxic elements can be extremely toxic as they damage nerves, bones and liver, and also block functional groups of vital enzymes. Some of the metals, like nickel, are also listed as possible human carcinogens and associated with reproductive problems and birth defects (Ewan and Pamphlett, 1996). Furthermore, a range of negative impacts on flora and fauna are as well documented. Frequently, these contaminants also hinder biological remediation processes due to metal sensitivity of the strain and necessitate additional combat strategies for efficient operation (Malik et al., 2001).

Why is it important to examine the content of humic acids?

During bioremediation of sites polluted with petroleum and its derivatives, substances similar to humic compounds are created - humification processes (Jednak et al. 2017). This is very important because humic substances have a tremendously positive influence on soil quality (Jednak et al. 2017). Humic substances, heterogeneous organic macromolecules, are composed of a fraction soluble at all pHs – the fulvic acids, are soluble in alkali and acid pHs, humic acids are soluble in alkali, but not in acid solutions, while the humin fraction is insoluble in both alkaline and acid conditions (Jednak et al. 2017). The essential components of humic substances are humic acids, and their study is important for understanding many processes in the environment, due to their crucial role in many physicochemical processes occurring in the global ecosystem (Salati & Papa, 2011). Since humic acids are of vital importance for soil quality, their quantity should always be determined in bioremediation processes (Žeradanin et al. 2020).

7 CONCLUSION AND CONTRIBUTION OF THE THESIS

All the applied analytical methods confirmed significant loss of organic matter in the soil after the bioremediation process. The loss of organic substances and carbonate for all the samples was significant. After bioremediation, a significant decrease in the content of total and organic carbon, as well as total hydrogen was registered, while it was not registered statistically significant difference in total sulfur content. The value of n-hexane extractable substances and total petroleum hydrocarbons content in the soil after bioremediation decreased by ~90%, which indicates a very high efficiency of the applied microorganisms in the process of bioremediation with a very favorable bioremediation potential. The soil sample at the initial state of bioremediation had a significant presence of hydrocarbons with 17C and 18C atoms (pristane and phytane), while after the completion of bioremediation, a significant decrease in the presence of this petroleum hydrocarbons in the soil was observed. The presence of a large number of microorganisms showed that intense aerobic and anaerobic processes take place in soil samples.

The obtained results indicated that this bioremediation procedure has successfully cleaned the soil contaminated with fuel oil.

The results of this research confirm the possibility of successful application of bioremediation (bioaugmentation) in the purification of soil contaminated with petroleum hydrocarbons. This is very important considering that the amount of waste that is in the environment today is too large to be removed naturally - by self-purification. Preservation of the purity and high quality of soil appears as one of the most on-going problem and at the same time the most complex problem. Soil protection is one of the biggest challenges that future generations will face, because we must not forget that soil is a heritage.

"All over the country [some soils are] worn out, depleted, exhausted, almost dead. But here is comfort: These soils possess possibilities and may be restored to high productive power, provided you do a few simple things".
C.W. Burkett, 1907.

(Magdoff F. & Van Es H., 2021.)

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8.1 The list of *on-line* figures sources

Figure 1.1: Example of Fenton reaction. https://www.chemistrylearner.com/fenton-reaction.html

Figure 3.1: Circulation of energy and matter in nature (Miller & Spoolman, 2012). https://www.slideshare.net/eyeznearz/ecosystems-how-do-they-work-8195195

Figure 3.2: Ecosystem components. https://www.takshilalearning.com/what-are-the-components-of-an-ecosystem-class-12th-biology/

Figure 3.3: Soil composition. http://www.prescriptionsoilanalysis.com/

Figure 3.4: Land profile. https://www.shutterstock.com/image-illustration/soil-layers-formation-horizons-mixture-plant-665526001

Figure 3.6: Nematodes - https://www.goodhousekeeping.com/home/gardening/a20705657/nematodes/;
Arthropods - https://www.oum.ox.ac.uk/thezone/animals/animalid/branch07.htm;
Anelide - https://edutorij.e-skole.hr/share/proxy/alfresco-noauth/edutorij/api/proxy-guest/c22bacc6-4a87-450d-9dc0-2acf43ade625/biologija-7/m04/j01/index.html;
Molluscs - https://www.dreamstime.com/photos-images/real-snail-isolated.html
Figure 3.7: *Acidithiobacillus ferrooxidans* - https://microbewiki.kenyon.edu/index.php/Acidithiobacillus_ferrooxidans;
Rhizobium sp. - https://www.alibaba.com/photo/rhizobium-biofertilizer-images.html;
Pseudomonas - https://www.biomerieux-industry.com/pharma-healthcare/resources/pharma-microorganisms-library/2020-03-02-how-does-pseudomonas;
Mushrooms - https://www.deviantart.com/chaseandlinda/art/Fungi-Mushroom-2-PNG-541534194;

Algae - https://krostrade.com/blog/how-to-control-green-algae-in-your-soil/;
Protozoa - https://www.sciencephoto.com/media/365146/view/ciliate-protozoans-from-soil

Figure 3.11: Stimulated bioremediation. (https://byjus.com/biology/bioremediation/)

Table 3.2. What is the difference between *in-situ* and *ex-situ* bioremediation? https://www.differencebetween.com/difference-between-in-situ-and-vs-ex-situ-bioremediation/

9 APPENDICES

Appendices 1

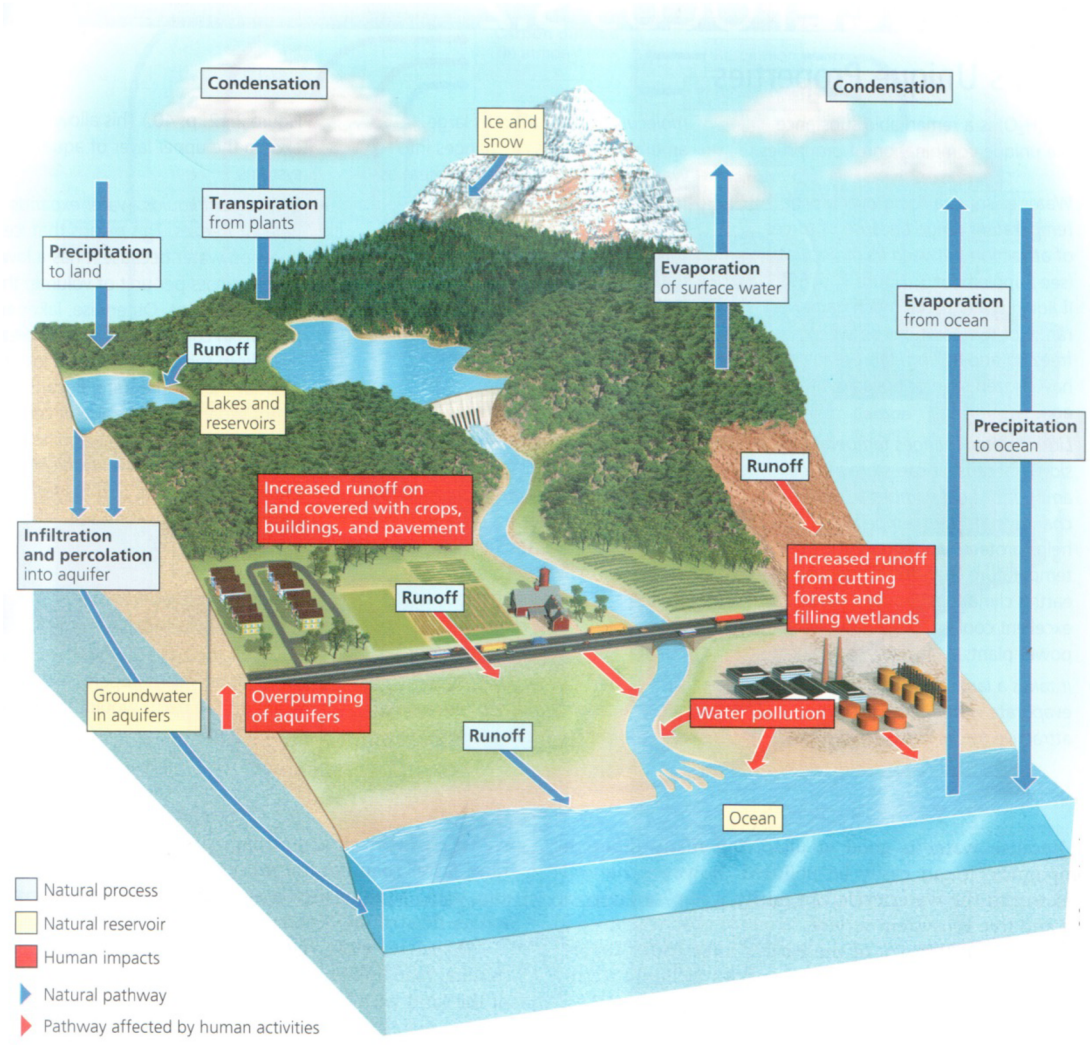

Figure 9.1: Simplified model of the ***water cycle***, or ***hydrologic cycle***, in which water circulated in various physical forms within the biosphere. The red arrows and boxes identify major effects of human activities on this cycle (Miller & Spoolman, 2012).

Appendices 2

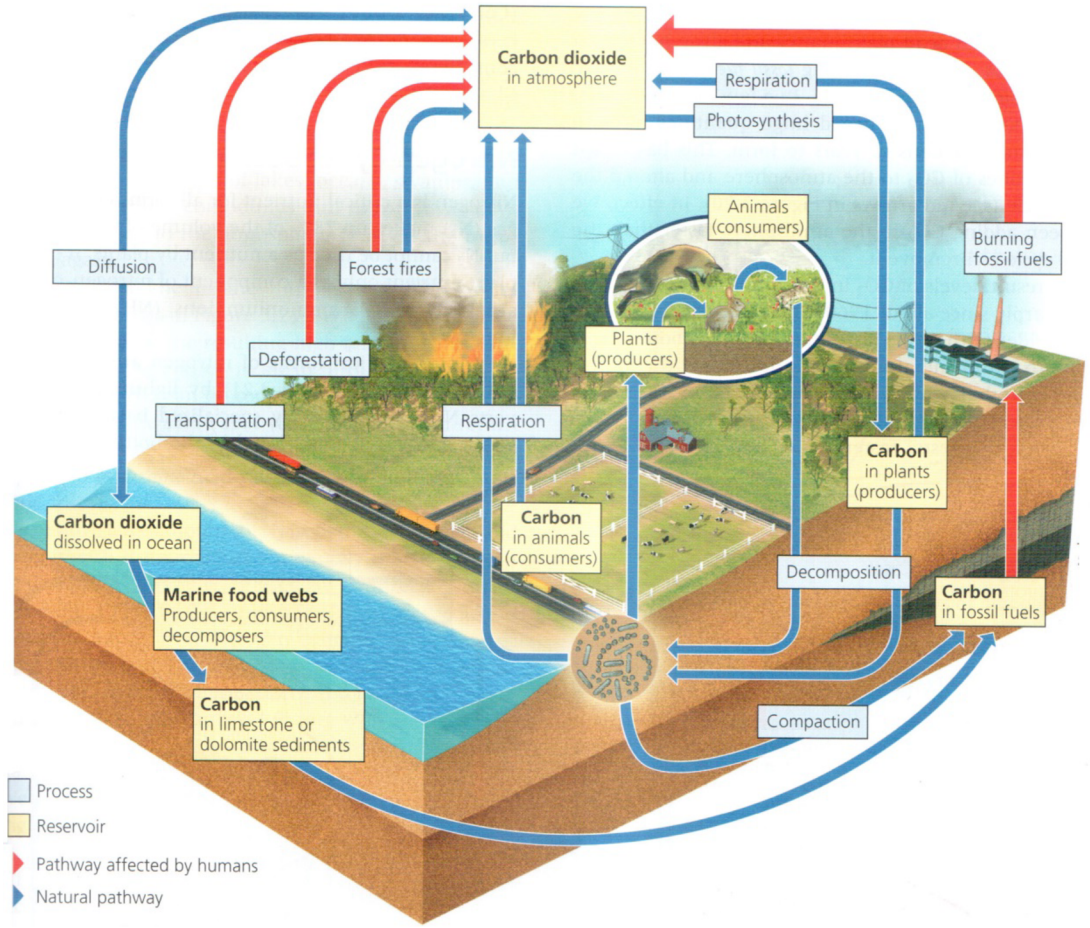

Figure 9.2: Simplified model showing the circulation of various chemical forms of carbon in the global ***carbon cycle***. Red arrows show major harmful impacts of human activities (Miller & Spoolman, 2012).

Appendices 3

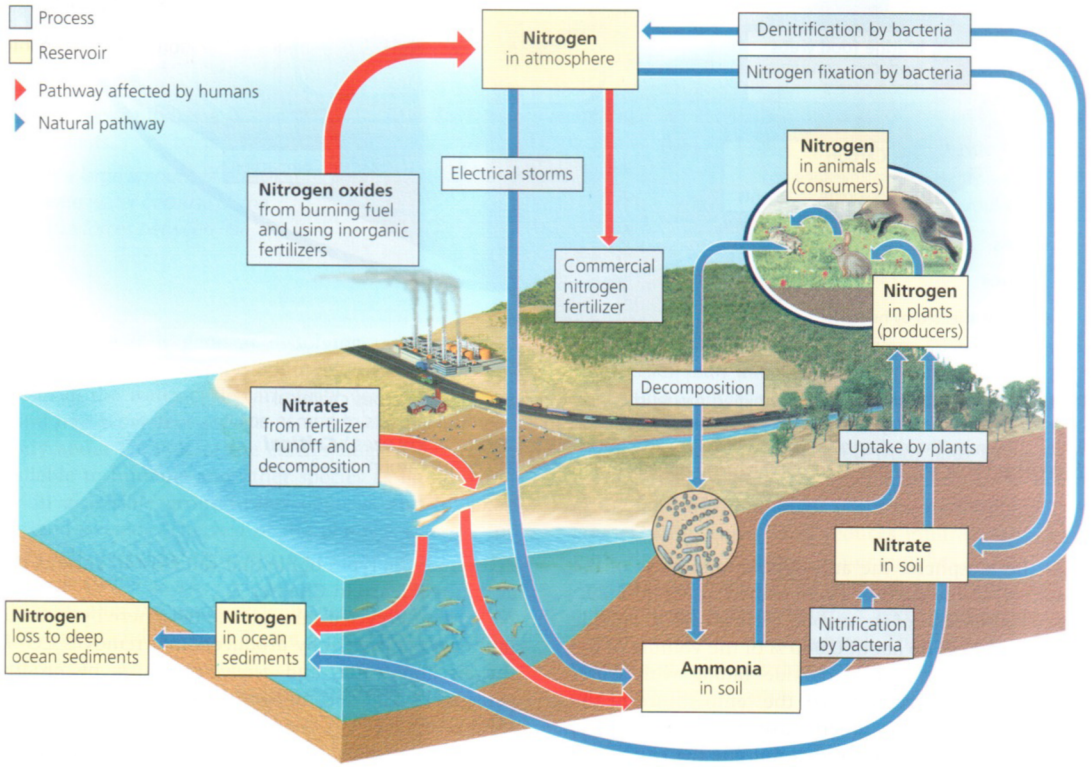

Figure 9.3: Simplified model showing the circulation of various chemical forms of nitrogen in the ***nitrogen cycle***, with major harmful human impacts show by the red arrows (Miller & Spoolman, 2012).

Appendices 4

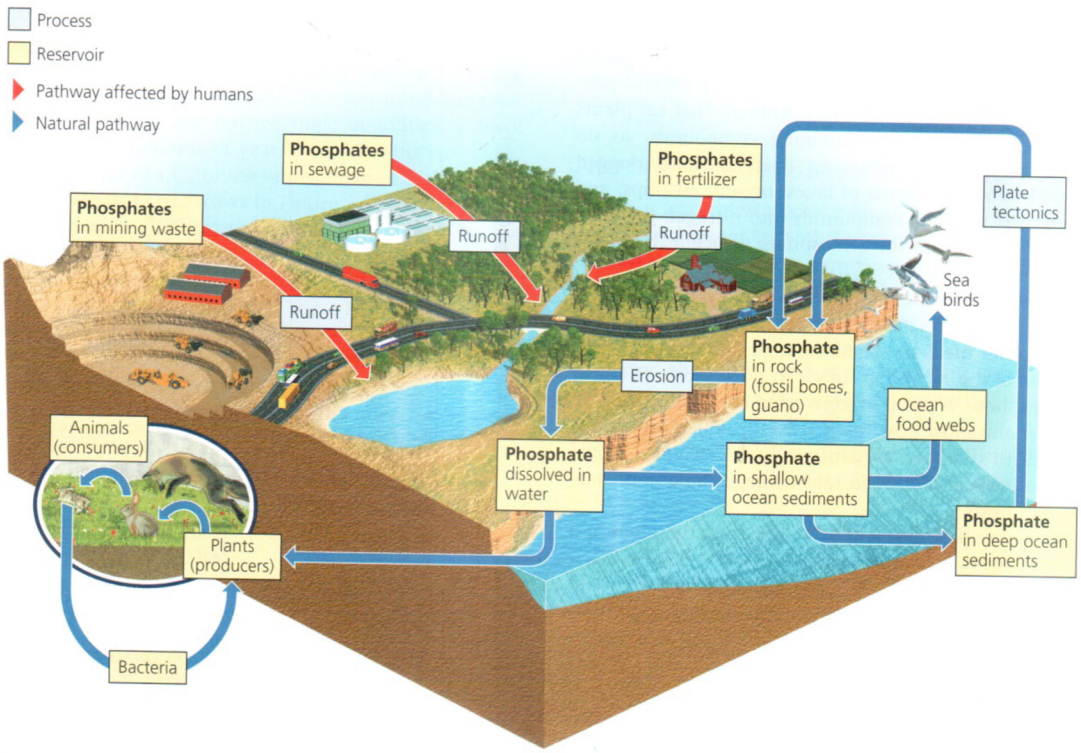

Figure 9.4: Simplified model showing the circulation of various chemical forms of phosphorus (mostly phosphates) in the ***phosphorus cycle***, with major harmful human impacts show by the red arrows (Miller & Spoolman, 2012).

Appendices 5

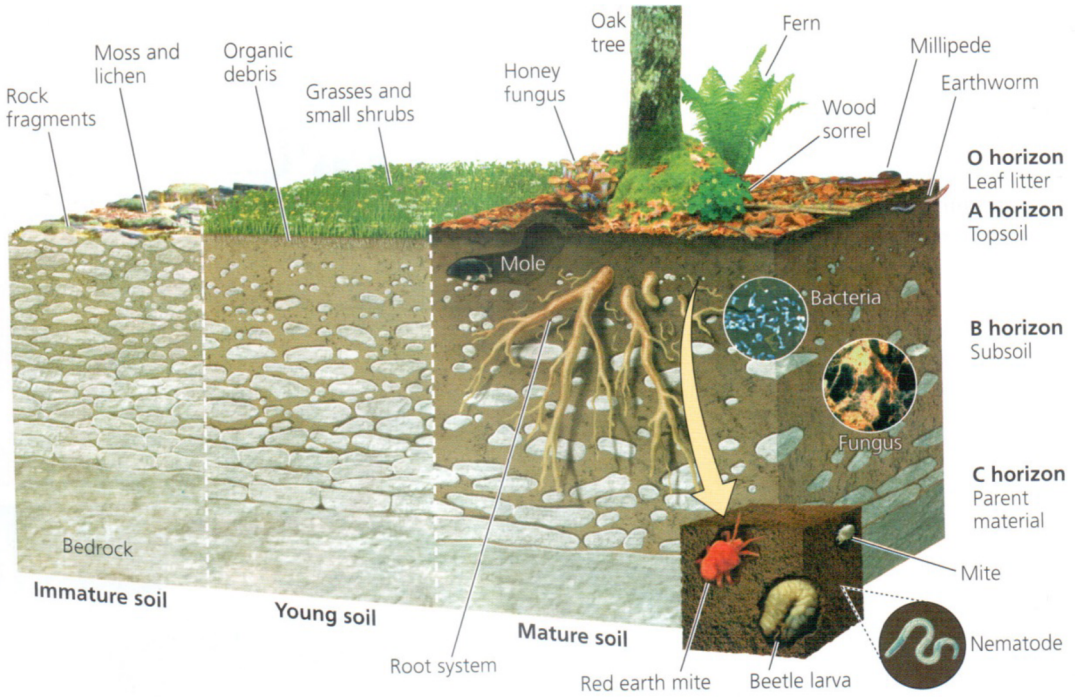

Figure 9.5: Generalized soil formation and soil profile (Miller & Spoolman, 2012).

Appendices 6

Table 9.1. Some 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-dichlorobiphe-nyl		+	Electrical manufacturing Power station Railway yards
Chlorinated phenol	Pentachlorophenol		+	Timber treatment Landfills
BTEX[1]	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 Glycophosphate Parathion Propham 2,4-D	+	+	Agriculture Timber treatment plants Pesticide manufacture Recreational areas Landfills

[1]BTEX - Soil contamination with benzene, toluene, ethylbenzene and xylene isomers

Appendices 7

Table 9.2. Summary of bioremediation strategies (Vidali, 2001).

Techno-logy	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	Biodegradative abilities of indigenous microorganisms Presence of metals and other inorganics Environmental parameters Biodegradability of pollutants Chemical solubility Geological factors Distribution of pollutants

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	Biodegradative abilities of indigenous microorganisms Presence of metals and other inorganics Environmental parameters Biodegradability of pollutants Chemical solubility Geological factors Distribution of pollutants Bioaugmentation Toxicity of amendments Toxic concentrations of contaminants

Appendices 8

Table 9.3. Correlation analysis of contents of moisture, ash, loss of organic matter and carbonate for samples in the initial state of bioremediation

Variable	Correlations (Spreadsheet5) Marked correlations are significant at p < .05000 N=3 (Casewise deletion of missing data)							
	Means	Std.Dev.	Moisture	Ash 550	Ash 800	pH	Organic supstantce	Carbonate 800
Moisture	30.32000	0.680000	1.00000	*-1.00000*	*-1.00000*	-0.866025	*-1.00000*	*-1.00000*
Ash 550	65.64000	1.100000	*-1.00000*	1.00000	*1.00000*	0.866025	*1.00000*	*1.00000*
Ash 800	63.46000	0.680000	*-1.00000*	*1.00000*	1.00000	0.866025	*1.00000*	*1.00000*
pH	6.96667	0.057735	-0.86603	0.86603	0.86603	1.000000	0.86603	0.86603
Organic supstantce 550	34.36000	1.100000	*-1.00000*	*1.00000*	*1.00000*	0.866025	1.00000	*1.00000*
Carbonate 800	36.54000	0.680000	*-1.00000*	*1.00000*	*1.00000*	0.866025	*1.00000*	1.00000

Numbers in italic marked correlations significant at p <0.05.
550 – 550°C; 800 - 800°C.

Table 9.4. Correlation analysis of contents of moisture, ash, loss of organic matter loss and carbonate for samples after bioremediation

Variable	Correlations (Spreadsheet5) Marked correlations are significant at p < .05000 N=3 (Casewise deletion of missing data)							
	Means	Std.Dev.	Moisture	Ash 550	Ash 800	pH	Organic supstantce	Carbonate 800
Moisture	29.58000	0.870000	1.00000	*1.00000*	*-1.00000*	-0.866025	*1.00000*	*-1.00000*
Ash 550	96.34000	0.640000	*1.00000*	1.00000	*-1.00000*	-0.866025	*1.00000*	*-1.00000*
Ash 800	95.79000	0.530000	*-1.00000*	*-1.00000*	1.00000	0.866025	*-1.00000*	*1.00000*
pH	7.86667	0.057735	-0.86603	-0.86603	0.86603	1.000000	-0.86603	0.86603
Organic supstantce 550	3.66000	0.640000	*1.00000*	*1.00000*	*-1.00000*	-0.866025	1.00000	*-1.00000*
Carbonate 800	4.21000	0.530000	*-1.00000*	*-1.00000*	*1.00000*	0.866025	*-1.00000*	1.00000

Numbers in italic marked correlations significant at p <0.05.
550 – 550°C; 800 - 800°C.

Table 9.5. Correlation analysis of contents of moisture, ash, loss of organic matter and carbonate for control samples

Variable	Correlations (Spreadsheet5) Marked correlations are significant at p < .05000 N=3 (Casewise deletion of missing data)							
	Means	Std.Dev.	Moisture	Ash 550	Ash 800	pH	Organic supstantce	Carbonate 800
Moisture	28.76000	0.250000	1.00000	*-1.00000*	*1.00000*	0.866025	*-1.00000*	*-1.00000*
Ash 550	65.18000	1.680000	*-1.00000*	1.00000	*-1.00000*	-0.866025	*1.00000*	*1.00000*
Ash 800	63.91000	0.010000	*1.00000*	*-1.00000*	1.00000	0.866025	*-1.00000*	*-1.00000*
pH	7.33333	0.057735	0.86603	-0.86603	0.86603	1.000000	-0.86603	-0.86603
Organic supstantce 550	34.82000	1.680000	*-1.00000*	*1.00000*	*-1.00000*	-0.866025	1.00000	*1.00000*
Carbonate 800	36.09000	0.250000	*-1.00000*	*1.00000*	*-1.00000*	-0.866025	*1.00000*	1.00000

Numbers in italic marked correlations significant at p <0.05.
550 – 550°C; 800 - 800°C.

Table 9.6. Correlation analysis of contents of n-hexane extractable supstance, total petroleum hydrocarbons, moisture, loss of organic matter and carbonate for samples in the initial state of bioremediation

Variable	Correlations (Spreadsheet5) Marked correlations are significant at p < .05000 N=3 (Casewise deletion of missing data)						
	Means	Std.Dev.	HES	TPH	pH	Carbonate 800	Organic supstantce
HES	24.02000	0.790000	1.00000	*1.00000*	-0.866025	*-1.00000*	*-1.00000*
TPH	22.82000	0.660000	*1.00000*	1.00000	-0.866025	*-1.00000*	*-1.00000*
pH	6.96667	0.057735	-0.86603	-0.86603	1.000000	0.86603	0.86603
Carbonate 800	36.54000	0.680000	*-1.00000*	*-1.00000*	0.866025	1.00000	*1.00000*
Organic supstantce 550	34.36000	1.100000	*-1.00000*	*-1.00000*	0.866025	*1.00000*	1.00000

Numbers in italic marked correlations significant at p <0.05. 550 – 550°C; 800 - 800°C.
HES -n-hexane extractable supstance. TPH - Total Petroleum Hydrocarbons.

Table 9.7. Correlation analysis of contents of n-hexane extractable supstance, total petroleum hydrocarbons, moisture, loss of organic matter and carbonate for samples after bioremediation

Variable	Correlations (Spreadsheet5) Marked correlations are significant at p < .05000 N=3 (Casewise deletion of missing data)						
	Means	Std.Dev.	HES	TPH	pH	Carbonate 800	Organic supstantce
HES	2.340000	0.170000	1.00000	*-1.00000*	-0.866025	*-1.00000*	*1.00000*
TPH	1.620000	0.140000	*-1.00000*	1.00000	0.866025	*1.00000*	*-1.00000*
pH	7.866667	0.057735	-0.86603	0.86603	1.000000	0.86603	-0.86603
Carbonate 800	4.210000	0.530000	*-1.00000*	*1.00000*	0.866025	1.00000	*-1.00000*
Organic supstantce 550	3.660000	0.640000	*1.00000*	*-1.00000*	-0.866025	*-1.00000*	1.00000

Numbers in italic marked correlations significant at p <0.05. 550 – 550°C; 800 - 800°C.
HES -n-hexane extractable supstance. TPH - Total Petroleum Hydrocarbons.

Table 9.8. Correlation analysis of contents of n-hexane extractable supstance, total petroleum hydrocarbons, moisture, loss of organic matter and carbonate for control samples

Variable	Correlations (Spreadsheet5) Marked correlations are significant at p < .05000 N=3 (Casewise deletion of missing data)						
	Means	Std.Dev.	HES	TPH	pH	Carbonate 800	Organic supstantce
HES	23.44667	0.875005	1.000000	*0.99999*	0.864371	*-0.99999*	*-0.99999*
TPH	22.13000	0.830000	*0.999995*	1.00000	0.866025	*-1.00000*	*-1.00000*
pH	7.33333	0.057735	0.864371	0.86603	1.000000	-0.86603	-0.86603
Carbonate 800	36.09000	0.250000	*-0.999995*	*-1.00000*	-0.866025	1.00000	*1.00000*
Organic supstantce 550	34.82000	1.680000	*-0.999995*	*-1.00000*	-0.866025	*1.00000*	1.00000

Numbers in italic marked correlations significant at p <0.05. 550 – 550°C; 800 - 800°C.
HES -n-hexane extractable supstance. TPH - Total Petroleum Hydrocarbons.

Table 9.9. Correlation analysis of contents of total/organic/inorganic carbon, total contents of nitrogen, hydrogen and sulfur for samples in the initial state of bioremediation

Variable	Correlations (Spreadsheet4) Marked correlations are significant at p < .05000 N=3 (Casewise deletion of missing data) Means	Std.Dev.	Ctotal	Corganic	Cinorganic	Ntotal	Htotal	Stotal
Ctotal	17.27000	0.290000	1.00000	*1.00000*	*1.00000*	*1.00000*	*1.00000*	*-1.00000*
Corganic	17.00000	0.280000	*1.00000*	1.00000	*1.00000*	*1.00000*	*1.00000*	*-1.00000*
Cinorganic	0.27000	0.010000	*1.00000*	*1.00000*	1.00000	*1.00000*	*1.00000*	*-1.00000*
Ntotal	1.05000	0.070000	*1.00000*	*1.00000*	*1.00000*	1.00000	*1.00000*	*-1.00000*
Htotal	3.45000	0.150000	*1.00000*	*1.00000*	*1.00000*	*1.00000*	1.00000	*-1.00000*
Stotal	0.07000	0.010000	*-1.00000*	*-1.00000*	*-1.00000*	*-1.00000*	*-1.00000*	1.00000

Numbers in italic marked correlations significant at p <0.05.

Table 9.10. Correlation analysis of contents of total/organic/inorganic carbon, total contents of nitrogen, hydrogen and sulfur for samples after bioremediation

Variable	Correlations (Spreadsheet4) Marked correlations are significant at p < .05000 N=3 (Casewise deletion of missing data) Means	Std.Dev.	Ctotal	Corganic	Cinorganic	Ntotal	Htotal	Stotal
Ctotal	1.290000	0.280000	1.00000	*1.00000*	*1.00000*	*-1.00000*	*1.00000*	*-1.00000*
Corganic	0.530000	0.260000	*1.00000*	1.00000	*1.00000*	*-1.00000*	*1.00000*	*-1.00000*
Cinorganic	0.760000	0.020000	*1.00000*	*1.00000*	1.00000	*-1.00000*	*1.00000*	*-1.00000*
Ntotal	1.150000	0.110000	*-1.00000*	*-1.00000*	*-1.00000*	1.00000	*-1.00000*	*1.00000*
Htotal	0.130000	0.020000	*1.00000*	*1.00000*	*1.00000*	*-1.00000*	1.00000	*-1.00000*
Stotal	0.080000	0.010000	*-1.00000*	*-1.00000*	*-1.00000*	*1.00000*	*-1.00000*	1.00000

Numbers in italic marked correlations significant at p <0.05.

Table 9.11. Correlation analysis of contents of total/organic/inorganic carbon, total contents of nitrogen, hydrogen and sulfur for control samples

Variable	Correlations (Spreadsheet4) Marked correlations are significant at p < .05000 N=3 (Casewise deletion of missing data) Means	Std.Dev.	Ctotal	Corganic	Cinorganic	Ntotal	Htotal	Stotal
Ctotal	17.51000	0.270000	1.000000	*0.999936*	0.981981	*1.000000*	*1.000000*	-0.866025
Corganic	16.79667	0.255016	*0.999936*	1.000000	0.979778	*0.999936*	*0.999936*	-0.860310
Cinorganic	0.71333	0.015275	0.981981	0.979778	1.000000	0.981981	0.981981	-0.944911
Ntotal	1.11000	0.040000	*1.000000*	*0.999936*	0.981981	1.000000	*1.000000*	-0.866025
Htotal	3.65000	0.050000	*1.000000*	*0.999936*	0.981981	*1.000000*	1.000000	-0.866025
Stotal	0.06667	0.005774	-0.866025	-0.860310	-0.944911	-0.866025	-0.866025	1.000000

Numbers in italic marked correlations significant at p <0.05.

"Without fertile soil, what is life"?
V. Shiva, 2008.

(Magdoff F. & Van Es H., 2021.)