Chapter 5

The Molecular-Based Methods Used for Studying Bacterial Diversity in Soils Contaminated with PAHs (The Review)

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Additional information is available at the end of the chapter

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Abstract

Soil contamination could adversely affect microbial diversity, and perhaps also above- and below-ground ecosystem functioning. It is important to study microbial diversity not only for basic scientific research, but also to understand the link between diversity and community structure and function in the pollution site. The study of microbial diversity and their function in contaminated soil creates a serious problem because they observed significant limitations in methodology and taxonomy of this group. Methodology for the determination of bacterial diversity does not include their function in the soil and other environment areas. Microbes are known for their catabolic activity in bioremediation, but changes in microbial communities are still unpredictable. The bioremediation of a pollutant and its rate depend on the environmental conditions, number and type of the microorganisms, nature and chemical structure of the chemical compound being degraded. However, molecular methods have been used to study soil bacterial communities. While many anthropogenic activities, such as city development, agriculture, and use of pollution, can potentially affect soil microbial diversity, it is unknown how changes in microbial diversity can influence below-ground and above-ground ecosystems. There are problems associated with studying bacterial diversity in soil. These arise not only from methodological limitations, but also from a lack of taxonomic knowledge. Methods to measure microbial diversity in soil can be categorized into two groups: biochemical-based techniques and molecular-based techniques. But more common for studying microbial diversity in soil contaminated with polycyclic aromatic hydrocarbons are the molecular methods.

Keywords: bacterial diversity, soil contamination, PAHs, trace elements, molecular methods, DGGE, NGS

1. Introduction

The oil refinery industry is involved in the global processes of exploration, extraction, transporting (often with oil tankers and pipelines), and marketing petroleum products. The products of largest volume of the industry are oil and gasoline [1, 2]. Crude oil and petroleum are also the raw materials for many chemical products, including pharmaceuticals, solvents, fertilizers, pesticides, and plastics. Oil and its derivatives (such as polycyclic aromatic hydrocarbons, PAHs) are among very significant and dangerous sources of ecosystem contaminants. Oil derivatives that contaminate soil are a threat to human health as well as a hazard to all living beings [2].

Polycyclic aromatic hydrocarbons (PAHs) are a large group of carcinogenic compounds emitted into the atmosphere by incomplete combustion of fossil fuel or biomass. As semi-volatile chemicals, PAHs can be transported over long distances in the atmosphere. In general, 3-4-5 ringed PAHs are largely predominant in air wherever the sampling was established, whether in rural, suburban, or urban areas. PAHs can pass from air to water, soil, and vegetation, through dry gaseous, dry particle-bound, and wet depositions [3]. They are persistent in various environmental media and can subsequently enter the food chains. Nowadays, it is well known that human exposure mainly occurs by ingestion of contaminated agricultural and natural food [4, 5]. Using plant in bioremediations is more popular and common. Plants are capable of accumulating PAHs from the soil, water, and air. In the ryzosphere of plants, we have a very higher activity of microorganism capable of using PAHs as the only source of carbon and energy [2, 5, 6].

The main source of hydrocarbons (PAHs) is incomplete combustion of organic different material. Polycyclic aromatic hydrocarbons are colorless, white, or yellow solids. They present low solubility in water and also low vapor pressure [7]. They arise mainly from anthropogenic sources (forest fires, oil seeps, and volcanic eruptions). Other sources of PAHs are burning of fossil fuel, coal tar, wood, oil derivatives, petroleum spills, and discharge. These substances are very toxic, mutagenic, and carcinogenic [8]. The remediation and bioremediation of PAHs are very longer and technically hard. Their persistence in soil increases with increase in molecular weight of PAHs. It is estimated that more than 90% of the total burden of oil derivatives such as PAHs reside in the surface layer of soils where they accumulate the most. Recent determinations of PAHs in agricultural soils in Poland indicate that the content of these contaminants in the majority of the soils is low but in long-term contaminated soils, this content is very higher [9, 10].

Several techniques of remediation of PAHs are known: volatilization, photooxidation, chemical oxidation, adsorption on soil particles, and microbial biodegradation. The main popular techniques are expensive and very time-consuming. Otherwise, the effect of that remediation in many cases transfers the pollutant from one phase (soil, water, or air) to another [2, 4].

Bioremediation process is much less dangerous, and the results (products) of this process are safe for the environment such as inorganic minerals, H_2O, CO_2 (aerobic), or CH_4 (anaerobic) [1, 11].

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Microbes are known for their catabolic activity in bioremediation, but changes in microbial communities are still unpredictable [1, 11]. The most popular PAH-degrading microorganisms are bacteria and fungi. The bioremediation of PAHs very often depends on the environmental conditions (climates, number and type of the microorganisms, soil structure, plants). The extent of biodegradation process depends on many biotic and abiotic factors, including pH, temperature, oxygen, microbial population, degree of acclimation, accessibility of nutrients, chemical structure of the compound, cellular transport properties, and chemical partitioning in growth medium [2, 4, 12].

Overall, PAHs are immobile and persistent in soil and also more difficult to extract. PAHs are less accessible to living organisms (microorganism) when they come in contact with the aggregate soil structure [2]. There are many methods used to clean up PAH and oil derivatives in contaminated soils, but bioremediation using bacteria and fungi consortium is most popular [1, 2, 8].

However, molecular methods have been used to study soil bacterial communities in contaminated soil with PAHs and oil derivatives. While many anthropogenic activities, such as city development, agriculture, and use of pollution, can potentially affect soil microbial diversity, it is unknown how changes in microbial diversity can influence below-ground and above-ground ecosystems. The study of bacterial diversity in soil contaminated with PAHs has some problems. The problems arise not only from the methodological limitations, but also from a lack of taxonomic knowledge. This studies focuses on whole groups of microorganism (bacteria and fungi) and its function in in the contaminated sites.

2. Bacteria and nitrogen fixation microorganisms in bioremediation of contaminated soil

Microorganisms have some potential as an effective and inexpensive mean to remediation of contaminated soils [13]. The successful application of bioremediation techniques (bioaugmentation, phytoremediation) is largely dependent on the some capacity of plant growth-promoting microorganisms to efficiently colonize growing plants roots [14].

Bacteria are the class of microorganisms actively involved in the degradation of organic pollutants from contaminated sites especially from soils rhizosphere [13, 14]. A number of bacterial species are known to degrade PAHs (shown in **Table 1**). These bacteria very often are isolated from contaminated soil and have special potential to degradation of oil derivatives. The most carcinogenic and toxic from PAHs is benzo(a)pyrene. This hydrocarbon is a model contaminate in bioremediation study. Bacteria which can degrade benzo(a)pyrene grow well on alternative carbon source in liquid culture experiments [19–21].

Other authors [22] observed a 5 % decrease in benzo(a)pyrene concentration after 168 h during incubations with *Sphingomonas paucimobilis* strain of bacteria. They also noticed that resting cells of *S. paucimobilis* grown on nutrient agar supplemented with glucose resulted in significant evolution of 14 CO_2 (28%), indicating higher hydroxylation and ring cleavage. Some

Bacteria	Plant	Contaminant	Role of bacteria	Ref.
Azospirillum lipoferum	Wheat	Crude oil	Promoted development of wheat root system enhanced level of oil degradation	[14]
Azospirillum brasilense	Tall fescue	Polycyclic aromatic hydrocarbons (PAHs)	Increased plant tolerance to PAHs Promoted plant growth under stress	[15]
Azospirillum spp. *Pseudomonas stutzeri*	Meadow fescue Maize Winter rye	Polycyclic aromatic hydrocarbons (PAHs) Crude oil	Promoted development of plant root system enhanced level of oil and PAH degradation Increased plant tolerance to PAHs	[9, 10]
Enterobactor cloacae	Tall fescue	Total petroleum Hydrocarbons (TPHs)	Promoted plant growth in the presence of environmental contaminants such as TPHs	[14]
Pseudomonas fluorescens	Wheat	Trichloroethylene (TCE)	Degraded TCE with toluene o-monooxygenase	[16]
Pseudomona fluorescens	Alfalfa	Polychlorinated biphenyls(PCBs)	More effectively metabolized PCBs with bph gene cloned	[17]
Pseudomonas putida	Arabidopsis	PCBs	Utilized plant secondary metabolites	[18]

Table 1. Examples of bioremediation of organic contaminants in soil with bacteria species.

authors [14, 19] isolated 11 strains from a variety of contaminated sites (oil, motor oil, refinery derivatives) with the ability to degrade benzo(a)pyrene. Bacteria capable to PAHs degradation and using as the only source of carbon and energy belong to the main species *Pseudomonas*, *Agrobacterium*, *Bacillus*, *Burkholderia*, *Sphingomonas, and Phanerochaete chrysosporium* [23]. Other authors reported PAH degradation using other bacteria including *Rhodococcus* sp., *Mycobacterium*, and mixed culture of *Pseudomonas* and *Flavobacterium* species [20]. In study of Heitkamp et al. [24], the authors described about bacterial isolated from oil-contaminated soil which was capable of mineralizing the pyrene. *Pseudomonas aeruginosa* isolated from a stream heavily polluted by a petroleum refinery was very effective in degradation of phenanthrene [25]. *Pseudomonas aeruginosa* actively grow over high doses of phenanthrene with complete removal of the pollutant in a period of 30 days of the experiment. Other authors report that *Mycobacterium* species isolated from a PAH-contaminated soil were able to utilize pyrene as the only sole source of carbon and energy (up to 60% of the pyrene added (0.5 mg ml^{-1}) within 8 days at 20°C of temperature) [26]. Some products of this degradation pathway were analyzed (Cis-4,5-pyrene dihydrodiol, 4-5-phenanthrene dicarboxylic acid, 1- hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde, phthalic acid, and protocatechuic acid). In the study of Yuan et al. [11], the authors isolated strains of bacteria from a petrochemical waste which having the capacity

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of degrading acenaphthene, fluorene, phenanthrene, anthracene, and pyrene by 70–100% in a period of 40 days of the experiment. This bacteria belong to the *Pseudomons fluoresens* and *Haemophilus* species. Dean-Ross et al. [15] isolated two bacterial strains (*Mycobacterium flavescens* and *Rhodococcus* spp.) from some sediments. This bacteria were found to be capable of PAH degradation (pyrene mineralization by *M. flavescens* and anthracene mineralization by *Rhodococcus species*) [27]. The study also proposed the degradation pathway of fluoranthene. In both strains, metabolism of fluoranthene occurred on the fused ring of fluoranthene molecule, producing 9-fluorenone-1-carboxylic acid.

Microbial degradation is the mean to remove PAHs from contaminated soils, especially using strains of bacteria which are able to degrade PAHs and using them as a source of carbon and energy and fix free nitrogen such as the strains of *Azospirillum* spp. and *Pseudomonas stutzeri*. These strains are the diazotrophic bacteria capable of free nitrogen fixing, hydrocarbon degradation as an only source carbon, and energy and biosurfactant production. Bacteria of the genus *Pseudomonas* are known in the literature as the most active degraders of hydrocarbons in natural biotopes of polluted sites and within biotechnological preparations [9, 10, 69].

Diazotrophic bacteria such as *Azospirillum* spp. and *Pseudomonas stutzeri* are also using in bioremediation of crude oil derivatives in soils naturally and artificially polluted [9, 10]. Gałązka et. al. reported the study with three soils artificially polluted with PAHs (anthracene, phenanthrene, and pyrene) at the doses of 100, 500, and 1000 mg kg^{-1} d.m. of soil and diesel fuel at the doses of 0.1%, 0.5%, and 1% (v/v). In study was also used soil naturally contaminated with crude oil (brown soil). Grasses were inoculated with the mixture of bacteria strains *Azospirillum* and *Pseudomonas stutzeri* and applied in the bioremediation process in the amount of 1 ml per 500 g of soil.. The amounts of anthracene, phenanthrene, and pyrene were determined in soils artificially polluted and Σ15 PAHs in soils artificially polluted with diesel fuel, as well as in brown soil aged polluted with crude oil. It was found that the inoculation of plants with *Azospirillum* spp. and *Pseudomonas stutzeri* had a positive effect on bioremediation process either in soils artificially polluted with PAHs (decrease from 25–60% of the primary concentration comparing to the control) or in soils polluted with diesel fuel (decrease from 2–25%) [9, 10]. The slime of *Azospirillum* spp. and *Pseudomonas stutzeri* introduced to soil did not limit the development of indigenous bacteria consortia in the polluted soil; instead, progressive biodegradation of PAHs enabled major growth of total number of bacteria, *Actinomycetes* and their biological groups. The ability of *Azospirillum* spp. and *Pseudomonas stutzeri*, populating rhizosphere and the inside of grass roots, to free nitrogen fixing and the use of PAHs (phenanthrene, anthracene, and phyrene) as the only source of carbon and energy suggests that in the future, after the series of detailed analysis, it will be possible to invent preparation based on these species, suitable for bioremediation of soils polluted with PAHs, with very limited supplementation of environment with nitrogen fertilizers. The successful results were observed (an important decrease in the content of PAHs in soils) in soil inoculated with *Azospirillum* and *Pseudomonas stutzeri* after grass growth (maize, meadow fescue). This processes were especially effective in calcareous rendzina artificially polluted with PAHs and in soil long-term contaminated with crude oil [28, 29].

2.1. Bacterial diversity in soil contaminated with PAHs

Soil microorganisms play a big roles in various biogeochemical cycles and are responsible for the cycling of organic compounds especially oil derivatives and polycyclic aromatic hydrocarbons. Also they influence above-ground ecosystems by contributing to plant nutrition, plant health, soil structure, and soil fertility. Our knowledge on soil microbial diversity is limited in part by our inability to study soil microorganisms. It is known that in 1 g of soil there are 10^{30} different soil microorganisms [30]. Only 1% of this soil bacterial population can be cultured by classical methods. About 99% is unknown, and this group of microorganism is possible to measure only in using molecular methods [31, 32].

Various molecular methods have been used to study soil bacterial communities. Many biotic and abiotic factors play a big role to changes in microbial diversity (contamination, anthropogenic activities, plant growth). It is not known how changes in microbial community structure influence ecosystem functions. Study of microorganisms function is the need for reliable and accurate mechanisms of understanding their diversity and taxonomic [33–35].

Typically, diversity studies include the relative diversities of communities across a gradient of stress, disturbance, or other biotic or abiotic difference [35]. It is difficult with current techniques to study true diversity since we do not know what is present and we have no way of determining the accuracy of our extraction or detection methods. Species diversity consists of species richness, the total number of species present, species evenness, and the distribution of species [32].

Methods to measure microbial diversity in soil can be categorized into two groups: biochemical-based techniques and molecular-based techniques. But more common for studying microbial diversity in soil contaminated with polycyclic aromatic hydrocarbons are the molecular methods.

2.2. Limitations of molecular methods to study bacterial diversity in contaminated soils

Molecular techniques based on polymerase chain reaction (PCR) have been used to overcome the limitations of culture-based methods; however, they are not without their own limitations [32, 34].

Soil microorganisms (especially bacteria) are located between soil aggregates. There is a very big problem with separating these from micro- and macro-components of soil structure. The study bacterial biodiversity requires isolated genomic DNA from bacterial cells [35]. This process is dependent on bacterial cells (gram-negative or gram-positive bacterial cells). Gram-negative cells would be lysed when the cell extraction is sensitive, but the gram-positive cells may be lysed in stronger conduction, but in this case DNA may be disintegrated [32]. The special method of DNA or RNA extraction from bacterial cells used can also bias biodiversity studies. The harsh and drastic DNA extraction methods (bead beating) can shear the nucleic acids, leading to some problems in subsequent PCR detection products [36]. With soil samples, it is necessary to remove some inhibitory substances (fulvic acids, humic acids). These substances can be coextracted and can strongly interfere with subsequent PCR and analysis. Second step of analysis can lead to loss of DNA or RNA in-

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hibitory of PCR. The most popular in bacterial biodiversity studies are primers which targeted typical regions coding genes present in all organisms such as 16S rRNA or ITS (internal transcribed spacer). This genes have well-defined regions for taxonomic classification of bacteria and are not subject to horizontal transfer and have sequence databases available to researchers.

Many authors [32, 34, 36, 37] discussed some issues surrounding differential PCR amplification including different affinities of primers to templates, different copy numbers of target genes, hybridization efficiency, and primer specificity. In addition, some sequences with lower G+C content are thought to separate more efficiently in the denaturing step of polymerase chain reaction and therefore could be preferentially amplified [32, 34]. There are known a few important points in optimalization of PCR such as amplification including different affinities of primers to templates, different copy numbers of target genes, hybridization efficiency, and primer specificity. The above discusses a few limitations of molecular-based methods, which can influence the analysis and interpretation of their community analysis. Molecular-based methods provide valuable information about the microbial community as opposed to only culture-based techniques.

3. Molecular techniques based on PCR methods to study bacterial diversity

The molecular methods of study bacterial diversity include some methods profiling of soil microbial communities, based upon culture-independent techniques (cloning, fingerprinting techniques, automated ribosomal intergenic spacer analysis (ARISA), or terminal/restriction fragment length polymorphism (TRFLP, RFLP) (**Table 2**) [32, 34, 35, 74, 73].

Application of these techniques yields information that can be used to assess how environmental factors contribute to changes in microbial community structure. Although a considerable amount is known about how culturable bacteria respond to anthropogenic agents, little is known about how organic compounds influence the structure of soil microbial communities in situ. It has been suggested that microbial community structure in polluted environments is influenced by the complexity of chemical mixtures present and time of exposure and is thought generally to lead to a reduction in microbial diversity. We do not know why the amount of PAH contamination together with the PAH compound present significantly affected microbial community structure in PAH-contaminated soils [35, 37].

DNA hybridization is a measure of genetic complexity of the microbial/bacterial community and has been used to estimate diversity in soil contaminated. The similarity between communities of two different samples can be studied by measuring the degree of similarity of DNA through hybridization kinetics [39]. Nucleic acid hybridization using specific probes is an important qualitative and quantitative tool in molecular bacterial ecology. These hybridization techniques can be done on extracted DNA or RNA, or *in situ*.

Method	Advantages	Ref.
G+C content	Not influenced by PCR biases	[38]
	Includes all DNA extracted	
	Quantitative	
	Includes rare members of community	
DNA hybridization	Same as nucleic acid hybridization	[39]
	Thousands of genes can be analyzed	
	If using genes or DNA fragments, increased specificity	
Denaturing and temperature gradient gel electrophoresis (DGGE/TGGE)	Large number of samples can be analyzed simultaneously	[40, 41]
	Reliable, reproducible, and rapid	
Restriction fragment length polymorphism (RFLP)	Detect structural changes in microbial community	[42]
Terminal restriction fragment length polymorphism (T-RFLP)	Simpler banding patterns than RFLP	[42]
	Can be automated; large number of samples	
	Highly reproducible	
	Compare differences in microbial communities	
Ribosomal intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA)	Highly reproducible community profiles	[43]

Table 2. Advantages of some molecular-based methods to study soil microbial diversity.

The known sequences of some oligonucleotide/polynucleotide probes ranging in specificity from domain to species can be tagged with markers at the 5'-end of DNA. The most popular markers are fluorescent markers that include derivatives of fluorescein or rhodamine. Quantitative dot-blot hybridization methods are used to measure the relative abundance of the special group of microorganisms (bacteria). In these methods, samples (bacterial culture) are lysed to release all nucleic acids. In dot-blot hybridization with specific and universal oligonucleotide primers, the rRNA sequences are quantified relative to total rRNA [32, 34, 35]. The changes in the activity and hence the amount of rRNA content or changes in the abundance in the population may represent the relative abundance is samples. Hybridization methods of studying bacterial biodiversity can also be conducted at the cellular level and can be done in situ (valuable spatial distribution information on microorganisms in environmental sample) [34]. The method, known as fluorescent in situ hybridization or FISH (fluorescence in situ hybridization), has been used successfully to study the spatial distribution of bacteria in biofilms [39]. The lack of sensitivity is the most limited point in the methods such as in situ hybridization or hybridization of nucleic acids extracted directly from soil samples. The some unless sequenced are present in very high copy and there are not detected in this methods. Polymerase chain reactions the methods which there is no this problem. DNA extracted

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directly from soil samples can act as a template for PCR or mRNA and can be reverse-transcribed into cDNA and then amplified using standard PCR methods [31, 32]. The use of mRNA in biodiversity studies will allow a snapshot of the active bacterial population in contaminated soil, whereas DNA extracted directly from this samples can represent active as well as dormant bacteria. The amplified PCR product can be hybridized with either oligonucleotide probes to provide specific information on the bacterial community in contaminated soil or with other samples to which bacterial community similarity is compared [35]. The PCR targeting the 16S rDNA has been used extensively to study prokaryote (bacteria) diversity and allows identification of prokaryotes as well as the prediction of phylogenetic relationships [26]. Initially, molecular-based methods for ecological studies relied on cloning of target genes isolated from environmental samples [44]. Although sequencing has become routine, sequencing thousands of clones is cumbersome [45].

3.1. The denaturing gradient gel electrophoresis methods to study bacterial diversity

The property of double-stranded DNA molecules allowing their separation in an electric field is used in many electrophoretic techniques. A standard electrophoresis consists in separating the DNA molecules by size. For this purpose, the agarose gel is prepared with the appropriate concentration, typically from 0.5 to 2%, and is connected to constant electric field. The DNA molecules pass through the small spaces within the gel and migrate at different rates depending on their size [46]. As a result, towards the end of the gel we observe DNA fragments of smaller sizes (less base pairs), and the large fragment will move slower, remaining closer to the top. In this way, it is possible to know the approximate size of the analyzed fragments [See **Figure 1**, gel on the left]. However, this method cannot be used to distinguish between each of the DNA molecules of the same size, differing only in the nucleotide sequence. The solution was developed in 1987 (See [47]). Method called denaturing gradient gel electrophoresis(DGGE) is based on the fact that only double-stranded DNA fragments move in the electric field, whereas single-stranded not have such ability, or at least their mobility is strongly reduced. Denaturation of the double-stranded structure of DNA into single strands is accomplished by treatment DNA using high temperature and denaturing agents, usually a mixture of formamide and urea [48]. The specific temperature and concentration of denaturant in which the DNA is denatured, also known as the melting point of DNA, are dependent on nucleotide sequence. This correlation means that even a single base mutation can change the melting point of DNA. What is important in understanding the phenomenon, it is not only the influence of bonds between paired bases, but also the interaction between neighboring pairs [49, 70]. This makes it possible to distinguish DNA fragments of the same size but with different nucleotide sequence [See **Figure 1**, gel on the right].

DGGE electrophoresis is usually performed at a constant temperature (usually 60°C) in the presence of two denaturing agents: formamide and urea, the concentration of which depends on the experiment and analyzed fragments. The analysis is carried out in polyacrylamide gel (6–12%), which consists of a mixture of acrylamide and bis-acrylamide, usually in a 37.5:1 ratio [50]. This polymer is resistant to high temperatures and denaturing agents, and also creates

the appropriate pores through which DNA can easily migrate. It is also characterized by a much higher resolving power with respect to agarose [51].

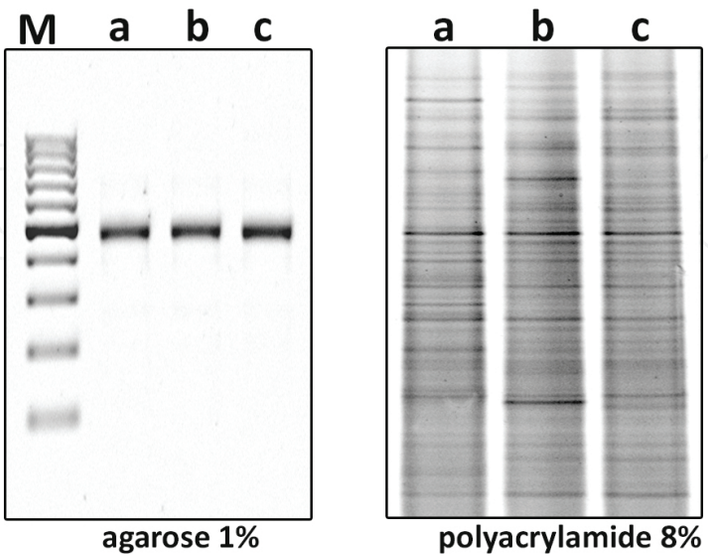

Figure 1. Comparison of agarose electrophoresis and DGGE. The letter M represents size marker of the DNA; the letters a–c are designations of samples. The same PCR products were placed on both gels for comparison.

Gel preparation and electrophoresis are in a vertical orientation, where the top of the gel is the lowest concentration of denaturing agents (usually from 0 to 30%) and the bottom of the gel fills the highest concentration (usually 50–80%). Between the extreme values, the concentration of denaturing agents creates an increasing gradient. Throughout the run electrophoresis is supplied a constant voltage, typically about 60V for 16 h [52]. In some cases, it can be applied a higher voltage of 130–150 V for 3–6 h, while the bands are then more blurred [53, 54]. This affects the image of electrophoresis. Electrophoresis in the gradient of denaturant allows the rapid identification of the different variants of genes (alleles), detection of mutations in medicine, and an overview of genetic diversity in any environment. Many studies using DGGE method is used for rapid diagnosis of disorders of human microbiota [55, 56] or to analyze the change in the composition of the bacteria in the fermenters or other dynamic biological systems [57]. DGGE limitation is the selection of appropriate fragments of DNA for analysis. This method keeps its resolving power in fragments size between 100 and 500 bp. The analyzed DNA fragments are always PCR products–amplicons, typically including the hypervariable regions of the 16S rDNA gene (in the case of bacteria) or ITS (internal transcribed spacer) in the case fungi. The ITS regions are situated between the small and large subunits of the ribosomal rDNA. The advantage of choosing these regions is the presence of both conservative and those highly variable sequences [58, 71, 72].

DGGE method has been known for more than 30 years but is continually improving. The first enhancement was the introduction of the GC-clamp. This is 20- to 60-nt-long DNA fragment that is added to one of the primers for PCR and contains only the G and C bases. It has been found to increase resolving power of the method by maintaining a small fragment of double-

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stranded structure, even at high temperatures (almost 100°C) and in high concentrations of denaturant [59].

Another improvement of the method is the use of specific markers (as a references). This involves selecting the reference strains of known origin and certified taxonomy, and then isolating the DNA. The next step is to prepare DGGE-PCR amplicons. Appropriately prepared amplicons are placed in an empty well of the polyacrylamide gel as a reference. Taking advantage of markers, it is possible to normalize gels and then compare different experiments with each other. The second application is to compare the quality and the quantity of bands in the analyzed wells, with those in the well marked as a reference in order to classify and the species composition in the sample, as well as their abundance [60].

It should be noted that this method has a broad spectrum of applications, from medicine to the currently developing metagenomics, and provides a complementary tool to traditional classical methods of exploring the composition of microorganisms. Although it does not provide as comprehensive and complete results as sequencing, the costs of its implementation and the time in which you can get to know the preliminary results are much smaller. This is a very good method for the presumptive identification of microorganisms as well as continuous monitoring of changes in the composition of microbial communities such as contaminated soil, water, bioreactors, or the composition of the human microflora.

It is worth mentioning also the limitations of DGGE. First of all, this method is based on PCR; therefore, the selection of appropriate conditions but also suitable polymerase is a key issue. Most of the problems with this method stems from mistakes at this stage. Polymerase chain reactions is always associated with the possibility of introducing errors by altering the genetic profile in the investigated samples. Occasionally, PCR products from different organisms, despite differing nucleotide sequences, may also have the same melting point. This causes the risk of missing some of the bands on the gel. On the other hand, there is a risk of nonspecific products in PCR (e.g., as a result of amplification of the chloroplast or mitochondrial DNA) to give false results. Often, in order to avoid such a situation there can be applied several-step PCRs (e.g., nested PCR), as well as touchdown PCR which is known to increase the specificity of the reaction [61].

3.2. Next-generation sequencing

Next-generation sequencing (NGS), otherwise high-throughput sequencing, resulted in a breakthrough in the automation and commercialization of the sequencing process.. In 2000, the company Lynx Therapeutics launched the first fully automated sequencing apparatus, the principle of which was still based on the Sanger method. In 2004, the company 454 Life Sciences has developed and successfully launched the sale of second-generation sequencer, which used discovered in 1996 pyrosequencing method. In addition to the huge success in the prevalence of the device, the cost of sequencing decreased sixfold in comparison with the device from 2000 [62, 63].

High-throughput sequencing is probably the fastest growing method used in the biology and biotechnology. To date emerged a series of modifications which resulted in the development of equipment relatively cheap and efficient.

On the market, there is a large selection of sequencing systems introduced by many other companies, but this chapter focuses on Illumina sequencing system. It is the most common method in the study of metagenomes different environments. Due to the a very dynamic development of the technology described herein, performance data and bandwidth become outdated several times a year.

DNA prepared for sequencing must meet several requirements. First of all, it must be free from contamination and PCR inhibitors such as humic acids, ethanol, and phenol compounds. A very important and crucial step in the preparation of biological samples is appropriate for DNA extraction and its purification. Commercially available kits provide high-performance elution of DNA, contain enzyme (such as DNase) inhibitors, and allow getting rid of impurities.

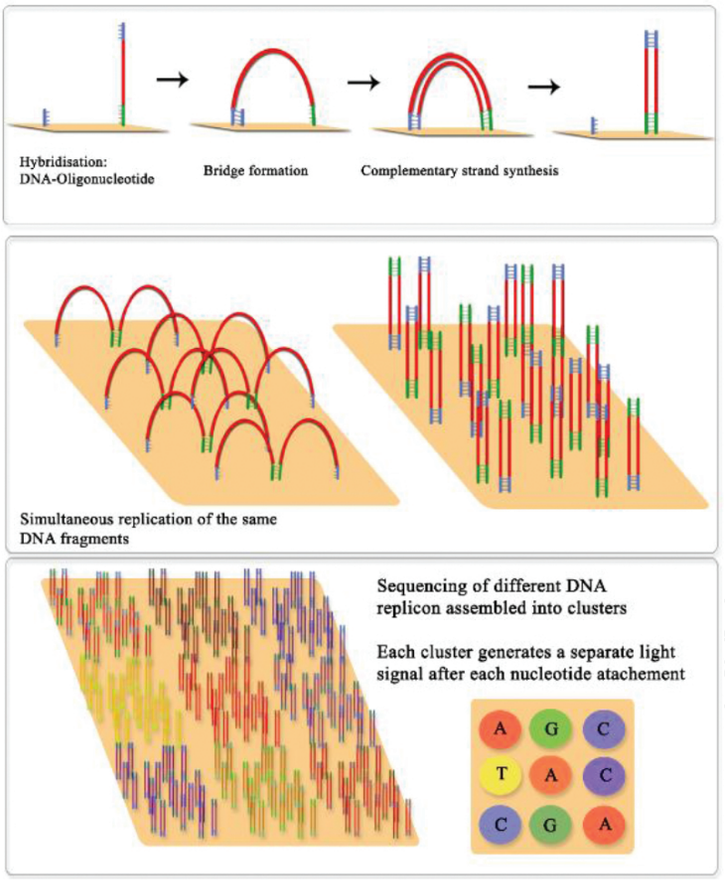

Figure 2. Cluster formation in Illumina NGS sequencing.

An important advantage is the ability to simultaneously sequencing of many samples at the same time. This is done by marking samples by attaching specific, short DNA fragments of known sequence treated as barcodes. The principle of the sequencing uses fluorescently la-

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beled nucleotides. During the attachment of one nucleotide, generation of a light signal occurs and the reaction is temporarily blocked. After registration signal, a fluorescent label is cleaved enzymatically allowing the connection of the next nucleotide. Each of the nucleotides (A, T, C, G) has a different type of fluorescent label recognized as a different wavelength. DNA is immobilized on the surface of the flow cell, which allows direct and equal access of polymerase to each of the each DNA molecule [64]. At a distance of less than one micron, there are more than a thousand copies of the same DNA fragments to form one cluster. Different DNA fragments form separate clusters, allowing for simultaneous sequencing of millions of DNA fragments [**Figure 2**].

The parameters of current devices are extremely high. Within 24 h, around 5 Gb (giga bases) of reads can be obtained, when reading 200–300 bp fragments (V3-V4 hypervariable regions for example). With exceptionally large genomic projects, there can be used the device with the highest performance (HiSeq series) allowing to generate up to 1 Tb of data within a few days [65].

Next-generation sequencing in combination with other molecular methods (including DGGE) is a very complex and indispensable method of testing microbiomes and the ecological. Metagenomic approach to the knowledge of the biodiversity present in difficult conditions, such as contaminated soil or sewage, sells out all other known methods, allowing the examination of not only a fraction of microorganisms, but also discovering new, previously unknown species [66–68].

4. Summary

The better understanding of the link between bacterial diversity and their community structure and function is very important to study microbial diversity in contaminated soil. This is not only important for basic scientific research but also to study biodiversity in soil contaminated with PAHs. Significantly higher amounts of 16S rRNA have been found in all microbial groups analyzed in fields that have never been cultivated than agricultural fields and also in soil contaminated with PAHs. This suggests a decrease in bacterial biomass or activity in cultivated fields. However, it is unknown what these reductions in diversity mean to ecosystem functioning, and it is important for the sustainability of ecosystems to examine and better understand the link between diversity and function. There are some limitations associated with studying organisms in contaminated soil. There are some taxonomic and methodological limitations. The methods to study bacterial diversity (numerical, taxonomic, structural) are improving for some group of bacteria and fungi. It is generally thought that a diverse population of microorganisms will be more resilient to biotic and abiotic stress and more capable of adapting with environmental changes (contamination). The knowledge of plant–microbe–soil-contaminant interactions is increasing, but the complexity of interacting biological, chemical, and physical factors means that much remains to be understood.

As new techniques are developed, our level of understanding increases and our knowledge expands.

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