Zuzanna SZCZEPANIAK[1], Justyna STANINSKA[2], Agnieszka PIOTROWSKA-CYPLIK[1], Paweł CYPLIK[2]
Poznań University of Life Sciences
[1] Institute of Food Technology of Plant Origin
ul. Wojska Polskiego 31, 60-624 Poznań, Poland
[2] Department of Biotechnology and Food Microbiology
ul. Wojska Polskiego 48, 60-627 Poznań, Poland
e-mail: zuzanna.szczepaniak@gmail.com

BIODEGRADATION POTENTIAL OF MICROBIAL CONSORTIUM ISOLATED FROM EASTERN CARPATHIANS SOIL

Summary

Polycyclic aromatic hydrocarbons (PAHs) pose a real threat towards all elements of the ecosystem due to their toxic properties. One of the methods reducing PAHs from the environment is bioremediation, including bioaugmentation. Bioaugmentation consists in introducing microorganisms capable of effective recultivation ground into contaminated areas. This kind of microorganisms (microbial consortia), are often isolated from places permanently contaminated with organic compounds due to their high potential for biodegradation. Genetic assays based on Next Generation Sequencing (NGS) indicate that K52 consortium obtained from crude oil contaminated site of eastern Carpathians is characterized by a high diversity of population with dominance of Pseudomonas genus. Polycyclic aromatic hydrocarbons (naphthalene, phenanthrene and fluorene) used throughout the currently described experiments were biodegradable both in the individual systems, and in the mixture, but the degree of biodegradation depended on the type of PAH. The most susceptible to biodegradation is naphthalene in the concentration of 20 mg/l, which undergoes completely decomposed after six days of experiments. In the system with initial concentration of 50 mg/l, biodegradation of naphthalene reached almost 80% after eight days. The results of flow cytometry confirm that the difficulties in PAHs decomposition increasing with amount of aromatic rings.
***Key words**: polycyclic aromatic hydrocarbons, microbial consortium, biodegradation, toxicity*

POTENCJAŁ BIODEGRADACYJNY KONSORCJUM MIKROBIOLOGICZNEGO IZOLOWANEGO Z GLEB WSCHODNICH KARPAT

Streszczenie

Wielopierścieniowe węglowodory aromatyczne (WWA) stanowią udowodnione zagrożenie dla wszystkich elementów ekosystemu ze względu na właściwości kancerogenne, teratogenne i mutagenne. Jedną z metod usuwania WWA ze środowiska jest bioremediacja, w tym bioaugmentacja zakładająca wprowadzenie do zanieczyszczonego środowiska mikroorganizmów zdolnych do efektywnej rekultywacji gruntów. Takie mikroorganizmy (konsorcja mikrobiologiczne) często izolowane są z miejsc trwale zanieczyszczonych związkami organicznymi ze względu na ich wysoki potencjał biodegradacyjny. Badania genetyczne opierające się na sekwencjonowaniu nowej generacji (NGS) wskazują, że wykorzystane w pracy konsorcjum mikrobiologiczne K52 pozyskane z trwale zanieczyszczonych olejem napędowym gleb wschodnich Karpat charakteryzuje się dużym zróżnicowaniem gatunkowym z dominacją bakterii z rodzaju Pseudomonas. Zastosowane wielopierścieniowe węglowodory aromatyczne (naftalen, fenantren i fluoren) wykazywały się biodegradowalnością zarówno w układach indywidualnych, jak i mieszaninie, przy czym stopień biodegradacji zależał od rodzaju WWA. Najbardziej podatnym na biodegradację jest naftalen, który ulegał całkowitemu rozkładowi w szóstym dniu inkubacji w układzie indywidualnym (20 mg/l). W układzie o stężeniu wyjściowym 50 mg/l biodegradacja naftalenu po ośmiu dniach inkubacji osiągnęła blisko 80%. Wyniki cytometrii przepływowej potwierdzają dane literaturowe wskazujące na ogólną tendencję wzrostu toksyczności WWA wraz ze wzrastającą liczbą pierścieni – naftalen posiada tylko dwa pierścienie i jak wynika z aktywności komórek mikroorganizmów jest dla nich mniej toksyczny niż trójpierścieniowy fenantren.
***Słowa kluczowe**: wielopierścieniowe węglowodory aromatyczne, konsorcjum mikrobiologiczne, biodegradacja, toksyczność*

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of over 100 homocyclic compounds composed of at least two aromatic rings in the molecule. Due to their mutagenic, carcinogenic and teratogenic properties, PAHs are a threat to all elements of the ecosystem, as well as the health and life of humans [1]. The PAHs sources in the environment could be both natural (natural pyrolysis processes such as forest fires, grassland) and anthropogenic (products of incomplete combustion of fossil fuels and wood during heating, transport, coking and refineries operations, and other). [2]. However, it is worth noting that natural sources of emissions are of marginal importance. The main reason for the accumulation of PAHs in ecosystems is human activity (anthropogenic sources). [3]

According to literature reports, plant cultivation and animal husbandry in contaminated areas can promote excessive accumulation of PAHs in raw materials and agricultural products [4]. Threat to the crops may be associated with the collection of PAH from soil and water through the roots, and their adsorption on the surface of vegetables and fruits. A danger to animals results from, for example, uptake of PAH with food plant origin and soil during grazing. In this way, PAHs can accumulate in the fat of milk, which in turn significantly affects the safety of its consumption by people [5].

Organic contaminants, such as PAH can be removed from the environment by both physico-chemical and biological methods [6]. Particularly noteworthy are the biological methods - bioremediation techniques, using microorganisms to degrade xenobiotics. However, the low solubility of PAHs is a limiting factor for their degradation, due to the low bioavailability for microorganisms. Moreover, high toxicity of organic pollutants inhibits the growth of soil microorganisms, changes their population and disturbance the ecosystem balance [7]. Therefore, there are a number of methods enabling bioremediation for example using additional substances, such as surfactants, biogenic elements or oxygenation. [8]. Another way to improve degradation of PAHs is bioaugmentation. During bioaugmentation process, additional microorganisms capable to efficient decomposition of organic compounds are introducing into contaminated area. It is very common to take advantage of microbial consortia obtained from permanently contaminated areas. Studies indicate that microorganisms isolated from contaminated areas have developed mechanisms responsible for the biodegradation of toxic compounds [9]. The use of biotechnological methods for the remediation of agricultural soils classified as contaminated can raise their quality by improving biological properties and profitability.

However, it is necessary to asses a natural self-purification capacity of the environment to determine an optimal implementation.

The aim of the study was to determine the potential to PAHs biodegradation of microbial consortium isolated from the eastern part of the Carpathian Mountains – areas permanently contaminated with organic compounds. The obtained results could be helpful in assessing the potential of the environment to self-purification and possible use of isolated consortia in *in situ* bioremediation of contaminated land, including agricultural soils. In addition to the characteristics of the consortium (using genetic methods), the degree of PAHs biodegradation and metabolic activity of microorganisms in systems of PAHs were also determined.

2. Materials and methods

2.1. Chemical reagents and microorganisms

Policyclic aromatic hydrocarbons: phenanthrene, naphthalene and fluorene were purchased from Sigma Aldrich. All the reagents were of the highest purity available. The microbial consortium, assigned K52 has been isolated from a crude oil-contaminated soil collected in Kryg (Poland).

2.2. The experimental variants

The metabolic activity and biodegradation assessment were performed in 300 ml flasks with the following sets of experiments: 1) tap water 100 ml + phenanthrene 20 mg/l + K52 2ml; 2) tap water 100 ml + phenanthrene 50 mg/l + K52 2ml; 3) tap water 100ml + fluorene 20 mg/l + K52 2 ml; 4) tap water 100 ml + fluorene 50 mg/l +K52 2 ml; 5) tap water 100ml + naphthalene 20 mg/l + K52 2 ml; 6) tap water 100 ml + naphthalene 50 mg/l + K52 2 ml; 7) tap water 100 ml + mix 20 mg/l + K52 2 ml; 8) tap water 100 ml + mix 50 mg/l + K52 2 ml;
Internal standard without microorganisms was used in order to assess the degree of non-biological degradation of PAHs.

2.3. Cultivation conditions

Cultivation was carried out for 8 days. Samples were taken sequentially at the beginning (day 0), after 2, 4, 6 and 8 days. Culturing was carried out under aerobic conditions at 28°C.

2.4. Genetic assays

Analysis of the consortium were conducted with the use of metagenomic assays. DNA isolation was performed using SoilMaster™DNA Extraction Kit. The obtained genetic material was free from contamination and PCR inhibitors. In a further step the primers specific for regions V3/V4 were used. To compose the library and NGS sequencing, the commercially available The Nexter XT DNA Sample Preparation Kit and MiSeq Reagent Kit v3 were used. The analysis was carried out with BaseSpace and MiSeqReporter software.

2.5. HPLC analysis

The HPLC analyses were based on the methodology described in the PN-EN ISO 17993 “Determination of 15 polycyclic aromatic hydrocarbons (PAH) in water by HPLC with fluorescence detection after liquid-liquid extraction”.

Identification and quantification were carried out by detecting UV-VIS / DAD.

2.6. Microbial activity of consortium

Metabolic analysis of microbial cells was carried out using a set of BacLight™ Redox Sensor™ Green Vitality Kit from Invitrogen. Cell metabolic activity is expressed as the oxidation-reduction potential of the bacterial strains. Determination of the redox potential of analyzed bacterial strains, were obtained by adding a fluorescent reagent RedoxSensor™ Green. Determination of cell viability was obtained by adding propidium iodide. Parameters waere measured using flow cytometer BD FACS Aria™ III (Becton Dickinson Biosciences).

3. Results and discussion

3.1. Identification of microorganisms within microbial consortium K52

Analysis of metagenomes (sequencing whole genetic material from ecological niche) allowed a full, complete taxonomic evaluation. 14 classes, 48 families and 175 species were identified, and the most numerous of them are presented in figures 1 and 2.

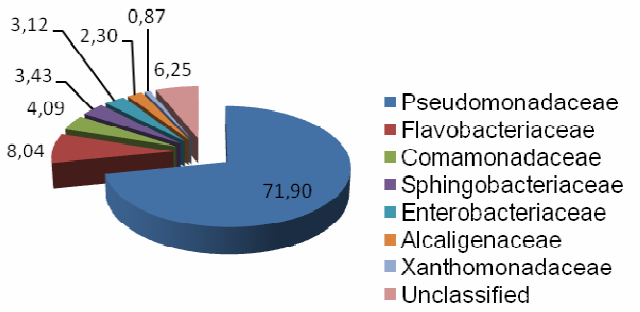

Figure 1. Metagenomes classification with reference to families

Despite the high level of contamination, a large variety of microorganisms could be observed. This may be associated with a huge potential of microorganisms to adapt to

high concentrations of pollutants. The results clearly indicate domination of *Pseudomonas* and *Sphingobacterium* (species *P. alcaligenes, P. aeruginosa, P. fluorescens, S. multivorum*). The domination of the family *Pseudomonadaceae* may results from their unique ability to metabolize petroleum products as sources of carbon, unavailable for other indigenous groups of microorganisms and a high level of resistance to environmental stress. [10] It can be assumed that the long-term contamination resulted in separating of autochtonic microflora with genes responsible for the synthesis of dioxygenase enzymes (with potential application in bioremediation technologies).

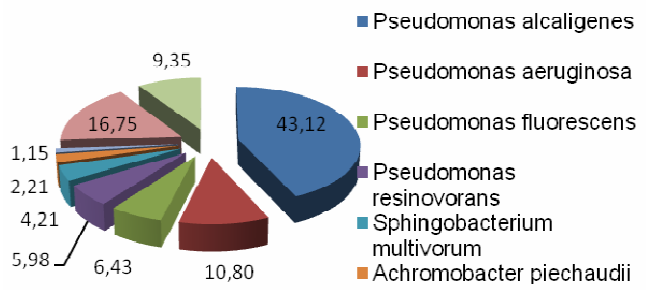

Figure 2. Metagenomes classification with reference to species

3.2. Evaluation of the PAH biodegradation efficiency by a K52 consortium

In all studied systems, a biodegradation of PAHs was observed, and the biological decomposition efficiency depended heavily on the type of PAH. Analyses of internal standards excluded non-biological degradation of PAHs. Studies indicate that the most susceptible to biodegradation is naphthalene, which undergoes completely decomposition after six days of incubation in the individually system (20 mg/l) (Fig. 3). In the sample with concentration of 50 mg/l, the naphthalene biodegradation after eight days reached almost 80%. Biodegradation of phenanthrene and fluorene was significantly lower than naphthalene for all tested samples. At a concentration of 20 mg/l biodegradation of fluorene was slightly higher than phenanthrene and for the concentration of 50 mg/l phenanthrene and fluorene reduction was similar (nearly 25% (Fig. 4)). This phenomenon can be explained by the fact that the concentration of phenanthrene and fluorene 50 mg/l is highly toxic and significantly inhibit the enzymatic activity. Literature reports confirm that the difficulties in PAHs decomposition increasing with amount of aromatic rings. [11].

Biodegradation of the most difficult degradable tested PAHs - phenanthrene and fluorene - in mixed systems showed higher level than in individual systems with corresponding concentrations. This can be explained by the fact that PAHs are biodegradable in a cometabolism way. [12]. It is also worth noticing that the PAHs in mixed systems had access to more easily assimilable carbon source which is naphthalene. In this case, the naphthalene as an easily assimilable carbon source could be a factor that stimulate and facilitate the biodegradation of other PAHs.

The dynamics of biodegradation will be discussed using naphthalene as an example. Both in the mixture and in the individual system 20 mg/l (Fig. 3, Fig. 5), the highest loss was observed in the first two days, when the reduction reached 50% of the initial amount (logarithmic phase). After six days the concentration of PAH was close to zero (stationary phase). In the case of an individual system with beginning concentration of 50 mg/l (Fig. 4) in the first two days, the biodegradation amounted only 20%. The fastest decrease in the amount of naphthalene in this sample occurred between the second and fourth day culture which is associated with the fact of the delayed input the microorganisms into the logarithmic phase of growth as a result of necessity to adapt to extreme conditions.

3.3. Evaluation of the microorganisms metabolic activity during the PAH biodegradation

Directly after the addition of PAHs at the concentration of 20 mg/l, the number of active cells diversified depending on the specific PAH: from 5000 relative fluorescence units (RFU) for naphthalene by approx. 4600 RFU for phenanthrene, until 4400 RFU for fluorene and 4300 RFU for mixture of PAHs (Figure 6).

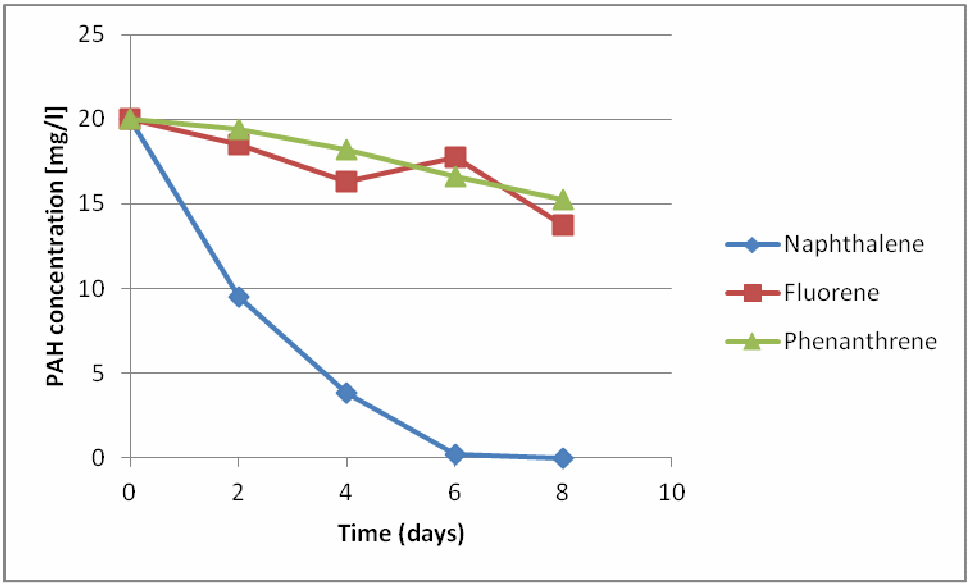

Figure 3. Changes in the concentration of naphthalene, fluorene, and phenanthrene at an initial concentration of 20 mg/l

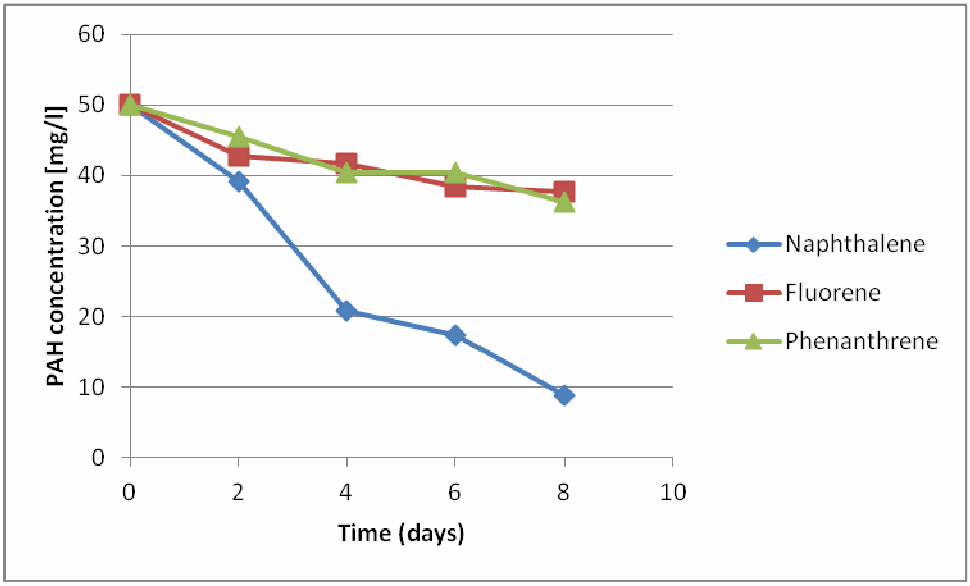

Figure 4. Changes in the concentration of naphthalene, fluorene, and phenanthrene at an initial concentration of 50 mg/l

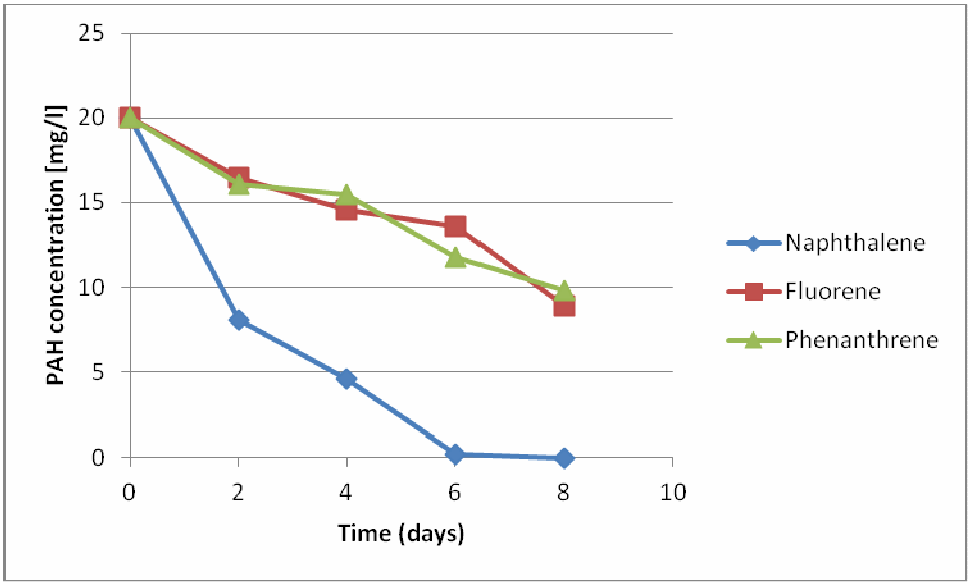

Figure 5. Changes in the concentration of naphthalene, fluorene, and phenanthrene in mixture at an initial concentration of 20 mg/l

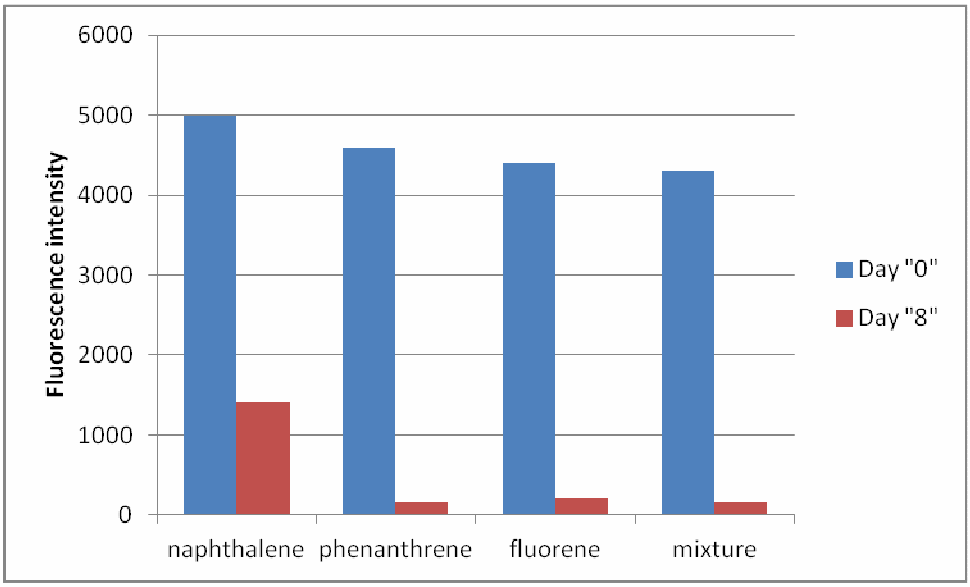

Figure 6. The metabolic activity of the K52 consortium at the beginning and after 8 days of experiment at a concentration of 20 mg/l

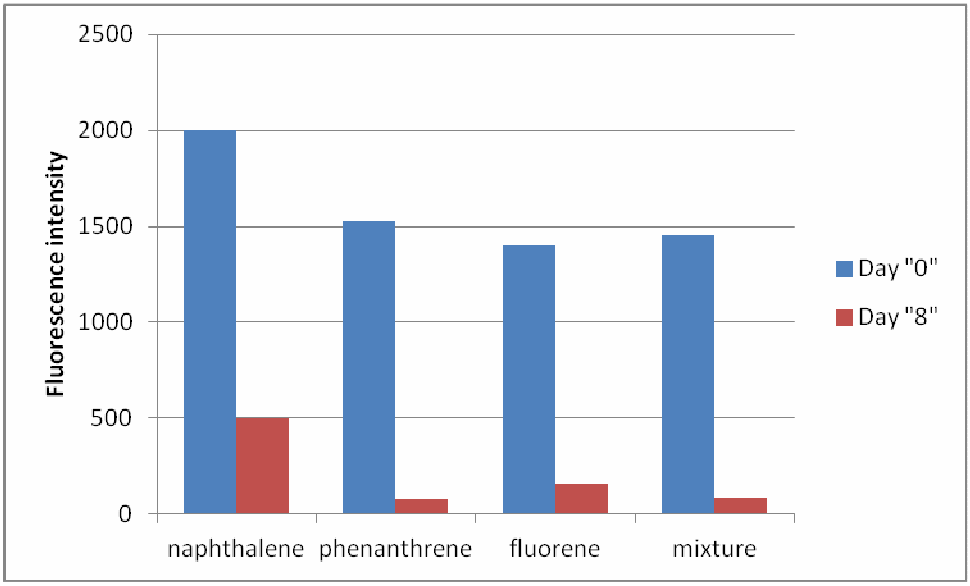

Figure 7. The metabolic activity of the K52 consortium at the beginning and after 8 days of experiment at a concentration of 50 mg/l

This means that the toxicity of naphthalene towards microbial consortium is smaller than toxicity of other PAHs used in the experiment. Metabolic activity tests performed immediately after the addition of PAHs at the concentration of 50 mg/l, showed a fluorescence intensity of 2000 RFU for naphthalene to 1400 RFU for fluorene (Figure 7). These results indicate that the concentration of 50 mg/l is much more toxic to microorganisms than the concentration of 20 mg/l.

Measurement of active microbial cells after eight days for the samples at a concentration of 20 mg/l, showed the fluorescence intensity for the naphthalene approx. 1400 RFU, for fluorene approx. 200 RFU, and for the remaining less than 100 RFU (Figure 6). After eight days for samples with initial concentration of 50 mg/l the number of active cells for the naphthalene reached approx. 500 RFU, the fluorene approx. 150 RFU, while for the rest below 100 RFU (Figure 7).

Significantly lower cell activity, even for the least toxic PAH - naphthalene - at a concentration of 50 mg/l suggests that this concentration is probably close to the limit of toxicity for microorganisms of the consortium K52. In other cases (phenanthrene, fluorene and mixture) after 8 days of experiment, the activity of living cells was close to zero, which may indicate that the concentrations of PAHs were too high. It is in agreement with results obtained by Yessica Gonzalez-Paredes et al. [13]. The author found the 40% inhibition of soil bacteria *Rhizobium Tropici* growth after 24 h of exposure to phenanthrene at a concentration of 40 mg/l. It is also worth noting that the model conditions differ significantly from natural soil conditions, where PAHs may undergo adsorption onto soil organic molecules. Due to the diverse nature of the soil, field studies are essential for the optimization of bioremediation processes and will be the subject of further research.

The obtained results confirm the literature data indicating the general trend of increased toxicity of PAHs with increasing number of rings [14]. Naphthalene consists of only two rings and is less than tricyclic phenanthrene.

Rapidly decreasing the activity of the microbial cells in the system with relatively low toxic naphthalene (after eight days of experiment, a decrease of active cells from the initial amount by 60% for a concentration of 20 mg/l and 90% for a concentration of 50 mg/l) may result from the fact that naphthalene was completely degraded after 6 days. The decrease of metabolic activity is associated in this case, with the lack of carbon source needed for microbial growth.

4. Conclusion

Microbial consortium isolated from permanently contaminated areas is characterized by high species diversity, and the metagenomic assays confirm the prevalence of bacteria of the genus *Pseudomonas*. Both the results obtained using flow cytometry and liquid chromatography indicates the fact that the consortium isolated from eastern Carpathians soils has the potential to biodegrade naphthalene, and has a large restriction in the direction of biodegradation of PAHs with more than two rings. This is an evidence that the environment permanently contaminated with petroleum products (including agricultural land excluded from use due to contamination) have insufficient capacity for complex biological self-cleaning and require the use of additional biotechnological methods to improve these processes.

5. References

[1] Yang F., Zhang Q., Guo H., Zhang S.: Evaluation of cytotoxicity, genotoxicity and terstogenicity of marine sediments from Qingdao coastal areas using in vitro fish cell assay, comet assay and zebrafish embryo test; Toxicologi in Vitro, 2010, 24, 2003-2011.

[2] Haritash A.K., Kaushik C.P.: Biodegradation aspects of Policyclic Aromatic Hydrocarbons (PAHs): A review; Journal of Hazardous Materials, 2009, 169, 1-15.

[3] Smolik E.: Wielopierścieniowe węglowodory aromatyczne WWA. Instytut Medycyny Pracy i Zdrowia Środowiskowego, Sosnowiec, 2001. (www.ietu.katowice.pl).

[4] McLachlan M.S.: Bioacumulation of hydrofobic chemicals in agricultural food chain. Organohalogened Compounds, 1995, 22, 81-103.

[5] Kubiak M.S.: Wielopierścieniowe węglowodory aromatyczne (WWA) – ich występowanie w środowisku i w żywności; Problemy Higieny i Epidemiologii, 2013, 94, 31-36.

[6] Gan S., Lau E.V., Ng H.K.: Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs); Journal of Hazardous Materials, 2009, 172, 532-549.
[7] Eom I.C., Rast C., Veber A.M., Vasseur P.: Ecotoxicity of polycyclic aromatic hydrocarbons (PAH)-contaminated soil. Ecotoxicol. Environ. Safety, 2007, 67, 190-205.
[8] Kanaly R.A., Harayama M.D.: Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J. Bacteriol., 2000, 182, 2059-2067.
[9] Wickle W.: Polycyclic aromatic hydrocarbons (PAHs) in soil – a review. I. Plan. Nutr. Soil.Sci. 163, 229-248.
[10] Mueller J.G., Chapman P.J., Pritchard P.H.: Action of a fluoranthene-utilizing bacterial community on polycyclic aromatic hydrocarbon components od creosote. Appl. Environ Microbiol., 1989, 55, 3085-90.
[11] Atlas R., Bartha R.: Ecologia microbiana y microbiologia ambiental, fourth ed. Editorial Prentice Hall, Espana, 2002, 677pp.
[12] Johnsen A. R., Karlson U.: Diffuse PAH contamination of surface soils: environmental occurence, bioavailability, and microbial degradation. Appl. Microbiol Biotechnol., 2007, 76, 533-543.
[13] González-Paredes Y., Alarcón A., Ferrera-Cerrato R., Almaraz J. J., Martínez-Romero E., Cruz-Sánchez J. S., Mendoza-López Ma. R., Ormeno-Orillo E.: Tolerance, growth and degradation of phenanthrene and benzo[a]pyrene by Rhizobium tropici CIAT 899 in liquid culture medium. Applied Soil Ecology, 2013, 63, 105-111.
[14] Tikilili P.V, Nkhalambayausi-Chirwa E.M.: Characterization and biodegradation of polycyclic aromatic hydrocarbons in radioactive wastewater. J. Hazard Mater. 2011, 15, 192(3), 1589-96.