Flow cytometry as a modern tool in evaluation the activity of microorganisms degrading polycyclic aromatic hydrocarbons (PAHs) in bioremediation process

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ABSTRACT

Environmental pollution by organic compounds can be reduced by the use of bioremediation methods e.g. bioaugmentation enhanced by the addition of biosurfactants. The aim of this study was to determine the metabolic activity of both autochthonous microorganisms and microbial consortia added into system in the presence of polycyclic aromatic hydrocarbons (PAHs) and biosurfactants (rhamnolipids) in the soil system. Metabolic activity was determined using flow cytometry. The results indicate both the competition between additional and autochthonous microorganisms after three months, as well as the dominance of additional microorganisms over native microorganisms after six months after start of the experiment. Moreover, the results do not indicate any effect of rhamnolipids on the value of %Q2.

Cytometria przepływowa jako nowoczesne narzędzie stosowane w ocenie aktywności mikroorganizmów biodegradujących wielopierścieniowe węglowodory aromatyczne (WWA) w procesie bioremediacji środowiska

Słowa kluczowe: ramnolipidy, WWA, cytometria przepływowa, aktywność metaboliczna

STRESZCZENIE

Zanieczyszczenie środowiska związkami organicznymi można zredukować m.in. poprzez metody bioremediacyjne z wykorzystaniem np. bioaugmentacji lub biosurfaktantów. Celem badań jest określenie aktywności metabolicznej konsorcjów mikrobiologicznych dodawanych na zasadzie bioaugmentacji oraz mikroorganizmów autochtonicznych w obecności wielopierścieniowych węglowodorów aromatycznych (WWA) i biosurfaktantów (ramnolipidów) w środowisku glebowym. Aktywność metaboliczna została wyznaczona dzięki zastosowaniu cytometrii przepływowej. Uzyskane wyniki wskazują zarówno na konkurencję pomiędzy mikroorganizmami wprowadzonymi dodatkowo a mikroorganizmami autochtonicznymi już po trzech miesiącach trwania eksperymentu, jak również na zdominowanie układu przez dodatkowo wprowadzone mikroorganizmy po upływie sześciu miesięcy. Równocześnie wyniki nie wskazują na jakikolwiek wpływ dodanych ramnolipidów na wartość %Q2.

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds that consist of at least two aromatic homocyclic rings and usually have carcinogenic, mutagenic and teratogenic properties. Specific toxicological danger of PAHs is associated not only with their toxicity, but also with their rapid biotransformation (with the use of CYP1 enzymes), leading to a range of intermediate metabolites with a broad spectrum of activity [1]. Sources of PAHs in the environment can be classified both as natural (including e.g. forest fires, volcanic eruptions) and anthropogenic, e.g. the processing of fuels, coke industry and metallurgy [2]. Currently, both physico-chemical and biological methods are used to remove hydrophobic compounds, such as PAHs, from the environment [3]. The main disadvantage of physico-chemical methods is the lack of complete elimination of xenobiotics from the environment. These methods allow only for the transfer of contaminants to the storage or purification area. Biological methods – especially bioremediation which employs hydrocarbon-degrading microorganisms – enable for the full bioconversion of complex chemical compounds into simple molecules (in the final stage into carbon dioxide and water). However, there are several factors which limit efficiency of bioremediation, including low bioavailability of hydrophobic compounds to microorganisms [4]. Therefore, there are several techniques which support bioremediation, including bioaugmentation based on the addition of microorganisms that possess higher metabolic potential towards pollutants. Recently, a lot of researchers have focused on the microbial consortia isolated from permanently contaminated sites with theoretically high biodegradation potential. Also surfactants (including biosurfactants – surfactants or microbial origin) can also be applied into contaminated areas. Example of biosurfactants used in bioremediation process are rhamnolipids [5].

The aim of introducing exogenous microbial consortia into the environment (bioaugmentation) is to increase the efficiency of natural biodegradation processes occurring in particular ecosystem. Such microbial communities should be characterized by a high metabolic activity and ability to adapt and function in the environment with indigenous microflora. The activity of microorganisms could be determined using flow cytometry. Flow cytometry allows the analysis of the metabolic activity of individual cells, and thus to recognize the share of individual species in the microbial consortia during biodegradation. Moreover, it enables observation of both the metabolic activity and population variability of individual microbial species. The essence of the method involves dying the cells, which in the first stage of

the analysis are set in a row of thin stream and further moved to the measurement zone, where a laser beam irradiates single cells. Diffused light induces fluorochromes, and the obtained signals are analyzed by detectors. The last step of analysis contains the computer processing of the data [6]. According to Oleszczuk, the pollution of air and water is much smaller than in the case of soil which is a additionally more complex matrix [7]. Therefore, there is a need to put emphasis on the soil environment in the context of difficult selection of a proper method and technology enabling effective removal of organic pollutants.

The aim of the study was to determine the metabolic activity of both indigenous and additionally introduced exogenous microorganisms in the presence of polycyclic aromatic hydrocarbons (PAHs) and rhamnolipids using flow cytometry. The research was conducted in the soil environment.

2. EXPERIMENTAL

2.1 Chemicals

In the experiment, the polycyclic aromatic hydrocarbons (Sigma Aldrich) were used in the following proportions for each sample (a total of 200 mg PAH per sample): naphthalene 34.84 mg; phenanthrene 34.84 mg; fluorene 34.84 mg; anthracene 34.84 mg; 30 mg pyrene; fluoranthene 9.88 mg; acenaphthylene 9.88 mg; acenaphthen 9.88 mg; chrysene 0.2 mg; benzo[a]anthracene 0.2 mg; benzo[k]fluoranthene 0.2 mg; benzo[b] fluoranthene 0,2 mg; benzo[a]pyrene 0.2 mg. Rhamnolipids (AGAE) were characterized by a purity of 90% and 5% concentration.

2.2 Soil and microorganisms

The soil used throughout the currently described experiments came from the urban site – Poznań city. The preparation of soil included sieving with the use of laboratory metal sieve (4 mm). This allowed the elimination of larger particles, which could introduce significant abnormalities in determining the mass of the system. Microorganisms were added in the form of consortia derived from sites permanently contaminated with petroleum. Microbial inoculum was prepared as follows: 10 g of soil was collected from creosote- and petroleum-contaminated areas located in Solec Kujawski (Poland) and next shaken with 90 ml of saline for 15 minutes. Then microorganisms in extract (5 ml) were grown in an aqueous system comprising standard M9 mineral medium and a mixture of naphthalene, phenanthrene, and pyrene in the maximum concentration not exceeding the solubility limit. The addition of a PAHs mixture was necessary due to the need of selection of microorganisms capable of propagation in the PAHs presence as the sole carbon source. After pre-propagation step and centrifugation, the pellet was suspended in 5 ml of saline. Preinokulat has been subjected to the further propagation in the presence of diesel fuel (5 mL) and the standard mineral medium M9 (50 mL) and the online spectrophotometric (UV-Vis spectrophotometer, Thermo Scientific, 600 nm) evaluation of kinetics of growth consortium was carried out. Based on observations of the growth curve after the entry into the logarithmic phase, the pellet was centrifugated and resuspended in saline to yield a final concentration of 10^6 cfu/ml. The inoculum was directly spiked (in liquid form) into 50 g of soil and the remaining 150 g of the soil was added and mixed.

2.3 Experimental variants

Experiments were conducted with following experimental systems: 1) 200 g of soil (reference sample); 2) 200 g of soil + polycyclic aromatic hydrocarbons; 3) 200 g of soil + polycyclic aromatic hydrocarbons + additional microorganisms; 4) 200 g of soil + polycyclic aromatic hydrocarbons + rhamnolipids; 5) 200 g soil + polycyclic aromatic hydrocarbons + additional microorganisms + rhamnolipids.

Analyses were carried out after: 1) one month; 2) three months; 3) six months; 4) twelve months of the experiment.

In order to maintain the same test conditions, samples were stored at constant temperature (18-20^{o}C) and soil moisture was remained at the same level (31%).

2.4 Metabolic activity analyses

During metabolic activity analyses, the flow cytometer BD FACSAria™ III Becton Dickinson Biosciences was used. Samples for analysis were prepared based on the methodology proposed by Molecular Probes Invitrogen Detection Technologies (BacLight™ RedoxSensor™ Green Vitality Kit). The sample preparation consisted of the following steps: soil samples were extracted with di-

stilled water at a ratio of 10 g of sample: 100 ml of water. Then the samples were shaken for approximately 15 minutes and 2 ml of the suspension was filtered through a filter with a pore diameter 20 µm. In a next step the samples were centrifuged (4 000 rpm 6 min), the supernatant was decanted and the precipitate was washed with 0.5 ml PBS. Green reagent (1.5 µl) and propidium iodide (0.8 µl) as fluorochromes were added and samples were incubated at 38°C for 10 min. Green reagent is a fluorescent dye for measuring redox, while propidium iodide is used to measure cell viability on the basis of intensive enter into cells with damaged cell wall and lack of penetration (and hence the absence of dying) in intact cells. In the last step analyses on flow cytometer were performed.

2.5 Statistical analysis

All analyses were performed in triplicate, and standard errors of the mean were calculated.

3. RESULTS AND DISCUSSION

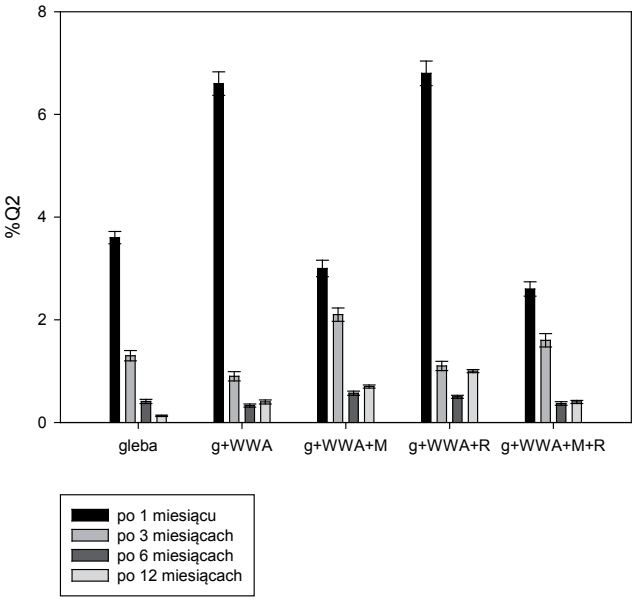

Figure 1 Percentage of active population (%Q2) in different variants

During the analyses of the differences in metabolic activity of individual variants, the parameter %Q2 should was measured (Fig. 1). This parameter determines the percentage of cells with the highest oxidation-reduction potential.

%Q2 has been determined by division the field on the dot plot charts into four parts, and consequently a selection of boundaries between the Q2 population and the other populations was possible. Dot plot charts of selected variants with determined divisions are shown in Figure 2.

After the first month of the experiment the most highly metabolically active cells (highest %Q2) were in the sample containing soil + PAH + rhamnolipids (6.8%) and slightly less in the sample with soil + PAH (6.6%). This could be explained by the presence of additional carbon source for the microorganisms in the form of PAHs and rhamnolipids, which enhanced their metabolic activity. It should be noted that in samples containing additional microbial consortium (soil + PAH + microorganisms and soil + PAH + microorganisms + rhamnolipids) the percentage of active population was the lowest (3% and 2.6% respectively). This is in disagreement with literature reports indicating a high ability to metabolize hydrocarbons by consortia isolated from areas permanently contaminated with petroleum [5]. On the other hand, antagonistic interactions – especially competition for food sources (carbon source) between autochthonous microorganisms and exogenous microbial consortium – could have negative effect on the activity of microbiota. This resulted in a decrease in the number of cells with the oxidation-reduction highest potential [8].

In addition, no significant differences in %Q2 between samples containing PAH and PAH + rhamnolipids, as well as PAH and PAH + microorganisms and PAH + microorganisms + rhamnolipids suggest a lack of additional effect of biosurfactants on the bioavailability of substrates. This is in the contrary to studies conducted by Sliwka et al. (2009), which proved beneficial effect of rhamnolipids isolated from *Pseudomonas* PS-17 on coal tar components biodegradation by increasing their bioavailability [9].

The results obtained after three months of experiment are characterized by another trend. In this case, the highest number of highly metabolically active cells was observed in variants with additional microorganisms i.e. variants of soil + PAH + microorganisms – (2.1%) and soil + PAH + microorganisms + rhamnolipids – (1.9%). This may be explained by complete adaptation and domination of exogenous microbial consortium. Thus, only three months after the beginning of experiment the thesis of high metabolic activity of consortia isolated from permanently contaminated sites was confirmed. Jacques et al. (2008) testing microbial consortium (bacteria: Mycobacterium fortuitum, Bacillus cereus, Microbacterium sp.,

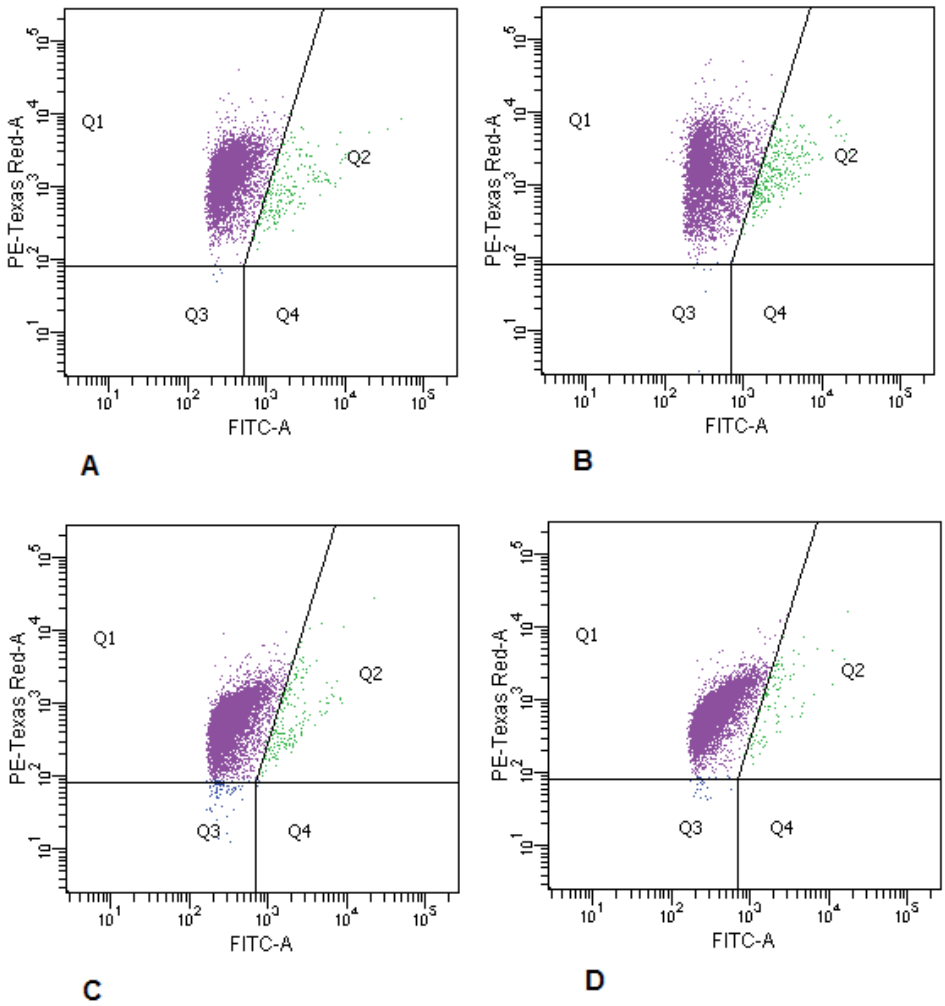

Figure 2 The dot plots charts showing the dependence of the fluorescence signal area from RedoxSensor reagent (FITC-A) and propidium iodide which allows differentiation of dead/live cell of variants: A) soil + PAH + microorganisms after 1 month; B) soil + PAH + rhamnolipids after 1 month; C) soil + PAH + microorganisms after 3 months; D) soil + PAH + rhamnolipids after 3 months

Gordonia polyisoprenivorans, Microbacteriaceae bacterium, Naphthalene-Utilizing bacterium and fungus Fusarium oxysporum) isolated from areas contaminated with polycyclic aromatic hydrocarbons within 70 days confirmed that this combination of microorganisms is characterized by a better ability to biodegradation (particularly in relation to anthracene, phenanthrene and pyrene with 99%, 99% and 96% of biodegradation respectively) compared to the autochthonous microorganisms [10].

Variants characterized by the highest value of %Q2 after first month i.e. soil + PAH and soil + PAH + rhamnolipids, after three months were marked by lower metabolic activity compared to the samples with additional microorganisms. Lower %Q2 may be related to the depletion of the additional carbon source (PAH and rhamnolipids).

After six and twelve months values of %Q2 were similar. Especially after six months there were no significant differences between the variants. Analyzing the differences in values %Q2 in function of time it is worth noting that during the first six months the number of cells highly active metabolically in different samples significantly decreased. This is probably the result of the gradual depleting of both carbon sources and other nutrients necessary for proper functioning of microorganisms. Over the next six months, there no significant differences in the values of %Q2 were noticed.

4. CONCLUSIONS

The obtained results demonstrate the complex interactions between the indigenous soil microflora and introduced microbial consortium as well as significant impact of additional microorganisms on the measured metabolic activity. Decrease in %Q2 can be explained by competition

between microorganisms. Toxic effects of PAHs is unlikely due to the increased metabolic activity of the variant containing soil and PAH compared with the control sample (soil) after 1 month. Moreover, the results do not indicate any effect of rhamnolipids on the value of %Q2.

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