CHEMICAL ENGINEERING TRANSACTIONS
VOL. 70, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Timothy G. Walmsley, Petar S. Varbanov, Rongxin Su, Jiří J. Klemeš

**ISBN** 978-88-95608-67-9; **ISSN** 2283-9216
DOI: 10.3303/CET1870297

# Biological Degradation of Pyrene and Fluoranthene in Porous Media and Attached Growth Systems by Biosurfactant Producing Bacteria

Evans M. N. Chirwa*, Tshilidzi B. Lutsinge

Environmental Engineering Group, Department of Chemical Engineering, Univ. Pretoria, Pretoria 0002, South Africa; evans.chirwa@up.ac.za

The dissolution and degradation of higher ring-number polycyclic aromatic compounds were tested in batch and attached growth medium using soil biopile and fixed-film bioreactor systems. Bioavailability of polycyclic aromatic hydrocarbons (PAHs) decreases with increasing ring-number. This means high ring-number compounds will survive longer in the environment and will therefore have a longer-term impact. In the current study, pyrene and fluoranthene were degraded using a biosurfactant producing culture of Pseudomonas aeruginosa grown in fixed-bed and soil biopile experiments. High ring-number PAHs prove to be recalcitrant in nature due to their hydrophobicity and poor partitioning to soil media fixed biomass in biofilm reactors. The use of biosurfactant could facilitate faster dissolution of the PAH and thus would increase bioavailability of the PAH compounds to PAH degrading bacteria. The culture used in this study was obtained from engine oil contaminated soil, that grow on HMW PAHs and are capable of degrading PAHs were enriched, isolated and characterize. The isolated microbial cultures were further characterised 16S rDNA gene sequencing and genotype fingerprinting. During baseline study, over 90 % fluoranthene was degraded as a model compound in biofilm reactor system. Over 88 % pyrene was degraded in soil biopiles. In both cases, increased biosurfactant dose improved degradation rate up to the point where the PAH concentration became inhibitory to biomass growth and PAH degradation.

## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are stable aromatic hydrocarbon molecules with two or more fused benzene and/or pentacyclic rings in linear, angular or cluster formation (Chauhan et al., 2008). The release of PAHs into the environment is wide-spread since these compounds are ubiquitous products of incomplete combustion and industrial activity throughout the world. Complete combustion of hydrocarbons in oxygen results in the complete oxidation of the carbon and hydrogen present to carbon dioxide and water, however, it is not as simple to completely combust organic substances. In the natural environment, it becomes more difficult to achieve complete combustion due to factors such as temperature and the presence of oxygen (Maliszewska-Kordybach, 1999). Incomplete combustion of organic substances at high temperatures and under pyrolytic conditions results in formation of PAHs (Figure 1) (Samanta et al. 2002). At temperature range of 400 - 5,000 $^{o}$C, organic components are partially cracked into smaller, unstable molecules which in turn recombine to form larger and more complex PAHs (Kabziński 2002).
PAHs are introduced into the environment not in pure form, but rather as a complex of compounds in both natural and artificial materials. One such material, creosote, has been used as a wood preservative for decades. Creosote is a complex mixture of over 200 compounds, predominantly PAHs, as well as phenolic and aromatic nitrogen and sulphur compounds. PAHs comprise 85 % of the creosote composition by weight and up to 30 different PAH species can be released from one creosote source (Melber et al., 2004). Materials painted with creosote emit PAHs slowly into soil causing long term pollution.
Several treatment strategies involving biological, physicochemical, and thermal processes have been developed to remediate contaminated sites. Methods such as incineration, excavation, landfilling and storage are expensive, inefficient, and often exchange one problem for another (Bustamante et al., 2012).

Please cite this article as: Chirwa E.M.N., Lutsinge T.B., 2018, Biological degradation of pyrene and fluoranthene in porous media and attached growth systems by biosurfactant producing bacteria , Chemical Engineering Transactions, 70, 1777-1782 DOI:10.3303/CET1870297

Biological treatment, on the other hand, offers low cost and more environmentally friendly alternative since the organic components that are responsible for the toxicity may be completely mineralized to $CO_2$ and $H_2O$ through known biological degradation pathways (Chirwa and Wang, 2000).
In this study, the balance between dissolution and biodegradability of PAHs was investigated using pyrene and fluoranthene as model compounds of high-ring number PAHs. The study was conducted in batch slurry bioreactors to establish degradation kinetics, continuous flow soil biopile to establish dissolution kinetics, and in biofilm reactors to evaluate possibility of an end-of-pipe bioremediation strategy.

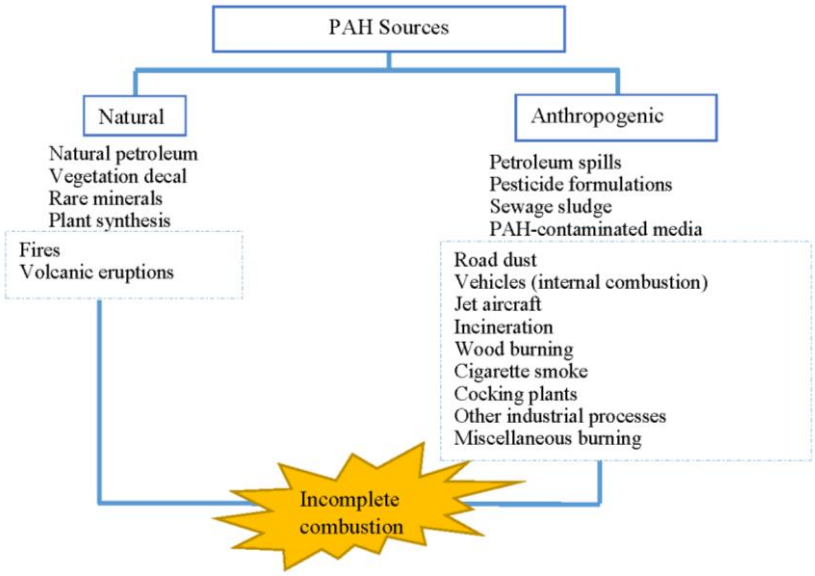


*Figure 1. Production and evolution of PAHs from natural and anthropogenic processes.*

## 2. Materials and Methods

### 2.1 Microorganisms (Microorganism isolation)

Samples of contaminated soil containing biosurfactant producing organisms were collected from crane service station sites which have been contaminated by spilt oil for decades. The enrichment and isolation of the PAH-degrading microbial consortium was performed by using naphthalene as the sole carbon and energy source. Initially, the bacterial consortium was enriched by adding 1 g of soil sample to 250 mL of minimal salt medium [MSM was composed of 6.0 g/L $(NH_4)_2SO_4$, 0.4 g/L $MgSO_4{\cdot}7H_2O$, 0.4 g/L $CaCl_2{\cdot}2H_2O$, 7.59 g/L $Na_2HPO_4{\cdot}2H_2O$, 4.43 g/L $KH_2PO_4$, and 2 mL/L of trace element solution. The trace element solution consisted of; 20.1 g/L EDTA (disodium salt), 16.7 g/L $FeCl_3{\cdot}6H_2O$, 0.18 g/L $CoCl_2{\cdot}6H_2O$, 0.18 g/L $ZnSO_4{\cdot}7H_2O$, 0.16 g/L $CuSO_4{\cdot}5H_2O$, and 0.10 g/L $MnSO_4{\cdot}H_2O$] in a 250-mL Erlenmeyer flask. The flask was shaken in an orbital shaker (150 rpm) at 30 °C for 5 days for microbial enrichment. After 5 days, an aliquot of 10 mL enriched culture was inoculated into another 250 mL conical flask containing 200 mL MSM with 250 mg/L PAH (Naphthalene) for the first enrichment. Five consecutive enrichments were carried out under the same condition to enrich a PAH-degrading microbial consortium. The bacterial colonies were isolated by streaking the enriched consortium on nutrient agar plates containing naphthalene as a carbon and energy source provided in a vapour form put on the lid. Morphologically distinct colonies were re-isolated by transfer onto naphthalene containing agar plates at least three times to obtain pure cultures.

### 2.2 Biosurfactant over-production

Purified cultures were grown using the method described in Lutsinge and Chirwa (2018) under nitrogen rich conditions to induce biosurfactant production. A two-step process was developed for producing biosurfactant in high amounts using resting Pseudomonas aeruginosa cells. The actual biosurfactant production was preceded by a culture for cell material production. This step was performed in shaking flasks with growth medium containing the carbon source. The biosurfactant production step was performed in another 2 L flask with the production medium under nitrogen free (no nitrogen source) growth limited production medium. Two culture media produced one for growth of the organisms, and the other one nitrogen free media for the growth limited overproduction of biosurfactant.

### 2.3 Biosurfactant characterisation

The composition of biosurfactant was determined using TLC plates and the method to characterize biosurfactants was adopted from Sharma et al. (2014). 1 mL aliquot of partially purified crude biosurfactant was separated on a precoated silica gel plate using chloroform:methanol:glacial acetic acid (65:15:2 v/v) as a solvent system. The plate was sprayed with a prepared ninhydrin solution (2 g of ninhydrin dissolved in 100 mL of acetone) and dried in an oven at 110 $^{o}$C for 2 min. The appearance of a pink spot on the TLC shows a positive reaction when biosurfactant reacted with ninhydrin and signifies the presence of peptide moieties that could be the lipopeptide.

### 2.4 Analytical method

PAHs in solutions was analyzed by HPLC system with a slightly modified EPA Method 8310 (U.S.EPA 1986). Chromatographic condition of a linear gradient elution condition from 70 % acetonitrile and 30 % ultra-pure-water to 100 % acetonitrile mobile phase over 10 min at a flow rate of 1 mL min$^{-1}$ was set up. Pyrene and fluoranthene was identified by its retention time and absorption spectrum and quantified by its absorbance compared with calibration curve prepared with the pyrene and fluoranthene standards. The detection limit of the HPLC system was 0.01 mg/L. All tests were conducted in triplicate with uninoculated controls to monitor the volatilization losses and total recovery of contaminants.

## 3. Results and discussion

### 3.1 Microbial culture characterisation

Eight morphologically different colonies were isolated, and 5 of them were found to produce biosurfactant as checked by “drop collapse” and “oil spread” test and the best biosurfactant producing strain was identified as pseudomonas aeruginosa|. The phylogenetic identification of this strain was based on the nucleotide sequence obtained by using 16S rRNA gene sequencing analysis. The results showed that pure culture that produced most biosurfactant and degraded naphthalene and phenanthrene, coly CB1, was most closely related to Pseudomonas aeruginosa strain H211A (Figure 2). This species was the most closely related species to the best performing pure culture with a similarity of 89.9 %. The strain 211A culture was thus preserved in the MSM broth with 30 % (w/v) glycerol at −80 °C for use in the rest of the experiments.

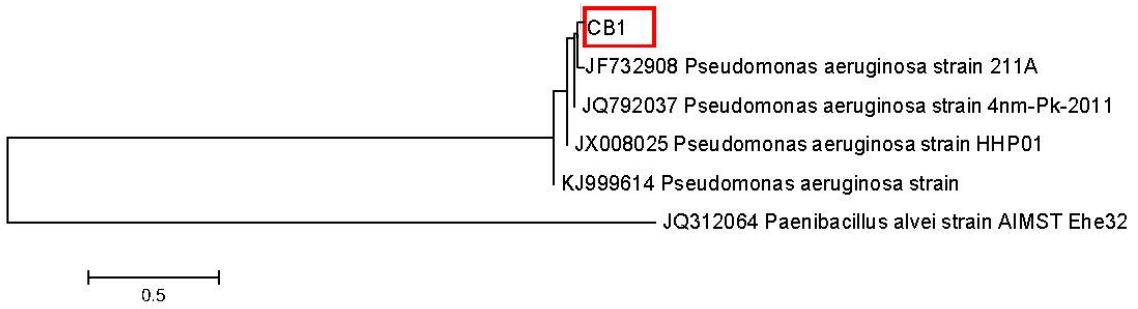


*Figure 2. Identification of the predominant biosurfactant producing, PAH-degrading microbial species as Pseudomonas aeruginosa strain 211A.*

### 3.2 Bioavailability and biodegradability of PAHs in material composites

This study uses creosote in soil as an example of the petrochemical composite material emitting PAHs. The water emitted from a creosote contaminated soil was analysed for a range of PAHs. Most of the PAHs were detected at the mg/kg level. In a separate experiment, the dissolution of the PAHs into the aquatic phase was enhanced by using a biosurfactant produced and purified from a pure culture of Pseudomonas putida 211A (Figure 3). Only abbreviations of the PAH compound names are shown in the figure ranked from lowest ring number (left) to highest ring number (right), i.e., naphthalene (Nap),fluorine (Flu), phenanthrene (Phe), anthracene (Ant),fluoranthene(Flr), pyrene (Pyr), benzo[a]anthracene (BaA), chrysene(Chr), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene(BkF), benzo[a]pyrene (BaP) ,dibenz[a,h]anthracene(D(a,h)A), benzo [g,h,i]perylene (B(g,h,i)P). The 57 % degradation was achieved after 45 days of exposure as shown in Figure 3c. The removal rate in each compound is limited by the dissolution index of each compound with high ring number compounds being typically less soluble and therefore more difficult to remove. In the main experiment, a pre biosurfactant was added to enhance dissolution of the PAHs. The addition of a biosurfactant at day two resulted in the enhancement of PAH removal in all categories (Figure 4).In the presence of the purified biosurfactant, degradation of PAHs improved to 79 % and 86 % after incubation for 25 days and 45 days, respectively, as shown in Figures 4b and c.

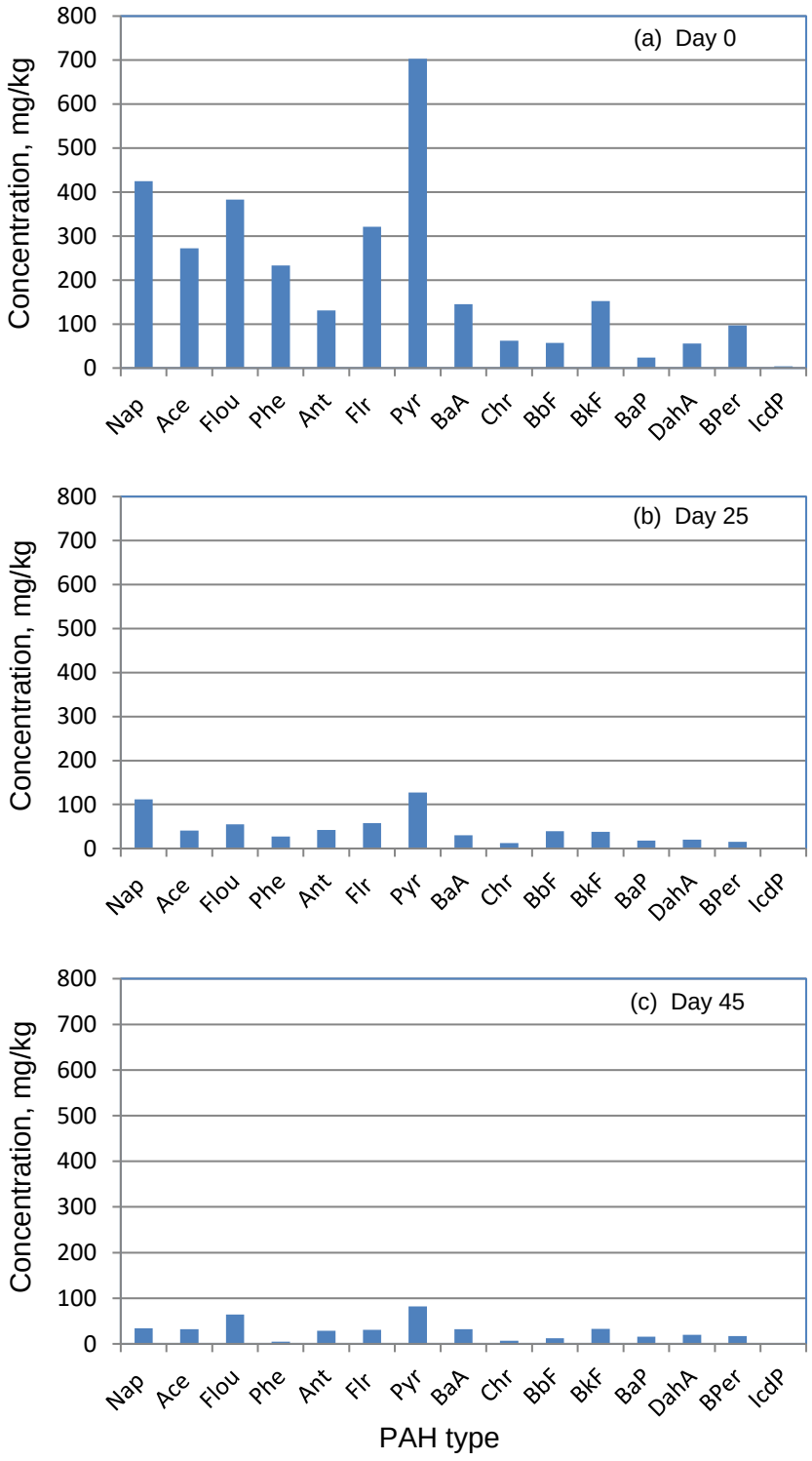


*Figure 3. Concentration of PAH in creosote contaminated soil exposed to cells only (biotic control) (a) initial concentration, (b) concentration at 25 days (42 % degraded), and (c) concentration at 45 days (57 % degraded).*

It was noted however that most of the removal occurred in the 3- to 4-ring PAHs such as fluorine (Flu), phenanthrene (Phe), anthracene (Ant), fluoranthene(Flr), pyrene (Pyr) as opposed to the anticipated decreasing order of removal from naphthalene to benzo [g,h,i]perylene. The reason for this discrepancy is not known. One indication might be that the observed values are transitional in nature.

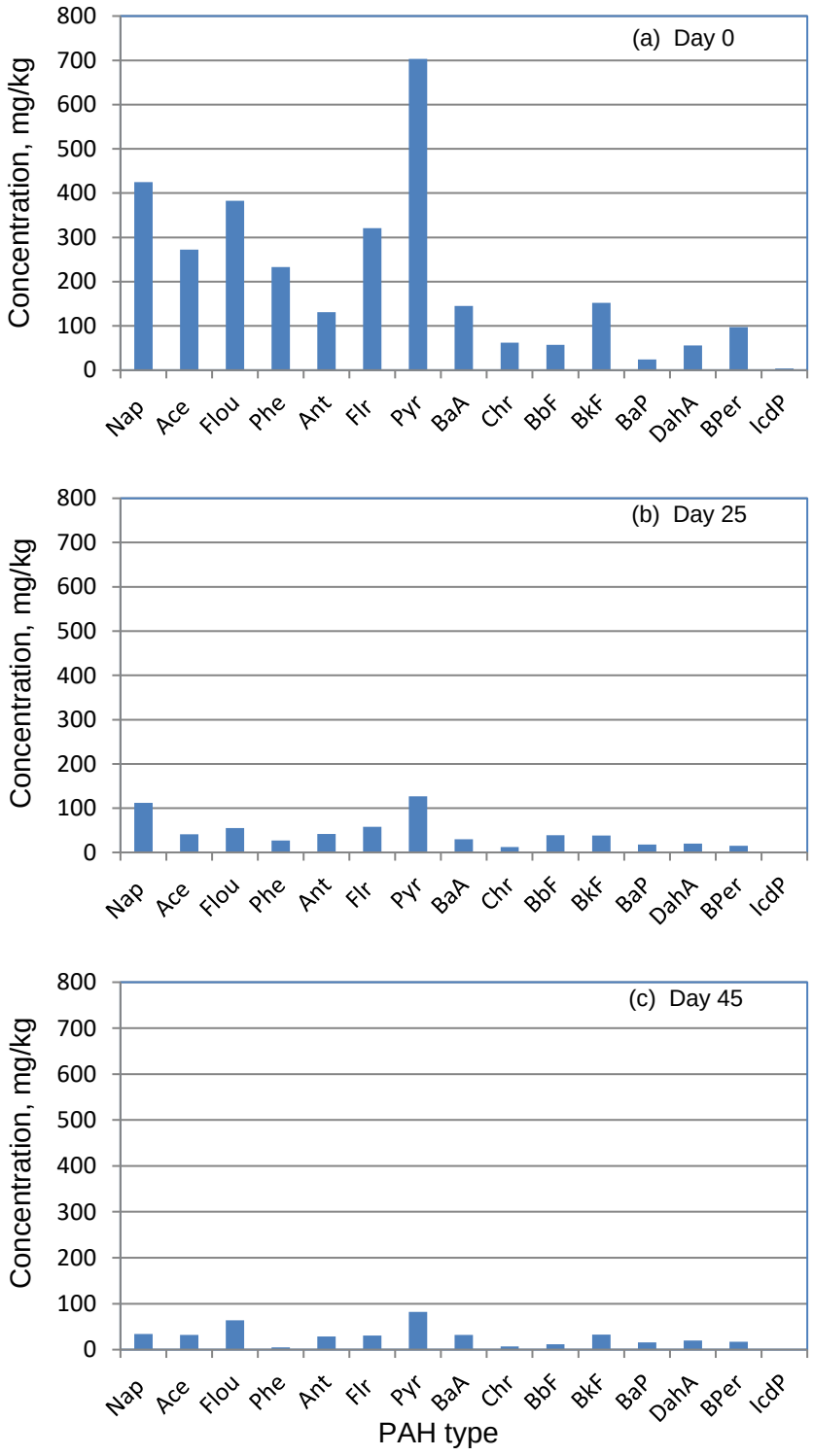


*Figure 4. Concentration of PAH in creosote contaminated soil exposed to cells + purified biosurfactant (biotic experiment) (a) initial concentration, (b) concentration at 25 days (79 % degraded), and (c) concentration at 45 days (86 % degraded).*

**3.3 Effect of ring number on biodegradability**

The effect of biosurfactants on the microorganisms depends on factors, such as: biosurfactant concentration and bioavailability, environmental and cultural conditions, and characteristic and properties

of microorganisms as cellular ultra-structure (Van Hamme et al., 2003). Other studies showed an enhanced degradation with different pseudomonas strains; study with the strain, Pseudomonas marginalis, indicated that the biosurfactant produced by the strain solubilized polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene and enhanced biodegradation (Burd and Ward, 1996).
The data in Figures 3 and 4 shows that very high ring number compounds (5- to 6-ring compounds) were detected at low concentration in creosote contaminated soil. The compounds were not removed much mainly due to their low solubility in water. Dissolution of the high ring-number PAHs into the aqueous phase is a common problem with many HMW_PAHs. The mid-range compounds “pyrene and fluoranthene” were the most easily degraded by percent mass change. However, the toxicity threshold of the compounds were seen to decrease from 800 mg/L for naphthalene, 640 mg/L for phenanthrene, 43 mg/L for pyrene, and 12.5 mg/L for fluoranthene. For this reason, overproduction of a biosurfactant in a solution with high loading of undissolved PAH can lead to inhibition of the degradation process. It is therefore necessary in future to optimise both cell growth and biosurfactant production to achieve the best results.

## 4. Conclusion

Biosurfactant enhanced biodegradation was achieved in soil containing creosote. The creosote contaminated soil was chosen as the model for soil contamination by complex petrochemical compounds. All the main PAH in the class of 2- to 6- ring compounds were detected at concentrations higher that the allowable levels in the contaminated soil. Among the PAHs, phenanthrene, pyrene, fluoranthene and chrysene were the most efficiently removed with removal percentiles of 98 % (Phe), 88 % (Pyr), 90 % (Flr) and 89 % removed. The results showed varied inhibition threshold coefficients across different chemical species which necessitates the development of kinetic models that will take into account the biosurfactant production rate to optimise dissolution and PAH degradation in biological systems.

### References

Burd G., Ward O.P., 1996, Involvement of a surface-active high molecular weight factor in degradation of polycyclic aromatic hydrocarbons by Pseudomonas marginalis, Canadian Journal of Microbiology, 42 (8), 791-797.

Bustamante, M., Durán, N., Diez, M.C., 2012, Biosurfactants are useful tools for the bioremediation of contaminated soil: a review. Journal of Soil Science and Plant Nutrition, 12 (4), 667-687.

Chauhan A., Oakeshott J.G., Jain R.K., 2008, Bacterial metabolism of polycyclic aromatic hydrocarbons: Strategies for bioremediation, Indian J. Microbiol.,48(1), 95–113.

Chirwa E.M.N., Wang Y-.T., 2000, Simultaneous Cr(VI) reduction and phenol degradation in an anaerobic consortium of bacteria. Water Research, 34 (8), 2376-2384.

Kabziński A., 2002, Determination of polycyclic aromatic hydrocarbons in water (including drinking water) of Łódź, Polish Journal of Environmental Studies, 11 (6), 695-706.

Lutsinge, B.T., Chirwa, E.M.N., 2018. Biosurfactant-assisted biodegradation of fluoranthene in a two-stage continuous stirred tank bio-reactor system using microorganism. Chemical Engineering Transactions, 64, 67-72.

Maliszewska-Kordybach B., 1999, Sources, concentrations, fate and effects of polycyclic aromatic hydrocarbons (PAHs) in the environment. Part A: PAHs in air, Polish Journal of Environmental Studies, 8 (3), 131-136.

Melber, C., Kielhorn, J., and& Mangelsdorf, I. (2004). Coal tar creosote. Concise international chemical assessment document, World Health Organization, Geneva, 2004.

Sharma, D., Mandal, S.M., Manhas, R.K., 2014, Purification and characterization of a novel lipopeptide from Streptomyces amritsarensis sp. nov. active against methicillin-resistant Staphylococcus aureus, AMB Express 4 (1), 50

Samanta S.K., Singh O.V., Jain R.K., 2002, Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends in Biotechnology, 20 (6), 243-248.

U.S.EPA, 1986, Method 8310: Polynuclear Aromatic Polynuclear Aromatic Hydrocarbons. <www.epa.gov/osw/hazard/testmethods/sw846/pdfs/8310.pdf> accessed 09.01.2018.

Van Hamme J.D., Singh A., Ward O.P., 2003, Recent advances in petroleum microbiology, Microbiology and Molecular Biology Reviews, 67 (4), 503-549.