International Journal of Recent Research and Review, Vol. VIII, Issue 1, March 2015
ISSN 2277 – 8322

Fluoranthene and Acenaphthene Metabolism by Chlorella vulgaris: Identity of Intermediates Formed During Degradation and Its Growth Effect

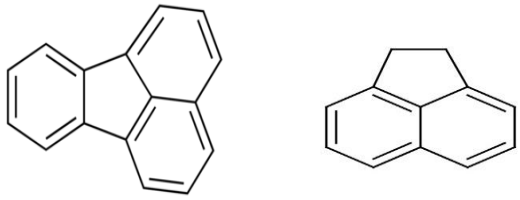
Miral S. Patel *, K. K. Tiwari
Sophisticated Instrumentation Center for Applied Research and Testing (SICART), Vallabh Vidya Nagar, Gujarat, India
Email: miral24@gmail.com, kktnature@gmail.com

***Abstract*- Fresh water microalge *Chlorella vulgaris* was grown in media amended with acenaphthene and fluoranthene. *Chlorella vulgaris* was identified as tolerating and effectively degrading polycyclic aromatic hydrocarbons that may be toxic in the environment. Based on LC50 value three different concetration was selectded for study. Decrease in total chlorophyll and protein content with increasing time and concetration showed the impact of PAHs on *C. vulgaris*. GC/MS analysis explained the degradation of these compounds by *C. vulgaris* and converted both acenaphthene and fluoranthene into non-toxic form. *C. vulgaris* colmpletely degraded acenaphthene at the 16th day of experiment with all three LC50 concentration while in fluoranthene maximum 93% reduction was seen at 6 mg L^{-1}.**

***Keyword*- Biodegradation, GC, LC50 value, total chlorophyll, protein.**

I. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are natural or anthropogenic compounds that are considered one of the largest groups of polluting compounds. PAHs can cause damage to the environment and to human health due to the production of genotoxic and carcinogenic derivatives by microorganisms and as a result of biotransformation in humans[1]. PAHs are found in plants, soil, sediments, natural and marine water and in the atmosphere. Thus, many polyaromatic hydrocarbons (PAH) contaminated sites lie in high value commercial land and remediation of such sites has been challenging due to the lack of appropriate cost effective technologies. Often the best solution for such sites has been excavation and transport to landfill sites which is now proving to be expensive due to the recent increases in the landfill levy [2]. As a rule, PAHs are biodegraded with more difficulty as their molecular weight increases because their water solubility is decreased. Unfortunately, this increased hydrophobicity usually correlates with increasing genotoxicity and decreasing biodegradability. Thus, the potential to detoxify PAHs containing three and four fused benzene rings by algae has been examined. However, the capacity of microalgae to degrade pollutants such as PAHs has been poorly investigated. The PAH selected in the present study were chosen due to their high recalcitrance in the environment and because of their toxicity: fluoranthene (four benzene rings) and acenaphthene (three benzene ring), (Figure 1) are recalcitrant molecules with a long half-life in the soil of 270 days to 5.2 years [3]. In addition, fluoranthene and acenaphthene are classified by the United States Environmental Protection Agency of the (USEPA) as a priority pollutant.



Fluoranthene Acenaphthene

Fig. 1. Molecular structure

PAHs can be degraded by photo oxidation, chemical oxidation or bioremediation. Since the 1970s, research on the biological capacity of organisms to degrade PAHs has demonstrated that bacteria and fungi have catabolic abilities that may be used for the soil and water remediation contaminated with PAHs [3]. In view of these different mechanisms for the initial oxidation of the aromatic hydrocarbons by bacteria and fungi, we

have initiated a study to determine whether microalgae have the capacity to degrade aromatic hydrocarbons.

The analytical methods commonly used to determine PAH degradation is gas chromatography with mass selective detection (GC-MS). The main advantages of using the GC-MS technique for the quantification of PAHs are its high selectivity when applied in single ion monitoring (SIM) mode and its quantification ability with simultaneous identification of the analyte.The objective of our study is to determine degradation of fluoranthene and acenaphthene by *C. vulgaris* using GC-MS and to identify by-products formed during degradation process which will able us to understand biotransformation of toxic PAH in to non-toxic by-products.

II. MATERIALS AND METHODS

A. Algae growth and axenic conditions

The *Chlorella vulgaris* was obtained from the Central Marine and Salt Research Institute, Bhavnagar, India. The growth media selected for microalgae *C. vulgaris* was develop in Zarrouks medium [4] under controlled illumination of 40µEm-2s-1 at 27±1°C in aerobic and static conditions. Before conducting experiment, microalgae were made axenic by treating with different concentration of streptomycin and benzyle penicillin for 24 hrs [5] and then streaking was done on nutrient agar medium to check bacterial contamination.

B. Experimental set-up

Seven-day-old cultures were homogenized by shaking on a wrist-action shaker using sterile glass beads. For each compound, 32 sugar tubes were maintained, each containing 2ml culture+18ml media. Zarrouks media were prepared by 1188 ml distilled water + 1188µl media and autoclaved. After sterilization, 0.1% stock solution of fluoranthene and acenaphthene was prepared in acetone with 0.05gm/ 50 ml for each PAH. Different doses of 0.1, 0.5, 1.0, 5.0, 10.0, 20.0, 40.0, 60.0, 100.0, 150.0 ppm of fluoranthene and acenaphthene were prepared to determine LC50 value and control is maintained separately without the addition of any chemical. A total of 64 tubes were prepared for the two PAHs (acenaphthen and fluoranthene) and triplicate set were maintained. Appropriate amounts of algal culture (approx 5 mg dry wt) was added to 18 ml fresh autoclaved medium. Triplicate tubes of the control and the PAH treatments were retrieved after 4, 8, 12 and 16 days of incubation to detect the LC50. LC50 was estimated in terms of total chlorophyll.

C. Pigment Analysis

Total chlorophyll was measured spectrophotometrically in cell lysates after extraction in 80% acetone according to Jeffrey SW and Humphrey GF [6].

D. Protein Estimation

The culture medium was discarded through centrifugation and the cells were thoroughly crushed in a mortar and pestle with 80% ethanol. The supernatant obtained after centrifugation was used for protein analysis according to Lowry OH [7] using bovine serum albumin as the standard.

E. Extraction of PAHs and metabolites from algal cultures

Based on LD50 value, three different doses were selected to study degradation of two PAHs. After 4^{th} and 16^{th} days of incubation periods, 5ml of Dichloromethane: methanol ratio (2:1) was added in each sugar tubes and kept for sonication for 15 mins. The medium was then transferred to centrifuge tube and was subjected to centrifugation at 10,000 rpm for 10 min (4°C). Anhydrous Sodium Sulfate was added in the DCM:methanolic extracts and samples were subjected for GC-MS analysis [8].

F. GC-MS analysis

The constituents were detected using an Auto System XL GC apparatus (Perkin Elmer, USA) attached to a PE-5MS fused silica capillary 5% diphenyl/95% dimethylpolysiloxane column (30 m x 50 m, 0.25 µm film thicknesses, Perkin Elmer). The column temperature was initially 80ºC, held for 5 min, then ramped from 80ºC - 290ºC at 10ºC/min. Helium (1.0 ml/min) was used as the carrier gas. Both Line and injector temperatures were set at 250ºC. 10 µl of each DCM:methanolic extract prepared were injected in the split mode (1:40). MS conditions were run in EI+ through a Perkin Elmer Turbo Mass spectrometer as follows: ionization energy -70 eV; scan rate 1.6

scans/sec; interscan delay 0.01 sec; source temperature 250ºC; mass range 30 to 650 m/z; solvent delay 3.00 min. The gas chromatogram as reproduced by the mass spectrometer identified the mass spectra scanned at each GC peak maximum. Data was thus obtained by comparing the mass spectra to those in the Wiley NIST Mass Spectral Library 2005.

III. RESULTS AND DISCUSSION

PAH molecular stability, hydrophobicity and low water solubility appear to be some of the main factors that contribute to their persistence in the environment. Some of these factors have also been correlated to the size of the molecule or total number of aromatic rings [9]. As it is widely known, HMW PAHs are sparingly soluble in water, electrochemically stable and may be acutely toxic, genotoxic, immunotoxic or act as agents of hormone disruption [10]. Due to their elevated octanol–water partition coefficients (Kow), HMW PAHs may separate into organic phases, soil and sediment organic matter and membranes of living organisms. They are also candidates for bioconcentration, bioaccumulation and sometimes biomagnifications through trophic transfer to food webs [11].

The Chlorella vulgaris strain was grown with 1.5ppm, 3.0 ppm and 6.0 ppm for fluoranthene; 1.25ppm, 2.5ppm and 5.0ppm for Acenaphthene. Above this concentration all strains lost pigmentation and died after few days. Further experiments were therefore conducted with LD50 concentration of fluoranthene and acenaphthene. The effect of fluoranthene and acenaphthene over the strain tested were measured by estimating total chlorophyll and protein concentration at various days interval with that of control. GC/MS analysis of fluoranthene and acenaphthene degradation was conducted with *C. vulgaris*.

A. Effect on growth

In our study, Acenaphthene treatments at various concentrations caused reduction in the total chlorophyll content of the cells, which was found significant after 4 days of treatment Total chlorophyll content reduced maximum by 82% at 16th day of acenaphthene treatment (5 ppm) while 84% reduction was shown with fluoranthene treatment (6 ppm) to *C. vulgaris.* The results were highly indicative of their inhibitory effects on photosynthetic activities of the cells. Fall of total chlorophyll content of the microalgae explained that the PAH not only accelerated the degradation but also blocked their pigment synthesis [12]. The similar result was also observed that growth decreased with increase in the concentrations of PAHs [13]. It has been reported that some organic pollutants such as 2,4-D pesticides can promote the contents of algae pigments, [14] however, this phenomena was not observed in the present study. We conclude that the decrease in growth is attributed to decrease in total chlorophyll content in microalgal cell when *C. vulgaris* is exposed to PAHs.

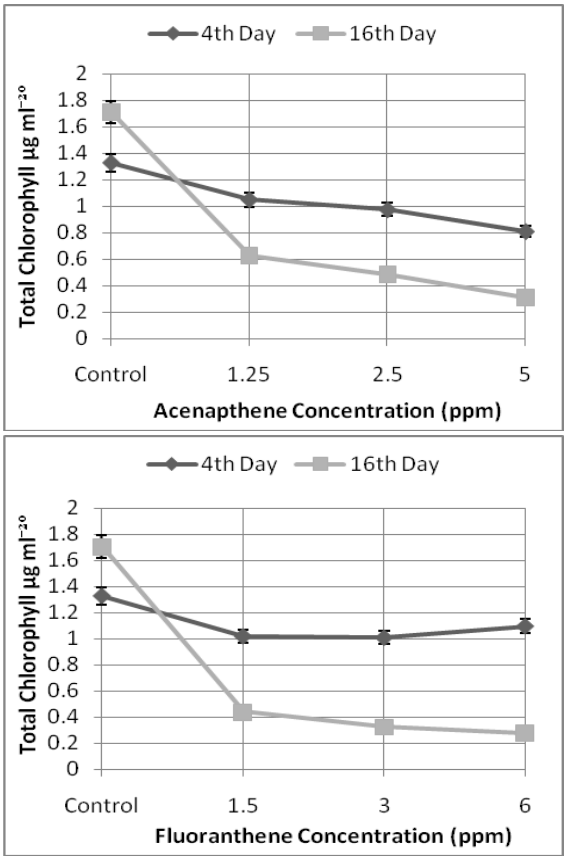


Fig.2a&2b: Concentration (µg ml$^{-2°}$) of total chlorophyll in *C. vulgaris* at different doses of acenaphthene and fluoranthene.

Acenaphthene and fluoranthene stress had a pronounced effect on the production of proteins in microalgae. The protein content from all experimental flasks treated with acenaphthene and fluoranthene was lower than that of control (fig.3a, 3b). Even though amount of protein in cultures treated with all three doses, increased up to certain time but it did not exceed

control values. It has been reported that PAHs stimulate production of some proteins, part of defenses and repair machinery of cells [15]. However PAHs at higher concentration of acenaphthene (5 ppm) in *C. vulgaris* showed a maximum reduction of 97% in the protein content after 16th day whereas 96% reduction was seen with fluoranthene. Kapoor *et al*. [16] also stated that the interruption of protein synthesis could be due to the inhibition of enzymes and structural proteins essential for growth of the organism.

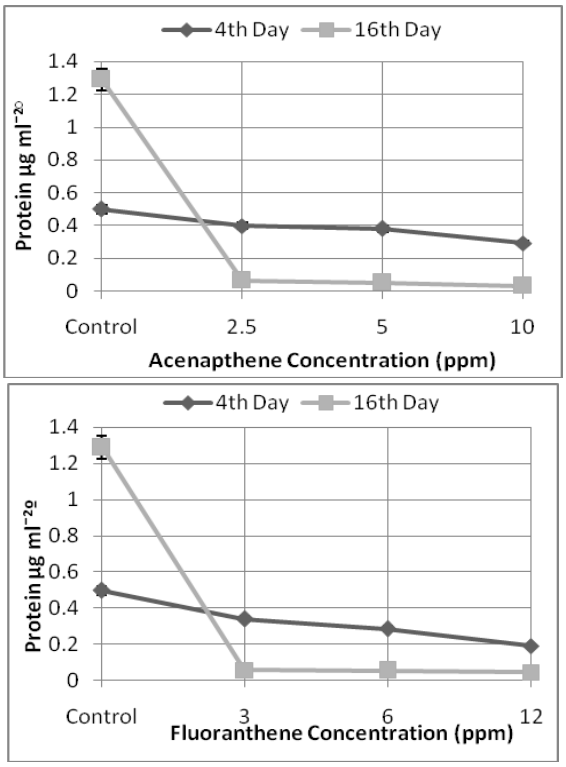
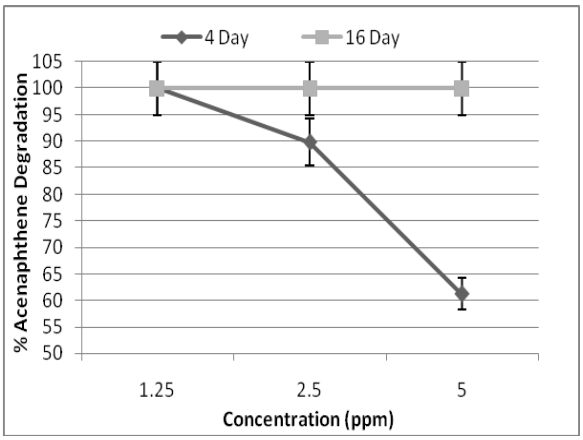


Fig.3a&3b: Concentration (µg ml⁻²°) of Protein in *C. vulgaris* at different doses of acenaphthene and fluoranthene.

B. Identificaton of Metabollites

GC/MS analyses of acenaphthene and fluoranthene degradation by C. vulgaris are shown in table I and II. The metabolites detected during acenaphthene and fluoranthene degradation were almost similar at the 4th and 16th days, despite the differences in PAH. The metabolites formed during degradation presumably arising by an inducible enzyme system. In this study, preliminary screening was made to optimize the concentration at which the selected strains can survive. Our data show that *C. vulgaris* strain tested have the ability to grow up to the concentrations of 0.006% which is similar to Raghukumar [17]. Oxidation of aromatic ring carbon is an energy providing process which often leads to complete degradation of the substrate. The intermediates formed during degradation describes that both the PAHs has converted to single chain compounds and fatty acids. No other compounds of PAHs were detected which able us to find out degradation pathway, prompting the possibility of complete degradation of all compounds of PAHs by *C. vulgaris*. The metabolites detected during the PAHs degradation, confirm biological transformation of the PAHs. The metabolites provided no evidence for substantially different degradation processes associated with different Pahs concentration. There are many reports which describes that marine microalgae can oxidize aromatic hydrocarbons under photoautotrophic growth conditions. Evidence supporting the biodegradation capacity of cyanobacteria/microalgae for the elimination of crude oil residues and PAHs are still very limited. Cultures of Microcoleus chthonoplastes and Phormidium corium were able to degrade n-alkanes [18]. Oscillatoria sp. and Agmenellum quadruplicatum oxidize naphthalene to 1-naphthol [19,20]. Oscillatoria sp. strain JCM oxidizes biphenyl to 4-hydroxybiphenyl [21] and A. quadruplicatum metabolizes phenanthrene into trans-9,10-dihydroxy-9,10 dihydro-phenanthrene and 1-methoxy-phenanthrene [22]. Several other strains can degrade crude oil and other complex organic compounds such as surfactants and herbicides [17,23,24].

C. PAHs Degradation



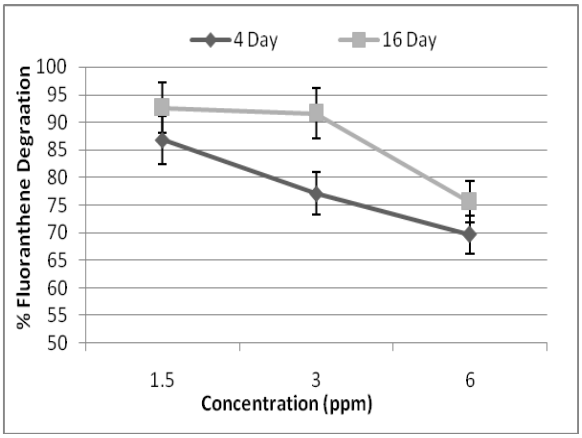


Fig.4a&4b: Biodegradatoin of acenaphthene and fluoranthene by *C. vulgaris*.

Acenaphthene is a three ringed strurcutred whereas fluoranthene is a four ringed compound, sparingly soluble and widely used as model HMW PAH. The PAH catabolic potential of *C. vulgaris* was assessed over a time course period in Zarrouk's medium supplemented with individual PAHs. The results obtained by GC-MS were calculated as a percentage of degradation and are presented in Figure 4a and 4b. The strain *C. vulgaris* presented degradation efficiencies of acenaphthene upto 90% in 2.5 mg L^{-1} and 62% in 5 mg L^{-1} at 4^{th} day. Completed degradation of acenaphthene with 1.25 mg L^{-1} was seen at 4^{th} day. Over the period of 16^{th} days, 100% degradation of acenaphthene with all three concentration was observed. The lower degradation results for fluoranthene compare to acenaphthene could be explained by its reduced availability for enzymatic attack due to its lower solubility in water. In general, the higher the molecular weight of the PAH molecule, the higher the hydrophobicity and toxicity, and the longer the environmental persistence of the molecule.1 Cerlinga. The concentration of fluoranthene decreased from an initial 1.5 mg L^{-1} to 0.3 mg L^{-1}, 3 mg L^{-1} to 0.4 mg L^{-1} and 6 mg L^{-1} to 1.3 mg L^{-1} after a remediation period of four days. Highest reduction was seen of 93% at 16^{th} day with 6 mg L^{-1} concentration. These results suggest that the biodegradation of acenaphthene and fluoranthene follow the same trend in the liquid medium except that comparatively, the degree of degradation was consistently higher in acenaphthene. This phenolmenon can be attributable to the fact that low molecular weights PAH degrade faster than those of higher molecular weight. [25].

IV. CONCLUSION

A microlagea strain was able to tolerate toxic PAHs upto certain concentration and effectively degraded. The ability of *C. vulgaris* to utilise PAHs such as acenaphthene and fluoranthene as a sole carbon source was demonstrated. Because the degradation of PAHs containing four or more benzene rings such as fluoranthene has been difficult to achieve, the microalgal *C. vulgaris* could be considered good targets for future studies. This microalgal strain not only degraded both the PAHs but would allow the detection and evaluation of the degradative enzymes they produce and the metabolites formed during the PAH degradation process. In present study, we could not find any metabollites of upper metabolic pathway of acenaphthene and fluoranthene degradation. Therefore, further study is required to characterize upper metabollic pathway of both the PAHs degradation.

V. REFERENCES

[1] P. Baborova, M. Moder, P. Baldrian, K. Cajthamlova, and T. Cajthaml, "Purification of a New Manganese Peroxidase of the White-Rot Fungus Irpex lacteus and Degradation of Polycyclic Aromatic Hydrocarbons by the Enzyme," *Res. Microbiol.*, vol. 157, pp. 248–253, 2006.

[2] (2002) EPA, Victoria, (Environment Protection Authority, Victoria), Landfill levies: information bulletin. Available http://www.epa.vic.gov.au/2009/0730

[3] A. L. Juhasz, and R. Naidu, "Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[*a*]pyrene," *Int. Biodeterior. Biodegrad.*, vol. 45, pp. 57–88, 2000.

[4] C. Zarrouk, :Contribution à l'étude d'une cyanophycée. Influence de divers' facteurs physiques et chimiques sur la croissance et la photosynthèse de Spirulina maxima," Ph.D. Thesis, Université de Paris, Paris,1966

[5] J. M. Ferris, and C. F. Hirsch, "Method for isolation and purification of cyanobacteria," *Applied and Environmental Microbiology*, vol. 57, pp. 1448-1452, 1991.

[6] S. W. Jeffrey, and G. F. Humphrey, "New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural populations," *Biochem. Physiol. Pflanzen*, vol. 167, pp. 191-194, 1975.

[7] O.H. Lowry, N. H. Rosenbrough, A. L. Farr, and R. J. Randall, "Protein measurements with folinphenol reagent," *J. Biol. Chem.*, vol. 193, pp. 265-275, 1951.

[8] M. Aseer, G. K. Seghal, J. Selvin, and M. V. N Panikkar, "Evaluation of seaweed bioactives on common aquatic floral and faunal weeds of shrimp ponds," *Thalassas*, vol. 27, pp. 47-56, 2010.

[9] C. E. Cerniglia, "Biodegradation of polycyclic aromatic hydrocarbons," *Biodegradation*, vol. 3, pp. 351–368, 1992.

[10] T. Van de Wiele, L. Vanhaecke, C. Boeckaert, K. Peru, J. Headley, W. Verstraete, and S. Siciliano, "Human colon microbiota transforms polycyclic aromatic hydrocarbons to estrogenic metabolites," *Environ Health Perspect*, vol. 113, pp. 6–10, 2005.

[11] R. A. Kanaly, and S. Harayama, "Advances in the field of highmolecular- weight polycyclic aromatic hydrocarbon biodegradation by bacteria," *Microbial Biotechnol.*, vol. 3, pp. 136–164, 2010.

[12] Miral Patel, J. I. Nirmal Kumar, and K.K. Tiwari, "An investigation on principle biochemical components, photosynthetic pigments, nucleic acis and enzymatic activities of axecinc culture of Scytonema sp. treated with two PAHS:Acenaphthene and Fluoranthene," *International Journal of Applied Science and Biotechnology*, vol. 2, pp. 34-40, 2014.

[13] P. Echeveste, S. Agusti, and J. Dachs, "Cell size dependent toxicity thresholds of polycyclic aromatic hydrocarbons to natural and cultured phytoplankton populations," *Environ. Pollut*., vol. 158, pp. 299–307, 2010.

[14] P. K. Wong, "Effects of 2,4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll-a synthesis of Scenedesmus quadricauda Berb 614," *Chemosphere*, vol. 41, pp. 177–182, 2000.

[15] S. Tukaj, and Z. Tukaj, "Distinct chemical contaminants induce the synthesis of Hsp70 proteins in green microalgae Desmodesmus subspicatus: heat pretreatment increases cadmium resistance," *Journal of Therm. Biol*., vol. 35, pp. 239–244, 2010.

[16] K. Kapoor, Leenta and Arora, "Observations on growth Responses of cyanobacteria under the influence of herbicides," *Pollut. Res.*, vol. 15, pp. 343-351, 1996.

[17] C. Raghukumar, V. Vipparty, J. J. David, and D. Chandramohan, "Degradation of crude oil by marine cyanobacteria." App. Micobiol. Biotechnol., vol. 57, pp. 433–436, 2001.

[18] R. H. Al-Hasan, D. A. Al-Bader, N. A. Sorkhoh, and S. S. Radwan, "Evidence for n-alkane consumption and oxidation by filamentous cyanobacteria from oil contaminated coasts of the Arabian Gulf," *Mar. Biol*., vol. 130, pp. 521–527, 1998.

[19] C. E. Cerniglia, and D. T. Gibson, "Algal oxidation of aromatic hydrocarbons: formation of 1-naphthol from naphthalene by Agmenellum quadruplicatum, strain PR-6," *Biochem. Biophy. Res. Commun*., vol. 88, pp. 50–58, 1979.

[20] C. E. Cerniglia, D. T. Gibson, and C. Van Baalen, "Oxidation of naphthalene by cyanobacteria and microalgae," *Journal of Gen. Microbiol*., vol. 116, pp. 495–500, 1980a.

[21] C. E. Cerniglia, D. T. Gibson, and C. Van Baalen, "Metabolism of naphthalene by cyanobacteria Osillatoria sp., strain JCM," *Journal of Gen. Microbiol*., vol. 116, pp. 485–494, 1980b.

[22] M. L. Narro, C. E. Cerniglia, C. Van Baleen, and D. T. Gibson, "Metabolism of phenanthrene by marine cyanobacterium Agmenellum quadruplicatum PR6," *Applied Environ. Microbiol*., vol. 58, pp. 1351–1359, 1992.

[23] S. S. Radwan, and Al-Hasan, *Oil pollution and cyanobacteria*, In: Whitton BA, Potts M (eds), The ecology of cyanobacteria. Kluwer, The Netherlands, pp 307–319.

[24] A. E. Mansy, and E. El-Bestway, "Toxicity and biodegradation of fluometuron by selected cyanobacterial species," *World J Microbiol Biotechnol*, vol. 18, pp. 125–131, 2002.

[25] S. E. Agarryt, R. O. Yusuf, and A. O. Ajanit, "Biodegradation of Anthracene and Naphthalene in soil," *Journal of the Nigerian Society of Chemical Engineers*, vol. 25, pp. 156-162, 2010.

Table I

Metabolites formed during Acenaphthene degradation study by GC-MS

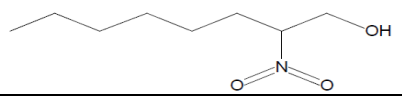
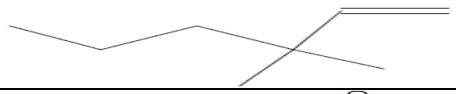
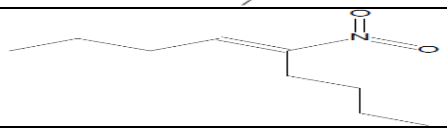
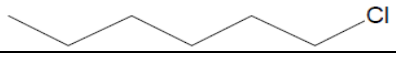
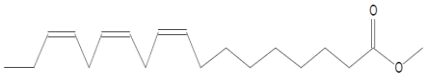
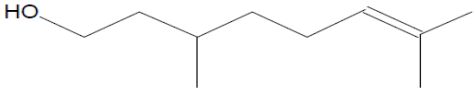
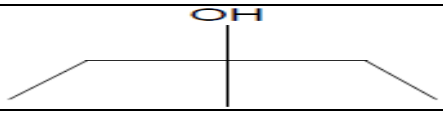
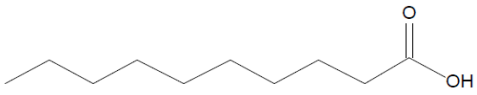
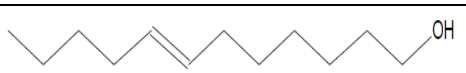
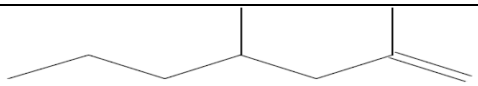
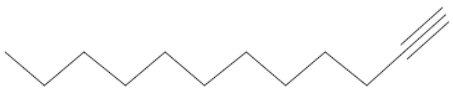
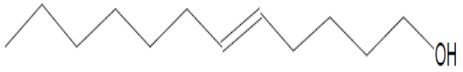
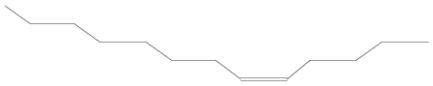
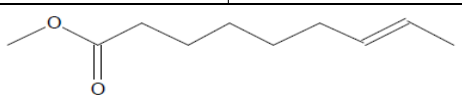
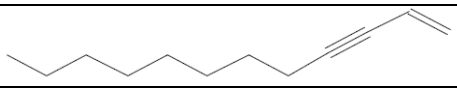
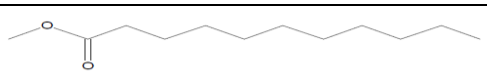
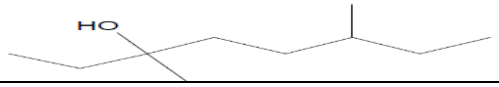
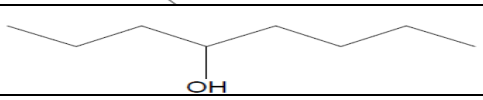
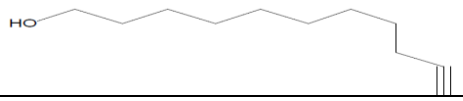
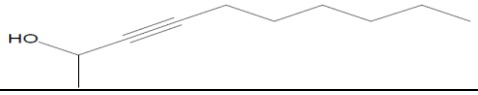
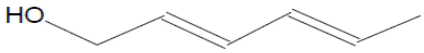
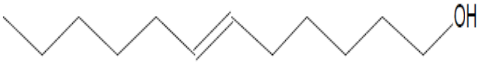
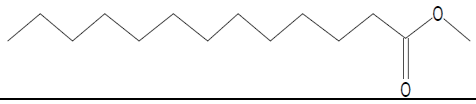
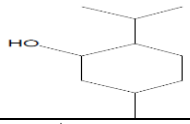
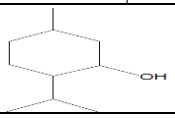
Compound Name	**Structure**
1-Octanol, 2-nitro	
1-Hexene, 3,3-dimethyl	
4-Nonene, 5-nitro	
Hexane, 1-chloro	
9,12,15-Octadecatrienoic acid, methyl ester	
6-Octen-1-ol, 3,7-dimethyl	
3-Pentanol, 3-methyl	
n-Decanoic acid	
7-Dodecenol	
2,4-Dimethyl-1-heptene	
1-Dodecyne	
5-Dodecenol	
5-Tridecene	
Neopentane	
7-Nonenoic acid, methyl ester	
6-Octadecenoic acid, methyl ester	
1-Dodecen-3-yne	

Table II

Metabolites formed during Fluoranthene degradation study by GC-MS

Compound Name	**Structure**
Undecanoic acid, methyl ester	
3-Octanol, 3,6-dimethyl	
4-Octanol	
10-Undecyn-1-ol	
4-Nonene, 5-nitro	
3-Decyn-2-ol	
Hexadecane	
2,4-Hexadien-1-ol	
6-Dodecenol	
Tridecanoic acid, methyl ester	
Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1α,2β,5α)	
Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1α,2α,5β)]	
4-Nonene, 3-methyl-, (Z)