INTERNATIONAL JOURNAL OF ENVIRONMENT
Volume-2, Issue-1, Sep-Nov 2013 ISSN 2091-2854
Received:25 October *Revised:11 November* *Accepted:12 November*

CHRONIC TOXICITY OF HIGH MOLECULAR WEIGHT POLYNUCLEAR AROMATIC HYDROCARBON- PYRENE ON FRESHWATER CYANOBACTERIUM *ANABAENA FERTLISSIMA* RAO

Jignasha G. Patel[1], Nirmal Kumar, J. I.[2*], Rita N Kumar[3], Shamiyan R. Khan[4]
[1,2,4]P.G.Department of Environmental Science and Technology, Institute of Science and Technology for Advanced Studies and Research,Vallabh Vidya Nagar- 388 120, Gujarat, India
[3]Department of Biological and Environmental Sciences, Natubhai V.Patel College of Pure and Applied Sciences, Vallabh Vidya Nagar- 388 120, Gujarat, India
[*]Corresponding author: istares2005@yahoo.com

Abstract

The aim of this work was to determine the consequences of Polynuclear aromatic hydrocarbon – Pyrene in response to growth, pigments and metabolic study on *Anabaena fertilissima* Rao. Test organisms were treated at different doses and encountered LC50 (Lethal concentration at which 50% growth reduction occur) concentration separately at 1.5 mg/l, 3.0 mg/l and 6.0 mg/l respectively for *Anabaena fertilissima* Rao. The influence of Pyrene on growth, pigments, release of metabolites such as carbohydrates, protein, amino acid, phenols was carried out. The test doses caused a concentration dependent decrease in pigments like carotenoids and phycobilliproteins and showed more sensitivity to pyrene. Depletion of carbohydrate by 13% to 81% and proteins by 47% to 93% was encountered with rise in pyrene concentrations after 16th day of exposure. However, phenols were found to rise by 27% to 50% with increased pyrene concentrations on the contrary, amino acids were reported to decline by 79% to 92%. This study therefore suggests high molecular weight pyrene that decreases in metabolite content and enzyme activity can be used as a signal of PAHs toxicity in cyanobacteria.

Key words: *Anabaena fertilissima* Rao, pyrene, LC 50, pigments, metabolic contents

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are toxic environmental pollutants. They are produced from incomplete combustion of organic materials, fossil fuels, petroleum product spillage, and various domestic and industrial activities (Johnsen *et al.* 2005). Contamination by PAHs is widespread, and has been detected in air, water, soil, and sediment (Juhasz *et al.*, 2000). Based on their ecotoxicity, the United States Environmental Protection

Agency has prioritized 16 PAHs as environmental pollutants (Kim *et al.*, 2005). High molecular weight PAHs are resistant to degradation and pose a concern to human health because of their carcinogenic potential. Low molecular weight PAHs do not pose a risk to human health as carcinogens, but are toxic to fish and other marine organisms as these compounds accumulate in their tissues (Law *et al*., 2002).

Cyanobacteria, a group of prokaryotic, oxygen evolving, photosynthetic Gram-negative bacteria, survive in a wide variety of extreme environmental conditions. Some cyanobacteria are also able to fix atmospheric nitrogen and are therefore especially inexpensive to maintain (Herrero *et al*., 2001). They are widespread in many ecosystems, including polluted ones. Marine cyanobacteria can oxidize aromatic hydrocarbons under photoautotrophic growth conditions. Evidence supporting the effect of PAHs - pyrene on cyanobacterial metabolites is still very limited. Good number of bacterial genera about 160 including *Pseudomonas, Alcaligenes,Vibrio Mycobacterium, Rhodococcus, Cycloclasticus* are degrade PAHs to derive energy (carbon source) (Berardesco *et al.*,1998, Nyer *et al.*, 2001).

Metabolic response and toxicity of each and every chemical differs from one to another organism therefore, it is essential to know about same by using the pyrene on *Anabaena fertilissima* Rao. However, to our knowledge, no previous report on toxicity of pyrene on the proposed work so as to establish the consequences on growth, photosynthetic pigmentation, metabolites content and enzymatic variation of selected cyanobacteria by pyrene. Moreover, Nirmal kumar *et al.*, (2010) explored chronic toxicity of chlorophenoxy herbicide on growth pigments, metabolites and enzymatic activities of *Anabaena fertilissima* Rao.

Materials and Methods

Pure cultures of *Anabaena fertilissima* Rao, a heterocystous form was procured from culture collection of the Centre for Conservation and Utilization of Blue Green Algae, IARI, New Delhi, India and grown photoautotrophically in BG11 medium (Rippka *et al.*, 1979) under controlled illumination of 800 lux light for 14:10 hours photo and dark period per day at 25±2°C. *A. fertilissima* Rao was maintained in nitrogen free BG11 medium and was subjected to different concentrations of pyrene response. The PAHs chosen for the study was four fused aromatic ring structure, hydrophobic, white crystalline, recalcitrant, molecular weight of 202.25 g/mol procured from Sigma-Aldrich chemistry, USA (98% purity). To analyze the effect of different concentrations of pyrene on growth, the experimental medium was prepared with 1.5 mg/l, 3.0 mg/l and 6.0 mg/l of pyrene (finalized the dose on the basis of LC50 values tested for different concentrations). Stock solutions (200µg/ml) of test PAH were prepared in minimal amount of acetone, sterilized by filteration and added to the culture medium to obtain their respective concentrations. Exponentially growing 2ml homogenous culture was inoculated and made upto 20ml with and without PAH (as control) constant. Samples were taken after every four days up to sixteen days for the determination of growth, pigments, and metabolites. Analytical grade (Merck Ltd, and Himedia Ltd,India) chemicals were used throughout the study. Each experiment was conducted in replicates of three and their ±SE values were calculated. The pigments included were chlorophyll *a* (Jeffrey & Humphrey., 1975), carotenoids (Parsons and Strickland., 1963) and phycobilin pigments

(Bennett and Bogorad., 1973). The changes in metabolites content like total carbohydrates (Hedge & Hofreitte., 1991), proteins (Lowry *et al*., 1951), amino acids (Lee & Takahanshi., 1966) and phenols (Malick & Singh., 1980) of *A. fertilissima* Rao exposed to pyrene were measured.

Statistics

Statistical differences were examined with ANOVA analysis for all data, using the software KY plot version 2 .0 beta 15.

Results and Discussion

Growth and Pigments

Growth in terms of Chlorophyll-*a* of the *A. fertilissima* Rao was adversely affected by pyrene at higher concentrations. The minimum growth of 0.13 µg/20ml was recorded at 6.0ppm followed by 0.21 µg/20ml at 3.0ppm and 0.43 µg/20 ml at 1.5ppm after intervals of 16 days. However, *A. fertilissima* Rao showed maximum growth of 0.55 µg/20ml on the 16th day in the control cells. Which is substantiated the findings of Chinnaswamy and Patel (1983) and Nirmal Kumar *et al.,* (2010) stated that the inhibition of chlorophyll synthesis by pesticides in *Anabaena sp.* The mechanism of toxicity is generally believed to be the disruption of biological membrane (Sikkema *et al.*, 1995). The lypophillic character of aromatic hydrocarbon can alter the membrane fluidity, permeability of the membrane, cause lipid bilayer disruption and diminish the energy transduction and affect activity of membrane associate proteins (Heipieper *et al*., 1995). Effect of the pyrene on various pigments of *A. fertilissima* Rao is represented in Figure. 1(a) to (d). It was observed that chlorophyll *a* content decreased with increased concentrations as the time progressed; a total decrease of 48%, 62%, and 74% was observed at 1.5 mg/l, 3.0 mg/l and 6.0 mg/l, respectively Figure.1 (a). After 16 days of treatment a low concentration (1.5 mg/l) of pyrene reduced chlorophyll-*a*, carotenoid, phycocyanin, phycoerythrin and allophycocyanin contents by 68%, 70%, 81%, 89% and 83%, respectively. The declining trend in the pigment content continued with the rising concentration of pyrene at 6.0 mg/l of PAH sharply lowered chlorophyll *a*, carotenoid, phycocyanin, phycoerythrin, and allophycocyanin contents by 96%, 80%, 93%, 95% and 94%, respectively after 16 days. The test PAH dose caused a concentration-dependent decrease in pigment contents. Phycocyanins are major reserves for nitrogen (Cohen-Bazire *et al.*, 1992) in cyanobacteria. The level of phycocyanin declined markedly with increasing concentrations of pyrene. Such a decline in phycocyanin level might be attributed to its possible degradation, thus leading to nitrogen starvation (Allen and Smith., 1969). Decline in pigment contents may be due to lysis of the cell-wall and disruption of the thylakoid membrane as known for *A. flos-aquae* (Rai *et al*., 1989). Phycocyanin, phycoerythrin and allophycocyanin located in phycobilisomes are the main accessory pigments in cyanobacteria (Xia., 2005). It is quite clear from our observations that the test PAH pyrene highly affected the synthesis of phycocyanin, phycoerythrin, and allophycocyanin pigments in *A. fertilissima* Rao. Thus the study supports the earlier findings of Mostafa and Helling (2002) who suggested that drop in chlorophyll-a, carotenoid and phycobiliprotein contents might be ascribed due to the inhibition of pigment synthesis directly by the insecticide or accelerated degradation of pigments due to increased Active Oxygen Species (AOS) formation at various

sites of the photosynthetic electron transport chain during stress. After 4 days high significance was determined between chlorophyll-a, carotenoids and phycocyanin (P<0.001) whereas phycoerythrin and allophycocyanin values were not significant after 16days of incubation and non-significant relation was observed between phycobilin pigments (Table1).

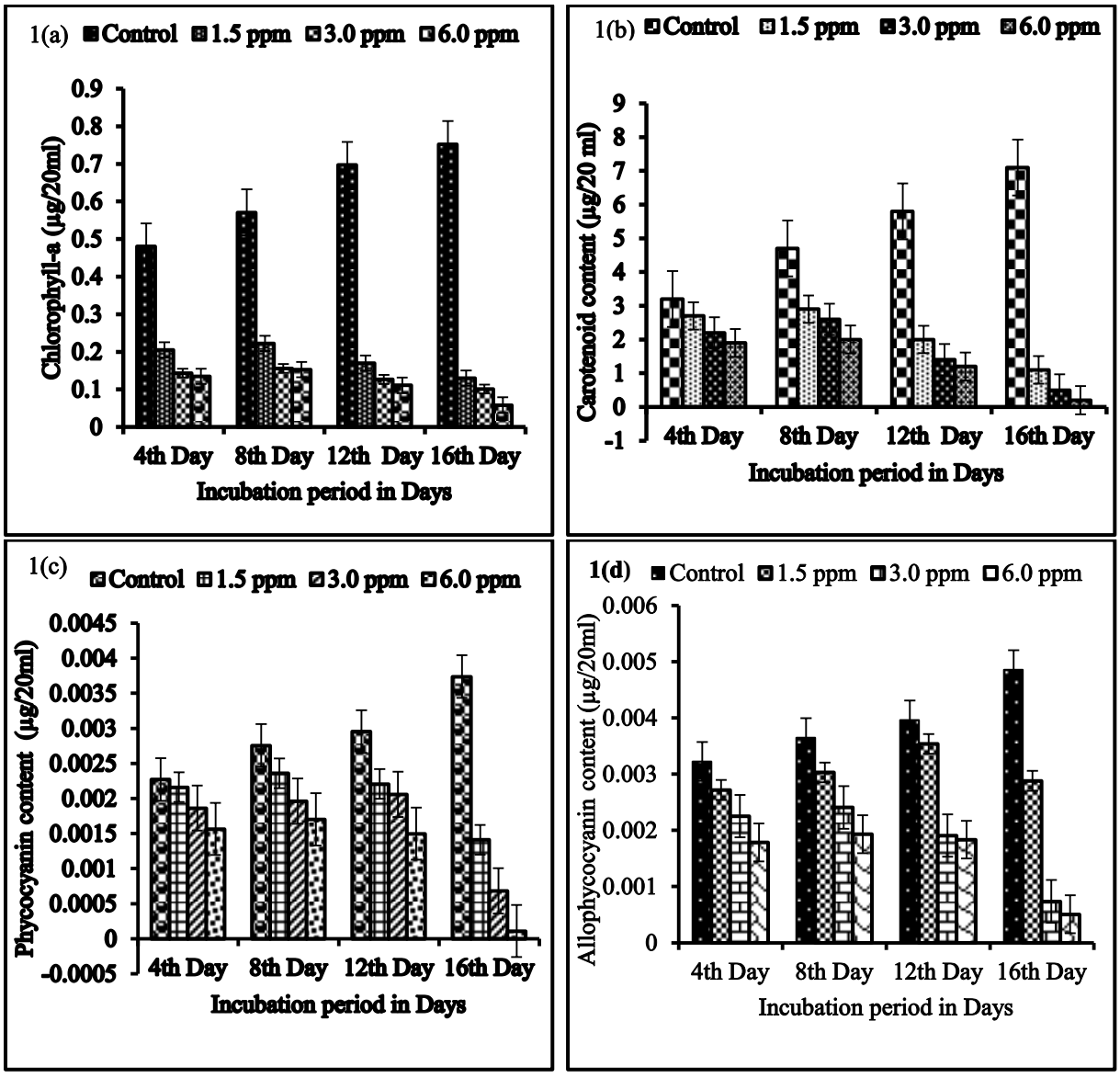

Figure.1 (a–d) shows effect of different concentrations of pyrene on different pigments of *A.fertilissima* Rao

Table1. Analysis of Variance (ANOVA) of *Anabaena fertilissima* Rao with reference to growth, pigments, and metabolites at three graded concentration of pyrene after 16 days of treatment

Parameters	*Anabaena fertilissima* Rao	
	F(cal)	P(F<=F(cal))
Chl.a	0.81703	0.50893
CA	0.110438 (NS)	0.952347
PC	0.545258 (NS)	0.660621
PE	0.456702 (NS)	0.717497
APC	0.171142 (NS)	0.913827
CAR	0.047801 (NS)	0.985471
PRO	0.12838 (NS)	0.941414
AA	0.164044 (NS)	0.918525
PH	7.397676 ***	0.004579

Values are significant at NS: None Significant (P > 0.05), ***P* ≤ 0.05; *** *P*≤ 0.01. Data in parentheses denotes non significant.

Abbreviations: Chl. a-Chlorophyll a, CA - carotenoid, PC-phycocyanin, APC-allophycocyanin, PE-phycoerythrene, CAR-carbohydrate, PRO-protein, AA- amino acids, PH-phenol

Carbohydrates

There was a decrease in carbohydrate content when the cultures were treated with 1.5 mg/l, 3.0 mg/l and 6.0 mg/l of pyrene. The highest amount of carbohydrate content was found in untreated cultures (0.34 mg/20ml) followed by 1.5 mg/l (0.30 mg/20ml) and 3.0 mg/l (0.25 mg/20ml) of pyrene treatment, whereas, lowest amount of carbohydrate content (0.06 mg/20ml) was observed at 6.0 mg/l (Figure. 2a). The gradual fall of carbohydrate concentration was registered by 13%, 42% and 81% at 1.5, 3.0 and 6.0 mg/l, respectively after duration of 16 days. The retardation of carbohydrate content might be due to the interference of chemicals with the photosynthesis process (Padhy Rabindran., 1985)

Proteins

The protein content increased in all the treatments as time progressed but the values were lower when compared with control (Figure. 2b). The reduction was observed in protein content after 16 days treatment by 47%, 66% and 93% in 1.5, 3.0 and 6.0 mg/l respectively.

The protein content of treated *A. fertilissima* Rao depleted with the progress of time could be possibly due to inhibition of enzymes and structural protein essential for the growth of cyanobacteria, which were confirmed with the findings of Shehata *et al.*, (2001) Similarly, Thiel (1990) reported decrease in protein content in *A. variabilis* under condition of starvation.

Amino acids

It was observed that the amino acid content decreased with increased concentrations of pyrene. As the time progressed, a total decrease of 79%, 84%, and 92% in 1.5, 3.0 and 6.0 mg/l, respectively was recorded after 16 days. At the end of 16 days treatment, the untreated culture showed higher amino acid content (0.02mg/20ml) than that of the treated cultures (Figure. 2c). Like proteins, amino acid concentration was sharply reduced with increasing concentrations of pyrene. Similar observations were recorded by Nirmal Kumar *et al.,* (2008) on treatment of *Tolypothrix tenuis* with fertilizer industrial effluents. Low amino acid content was recorded in control when compared to other metabolites. This might be due to production of relatively large quantities of combined nitrogen mainly in the form of polypeptides with lesser amounts of free amino acids during the growth of cyanobacteria (Santra, 1993)

Phenols

An increase in the phenol content of *A. fertilissima* Rao was noticed in applied treatments when compared to control (Figure. 2d). Highest amount of phenols was recorded in 6.0 mg/l (0.04 mg/20ml) after 16 days treatment, while the lowest was observed in control (0.03 mg/20ml). Phenols are important aromatic metabolites formed during stress conditions which trigger the various biochemical processes of the organisms. Initially, phenol content was noticed to increase at higher concentration as compared to control could be due to the chemical nature and composition of treated PAH. Nirmal Kumar and Rita Kumar (2002) also substantiate the findings that phenols could be act as protectants by the organisms under stress or drought conditions. Among metabolites, high significant relation was observed between proteins and amino acids ($P<0.001$) as compared to that between carbohydrates and proteins ($P< 0.01$) and amino acids and phenols ($P<0.05$) after 4 days of incubation. After 16 days, significance level between proteins and amino acids; amino acids and phenols was $P<0.05$ whereas between carbohydrates and proteins it was not significant (Table 1).

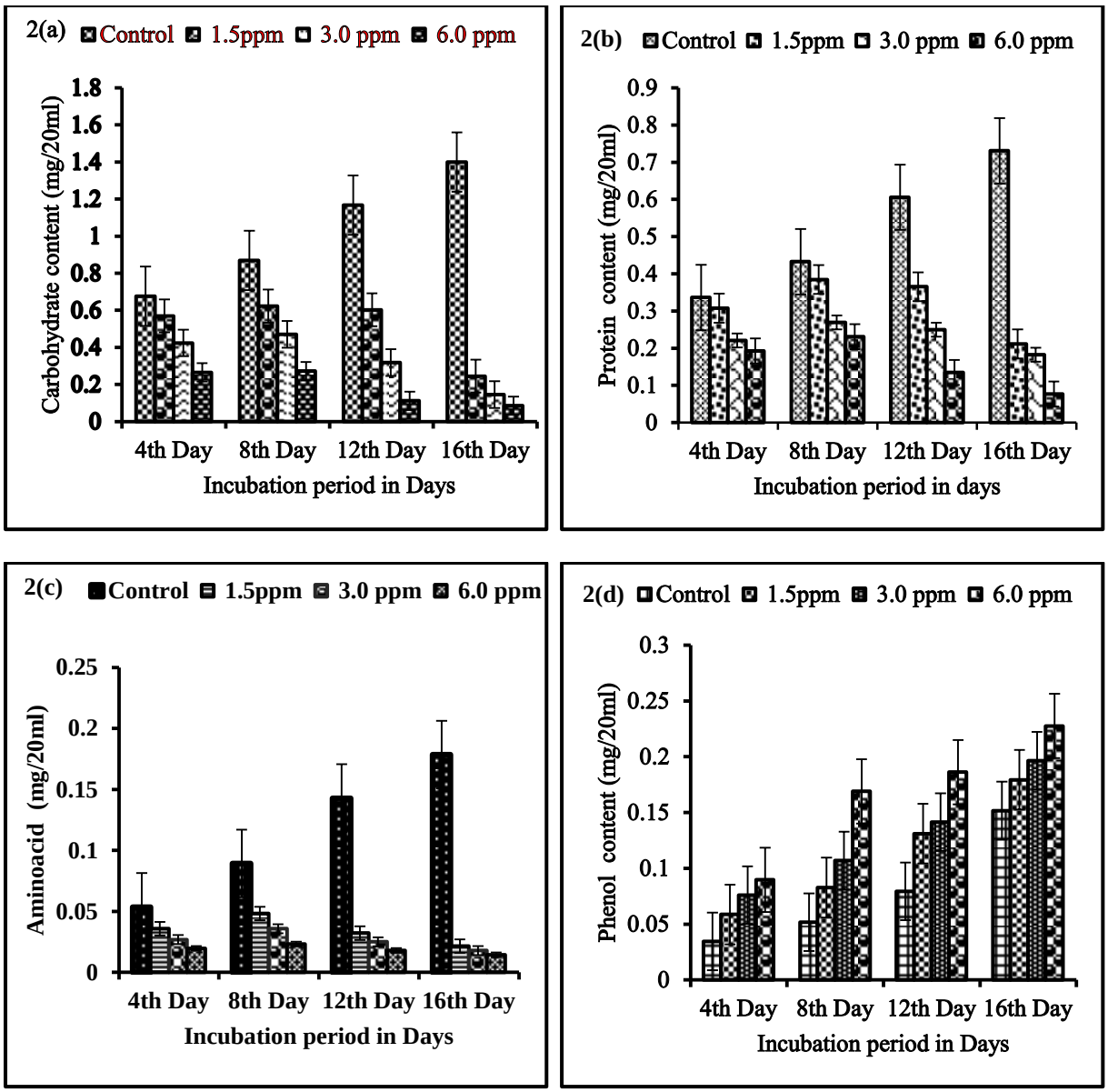

Figure. 2 (a-d) shows effect of different concentrations of pyrene on various metabolites and enzymes activity of *A. fertilissima* Rao

Conclusion

From the present investigation, it is clearly evident that the growth and metabolic activities of *A. fertilissima* Rao is adversely affected by pyrene, commonly found toxicants. Based on the inhibitory effect and growth arrest, the release of certain products, like carbohydrates, proteins, amino acids and phenols were also affected at 1.5 mg/l, 3.0 mg/l and 6.0 mg/l of pyrene, even at an earlier stage of treatment.

Acknowledgement

One of the author's Ms. Shamiyan Rahat Khan is highly thankful to University Grants Commission (UGC) for financial support by receiving Maulana Azad Fellowship. Authors

are also thankful to Sophisticated Instrumentation Centre for Advanced Research and Testing (SICART) for providing with the necessary infrastructural facilities.

References

Allen, M.B., Smith, A.J., 1969. Nitrogen chlorosis in blue green algae, Archives of Mikrobiology 69, 119-120.

Bennett, A., Bogorad, L., 1973. Complementary chromatic adaptation in a filamentous blue-green alga. Journal of Cell Biology 58, 419-435.

Berardesco, G., Dyhrman, S., Gallagher, E., Shiaris, M.P., 1998. Spatial and Temporal Variation of Phenanthrene - Degrading Bacteria in Intertidal Sediments, Applied and Environmental Microbiology 64 (7), 2560-2565.

Chinnaswamy, R., Patel, R.J., 1983. Effect of pesticide mixtures on the blue-green alga *Anabaena flos-aquae*. Microbiology Letters 24, 141–143.

Cohen-Bazire, G., Bryant, D.A.,1982. Phycobilisomes, Composition and structure, in, Carr, N.G., Whitton, S.C. (Eds.), The biology of cyanobacteria, Blackwell Scientific Publications, Oxford.

Hedge, J.E., Hofreitte, B.T., 1991. pp 8. Carbohydrates chemistry. In: Sadasivam S, Manickam A (eds) Biochemical Methods for Agriculture Sciences, Wiley Estern Ltd. Pub.

Heipieper, H.J., Loffeled, B., Keweloh, H., de Bont, J.A., 1995. The cis/tran isomerisation of unsaturated fatty acids in Psudomonas putida S12: an indicator for environmental stress due to organic compounds. Chemosphere 30, 1041-1051.

Herrero, A., Muro-Pastor, A.M., Flores, E., 2001. Nitrogen Controlling Cyanobacteria. Journal of Bacteriology 183, 411-425.

Jeffrey, S.W., Humphrey, G.F., 1975. New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural populations. 167, 191-194.

Johnsen A.R., Wick L.Y., Harms H., 2005. Principles of microbial PAH-degradation in soil. Environmental Pollution 133 (1), 71-84.

Juhasz, A. L., and Naidu, R., 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[*a*]pyrene. International Journal of Biodeterioration and Biodegradation 45, 57-88.

Kim, Y.H., Freeman J.P., Moody, J.D., Engesser, K.H., Cerniglia, C.E., 2005. Effects of pH on the degradation of phenanthrene and pyrene by *Mycobacterium vanbaalenii* PYR-1.Applied Microbiology and Biotechnology 67(2), 275-85.

Nirmal Kumar, J.I., Amb, M.K.., Bora, A., 2010. Chronic response of *Anabaena fertilissima* Rao, C. B. on Growth, Metabolites and Enzymatic Activities by Chlorophenoxy Herbicide, Pesticide Biochemistry and Physiology 98 (2), 168-174.

Nirmal Kumar, J.I., Kumar, R., 2002. Some metabolic observations of *Nostoc muscorum* to a Herbicide-Fluchloralin. Plant Archives 2 (2), 289-293.

Law, J.R., Kelly, C., Baker, K., Jones, J., McIntosh, A.D., Moffat, C.F., 2002. Toxic equivalency factors for PAH and their applicability in shellfish pollution monitoring studies. Journal of Environmental Monitoring 4(3), 383-388.

Lee, Y., Takahasi T., 1966. An imported colorimetric determination of amino acids with the use of ninhydrin**.** Analytical Biochemistry 14, 71-77.

Lowry, O.H., Rosenbrough, N.H., Farr, A.L., Randall, R.J., 1951. Protein measurements with folin phenol reagent. Journal of Biological Chemistry 193, 265-275.

Malick, C. P., Singh, M. B., 1980. Plant Enzymology and Histo Enzymology. Kalyani Publishers, New Delhi.

Mostafa, F.I., Helling, C.S., 2002. Impact of four pesticides on the growth and metabolic activities of two photosynthetic algae. Journal of Environmental Science and Health, Part A 37, 417-444.

Nyer, E.K., 2001. In Situ Treatment Technology, pp. 259-325. Lewis Publishers, BocaRaton.

Padhy Rabindran, N., 1985. Cyanobacteria and pesticides. Research and Review 95, 1-44.

Parsons, T.R., Strickland, J.D., 1963. Discussion of spectrophotometric determination of marine plant pigments with revised equations for ascertaining chlorophylls and carotenoids. Journal of Marine Research 21, 155-163.

Rai, L.C., Jensen, T.E., Rachlin, J.W., 1989. A morphometric and X-ray energy dispersive analysis approach to monitoring pH altered Cd toxixity in *Anabena flos-aquae.* Archives of Environmental Contamination and Toxicology 19, 479-487.

Rippka, R., Deruelles, S.,Waterbury, J.B., Herdman, M., Stanier, R.Y.,1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. Journal of General Microbiology 111, 1-61.

Santra, S.C., 1993. Biology of Rice Fields Blue Green Algae, Daya Publishing House.

Shehata, A.I., Arif, I.A.W., Aqeel, F.Al. 2001. Isolation and characterization of DNA and protein from endosulfan resistant mutant (endor) of the cyanobacterium anabaena flosaquae. Saudi Journal of Biological Sciences 8(1), 61-69.

Sikkema, J., de Bont, J.A., Poolman, B., 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiology and Molecular Biology Reviews 59, 201-222.

Thiel,T., 1990. Protein turn over and heterocyst differentiation in the cyanobacterium *Anabaena variabilis*-under condition of starvation. Journal of Phycology 26(1), 50-54.

Xia, J., 2005. Response of growth, photosynthesis and photoinhibition of the edible cyanobacterium *Nostoc sphaeroides* colonies to thiobencarb herbicide, Chemosphere 59, 561-566.