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CONSEQUENCES OF ENVIRONMENTALLY HAZARDOUS POLYCYCLIC AROMATIC HYDROCARBON- ANTHRACENE TREATMENT ON CYANOBACTERIA

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Abstract

The study was aimed to determine the chronic toxicity of Polynuclear aromatic hydrocarbon – Anthracene in response to pigments and metabolic study on three different cyanobacterial species such as *Synechocystis sp.*, *Anabaena fertilissima*, and *Nostoc muscorum*. Test organisms were treated at different doses and encountered LC50/Mean Lethal Concentration (at which 50% lethality/ growth reduction occur) separately at 7.0 ppm for *Synechocystis sp.*, 5.0 ppm for *Anabaena fertilissima* and 1.5 ppm for *Nostoc muscorum*. The influence of anthracene on pigments, metabolites and enzymes was carried out. The test doses caused concentration dependent and decreased pigments like carotenoids and phycobilliproteins. Depletion of carbohydrate by 65 to 80% and proteins by 58 to 78% was encountered with rise in Anthracene concentrations after 16th day exposure in case of *Synechocystis sp.* however, phenols were found to raise by 26 to 37% with increased anthracene concentrations. Similar trend also observed in other two tested blue green algae. Thus the *Synechocystis sp.* is more tolerant to anthracene treatments as compare to *Anabaena fertilissima* but *Nostoc muscorum* showed highest sensitivity to anthracene.

Key words: anthracene; photosynthetic pigments; metabolic contents; cyanobacteria.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic compounds with two or more aromatic rings. They are formed by incomplete combustion of fossil fuels and pyrolysis of organic matter derived from human activities and as a result of natural events like forest fires. The United States Environmental Protection Agency (USEPA) is more concerned to toxic, mutagenic and carcinogenic properties of PAHs, proposing some of them as priority pollutants including anthracene and phenanthrene (Simarro et al., 2011).

Anthracene is tricyclic aromatic hydrocarbons that are found in high concentrations in polycyclic aromatic hydrocarbon (PAH)-contaminated sediments, surface soils, and waste sites. Many past studies show that PAHs with lower molecular weight are toxic compounds, while high molecular weight compounds are significantly genotoxic (Juhaaz et al., 2000). These hydrophobic contaminants are widely distributed in the environment, occurring as natural constituents of fossil fuels and their anthropogenic pyrolysis products (Cerniglia 1992; Kanaly and Harayama, 2000). Despite they have been shown to be toxic to fish and algae (Sutherland et al., 1992). A variety of bacterial species have been isolated that have the ability to utilize anthracene or

phenanthrene as the sole source of carbon and energy (Sutherland et al., 1995). The fates of these compounds in the environment and the remediation of PAH-contaminated sites are, therefore, of high scientific and public interest. Cyanobacteria, a group of prokaryotic, oxygen evolving, photosynthetic Gram-negative bacteria, survive in a wide variety of extreme environmental conditions (Herrero et al., 2001). They are widespread in many ecosystems, including polluted ones (Sorkhoh et al., 1992). Marine cyanobacteria can oxidize aromatic hydrocarbons under photoautotrophic growth conditions. Evidence supporting the effect of anthracene on cyanobacterial metabolites is still very limited. Some bacterial genera including *Pseudomonas*, *Alcaligenes*, *Vibrio*, *Mycobacterium*, *Rhodococcus*, *Cycloclasticus* are degrade PAHs used as carbon source to derive energy (Berardesco et al., 1998).

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 anthracene on three selected cyanobacteria. However, to our knowledge, no previous report on toxicity of anthracene on the proposed work so as to establish the consequences and deleterious effects on growth, photosynthetic pigmentation, metabolites and enzymatic response of three species of cyanobacteria viz., *Anabaena fertilissima*, *Nostoc*

muscorum, and *Synechocystis sp.* by anthracene. Moreover, Nirmal kumar et al (2014) and Patel et al., (2013) explored chronic toxicity of pyrene in response to pigments, metabolic and enzymatic variation on certain sp. of cyanobacteria.

Materials and Methods

Organism and Culture Conditions

For the present study, axenic cultures of two filamentous, nitrogen-fixing, heterocystous cyanobacterium *Anabaena fertilissima*, *Nostoc muscorum* and one unicellular nonheterocyst forming cyanobacterium *Synechocystis sp.* procured from the Centre for Conservation and Utilization of Blue Green Algae, IARI, New Delhi, India. They were grown at 25±2° C in BG-11 medium (Rippka et al., 1979) under an illumination of 3,000 lux light with a photoperiod of 14:10 (Light/Dark). Exponentially grown cyanobacterial cells were used throughout the experiment. Each experiment was conducted in replicates of three and their ±SE values were calculated.

Anthracene Treatment

Anthracene (98% purity) was two aromatic ring structure pale yellow in colour and crystalline chemical, procured from Sigma Aldrich chemistry, USA. Exponentially growing 2 ml of the culture was inoculated to each effective dose and made up to 20.0 ml. For each experiment, the solution of anthracene was freshly prepared from 1 to 20 ppm doses. Growth retardation in terms of chlorophyll – a for LC₅₀ doses were selected in the culture treated with 5.0 ppm (*Anabaena fertilissima*), 7.0 ppm (*Synechocystis sp.*) and 1.5 ppm (*Nostoc muscorum*). Thus, three concentrations for each species were selected for the present investigation to carry out the response to selected cyanobacteria i.e one is LC₅₀ concentration, another is lower and the third is higher to LC₅₀ concentration. (table.1).Growth, pigments and biochemical response of three selected cyanobacteria to anthracene was studied at an interval of four days up to sixteen days. At the end of every four days, the treated as well as untreated cultures were assayed for various pigments, metabolites and enzymes. Analytical grade chemicals (Merck Ltd, and Himedia Ltd, India) were used throughout the study.

Growth and Photosynthetic Pigment Measurement

Growth was measured by determining the chlorophyll-a content (Jeffrey & Humphrey 1975). Cells were harvested by centrifugation, suspended in 80% acetone, and left in dark at 4°C overnight for extraction. The suspension was again centrifuged and the optical density of the supernatant was read at 630 and 663 nm.

Carotenoid was extracted in 80% acetone for 24 h at 4°C and determined according to Parsons and Strickland (1963). The phycobiliproteins pigments were estimated according to Bennett and Bogorad (1973). After extraction of chlorophyll-a using acetone, cells were suspended in 50

mM phosphate buffer (pH 6.7). Cyanobacterial cells were lysed by repeatedly freezing and thawing, and the crude extract was centrifuged at 12,000 g for 15 min at 4°C. The optical density of the supernatant was read at 562, 615, and 652 nm with phosphate buffer as the blank.

Estimation of Metabolites

Total carbohydrate release was determined spectrophotometrically by the anthrone method using glucose as the standard (Hedge & Hofreite 1991). The protein content of the crude cell-free extract was estimated according to Lowry and colleagues (1951) using bovine serum albumin as the standard. An improved colorimetric determination of amino acids with the use of ninhydrin was performed (Lee & Takahashi 1966). Phenol estimation was carried out with Folin-Ciocalteu reagent (Malick & Singh 1980).

Results and Discussion

LC₅₀ determination

LC₅₀ of *Synechocystis sp.*, *A. fertilissima* and *N. muscorum* for anthracene was found to be 7.0, 5.0 and 1.5 ppm respectively. Based upon the LC₅₀ doses, three different concentrations for each organism were selected for the study as represented in Table.1

Table 1: Anthracene treatments based upon determination of LC₅₀ on different Cyanobacterial species.

Organisms	<i>Synechocystis sp.</i>	<i>Anabaena fertilissima</i>	<i>Nostoc muscorum</i>
Lower to LC ₅₀ (ppm)	3.5	2.5	0.75
LC ₅₀ (ppm)	7.0	5.0	1.5
Higher to LC ₅₀ (ppm)	14.0	10.0	3.0 ppm

Growth in Terms of Chlorophyll-a

After 4-days exposure of high anthracene treatment, chlorophyll-a content showed a significant decrease by 75% at 3.0 ppm in *N. muscorum*, by 78% at 5.0 ppm in *A. fertilissima* and 67% *Synechocystis sp.* at 7.0 ppm in relative to control. The maximum reduction in chlorophyll-a content was recorded in *N.muscorum* (91% at 3.0 ppm), and the lowest in *Synechocystis sp.* (86% at 14.0 ppm) followed by *A. fertilissima* (90% at 10.0 ppm) by the end of 16th day of treatment (Fig. 1a). In present study, chlorophyll synthesis was found to be severely affected by the graded concentrations of anthracene and diminish in the growth of cultures between 6.0 to 14.0 ppm doses. Moreover, growth rate was decline more than 50% in the maximum concentrations of anthracene which is substantiated the findings of Sikkema et al, (1995) who stated that “the inhibition of chlorophyll synthesis by pesticides in *Anabaena sp.* believed to be the disruption of biological membrane”. The lipophilic 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). Anthracene showed the most deleterious effect on the growth of *Nostoc muscorum* among the three tested cyanobacterial species.

Pigment composition

Pigment response of all the three organisms to various concentrations of the low molecular weight anthracene has been represented in Fig 1 (b-e) Low concentration (3.5 ppm) of anthracene treated to *Synechocystis sp.* retarded carotenoid, phycocyanin, allophycocyanin and

phycoerythrin contents by 11,7,11 and 13%, respectively whereas 14.0 ppm of anthracene does sharply lowered carotenoid and phycocyanin, allophycocyanin and phycoerythrin contents by 90, 93,81 and 68 %, respectively . Similar observations were made while studying on the growth, photosynthesis, active oxygen species and antioxidants responses of paddy field cyanobacterium *Plectonema boryanum* to pesticide endosulfan stress (Prasad et al., 2005).

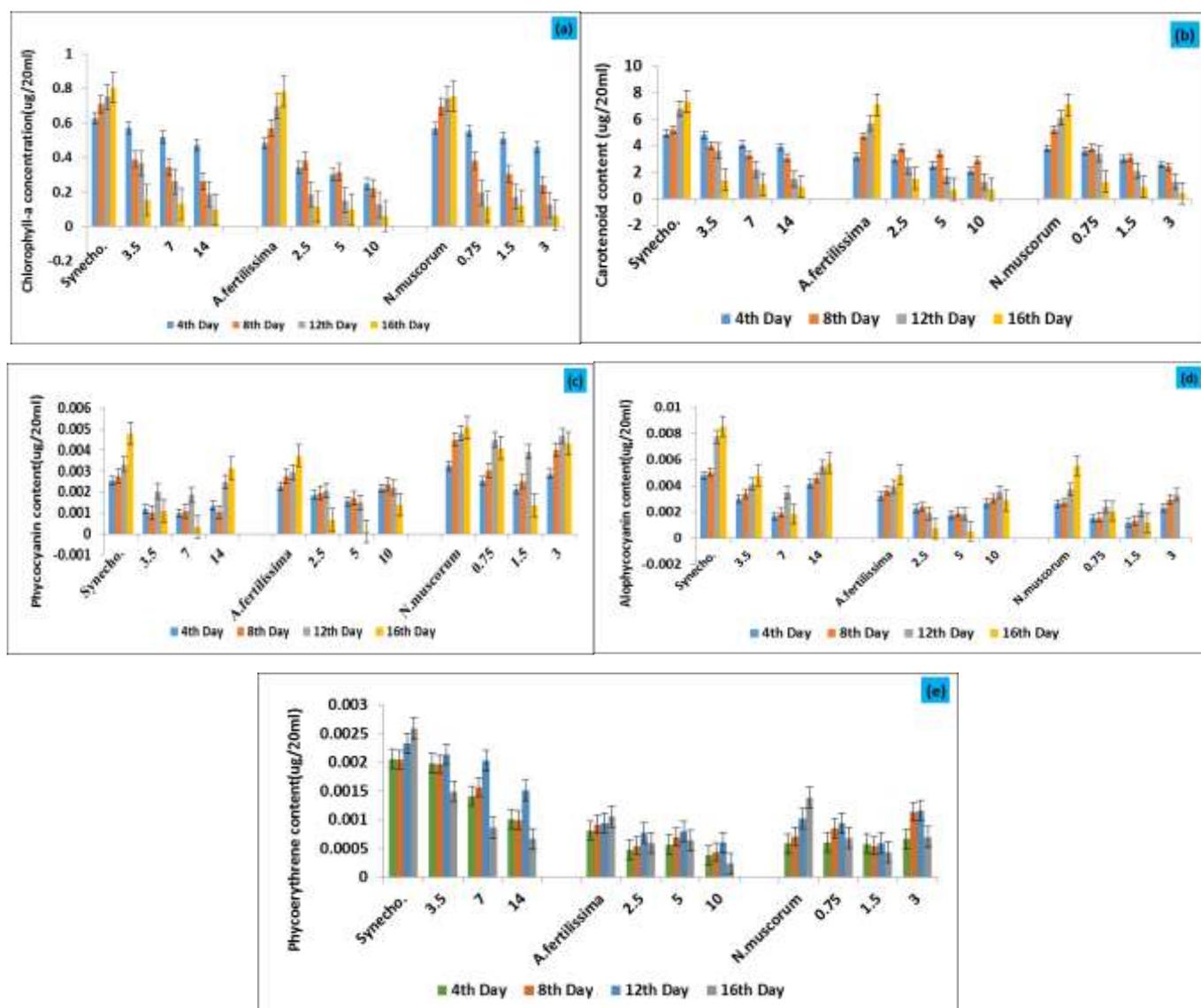


Fig. 1: (a) Chlorophyll-a content (µg /20ml) in *Synechocystis sp.*, *Anabaena fertilissima* and *Nostoc muscorum* at different doses of anthracene (in ppm); (b) Carotenoid content (µg /20ml) in *Synechocystis sp.*, *Anabaena fertilissima*.and *Nostoc muscorum* at different doses of anthracene (in ppm); (c) Phycocyanin content (µg /20ml) in *Synechocystis sp.*, *Anabaena fertilissima*.and *Nostoc muscorum* at different doses of anthracene; (d) Allophycocyanin content (µg /20ml) in *Synechocystis sp.*, *Anabaena fertilissima*.and *Nostoc muscorum* at different doses of anthracene; (e) Phycoerythrene content (µg /20ml) in *Synechocystis sp.*, *Anabaena fertilissima*.and *Nostoc muscorum* at different doses of anthracene

Photosynthetic and accessory pigment contents of *A. fertilissima* and *N. muscorum* decreased continuously with increasing anthracene concentrations and exposure (days). The reduction was significant at the highest anthracene concentration i.e. 10.0 ppm and 6.0 ppm to *A. fertilissima* and *N. muscorum* respectively. The pigments such as carotenoids, phycocyanin, allophycocyanin and phycoerythrin reduced by 93, 97, 89 and 87% respectively by 16 days in *N. muscorum* cultures. Similarly, the percentage reduction of carotenoids, phycocyanin, allophycocyanin and phycoerythrin were registered by 95, 92, 82 and 81% respectively in *A. fertilissima*. These water-soluble pigments were found to decline at a faster rate than those of chlorophyll-a and carotenoids. Mohapatra and Schiewer (2000) have demonstrated with organophosphorous insecticides that the toxicant-membrane interaction and dispersion of lipoprotein membrane are responsible for change in behavior and pigment content of *Synechocystis* PCC 6803 which also corroborated with the 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) and PAH - thylakoid membrane degradation”.

Biochemical Metabolites

The retardation of carbohydrate content might be due to the interference of chemicals with the photosynthesis process (Padhy et al., 1985). Significant reduction in carbohydrates of *Synechocystis* sp., *A. fertilissima* and *N. muscorum* was observed with increasing concentrations of anthracene (Fig. 2a). Significant reduction in carbohydrates upon raising the concentration of anthracene was observed by 80, 89 and 92% in *Synechocystis* sp., *A. fertilissima* and *N. muscorum* respectively after 16 days, depicting a concentration-dependent inhibition. Anthracene at 3.5, 7.0 and 14.0 ppm diminished the protein content of cyanobacterium *Synechocystis* sp. by 58, 70 and 78%, respectively on 16 days of treatment, which were confirmed with the findings of Shehata et al., (2001). Although, initial protein levels raised during 4th and 8th day in the other two test species *A. fertilissima* and *N. muscorum*, respectively in response to lower concentrations of anthracene. However the protein levels were fallen consistently by 70% and 84% at 10.0 ppm and 3.0 ppm after 16 days. However it has been noticed that anthracene suppressed the total protein content of all three organisms in comparison to control (Fig. 2b).

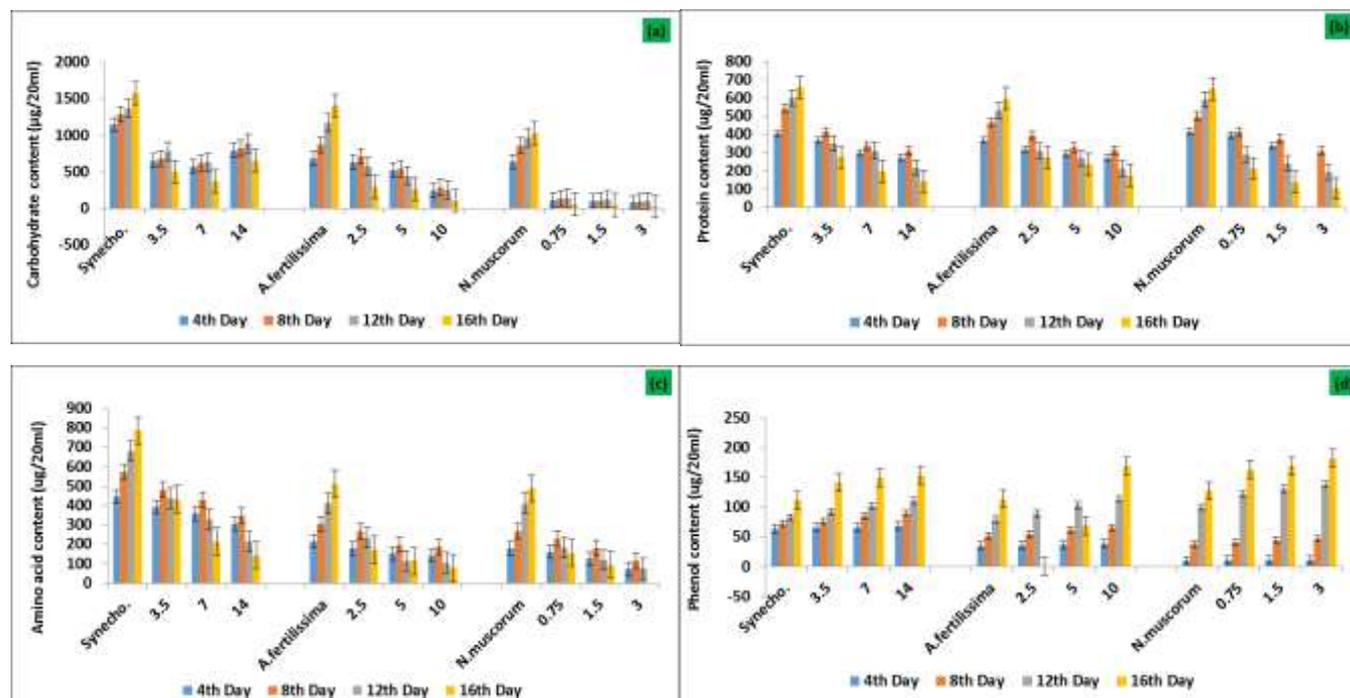


Fig. 2: (a) Carbohydrate content ($\mu\text{g}/20\text{ml}$) in *Synechocystis* sp., *Anabaena fertilissima* and *Nostoc muscorum* at different doses of anthracene; (b) Protein content ($\mu\text{g}/20\text{ml}$) in *Synechocystis* sp., *Anabaena fertilissima* and *Nostoc muscorum* at different doses of anthracene; (c) Amino acid content ($\mu\text{g}/20\text{ml}$) in *Synechocystis* sp., *Anabaena fertilissima* and *Nostoc muscorum* at different doses of anthracene; (d) Phenol content ($\mu\text{g}/20\text{ml}$) in *Synechocystis* sp., *Anabaena fertilissima* and *Nostoc muscorum* at different doses of anthracene (ppm).

Reduction in Amino acids of *Synechocystis sp.*, *A.fertilissima* and *N.muscorum* with respect to highest concentrations of anthracene were recorded by 81, 89 and 93% respectively after 16 days. After a consistent fall in the amino acids content of the tested organisms was registered in response to the treated concentrations of anthracene (Fig. 2c). Changes in amino acid concentration may be due to synthesis from endogenous precursors or inhibition of normal catabolism (Measures et al., 1975). Phenols are aromatic molecules, widespread in photosynthetic organisms, which are produced during the stress conditions. Phenol content enhanced in respective to treated cells of *Synechocystis sp.*, as compared to the control until 8th day (Fig. 2d). However, from 12th day onwards, phenol levels shot up with respect to higher concentrations of the anthracene as well as exposure periods. Moreover, phenol content of *Synechocystis sp.* was registered to increase by 37% when treated with 14 ppm after 16 days. Besides, phenolic concentration in the other two species, *A.fertilissima* and *N.muscorum* raised by 42 and 48% respectively at 10.0 and 3.0 ppm concentrations of the anthracene Pradnya et al., (2004); Calow et al., (1990) suggested that “all types of toxic stress induce metabolic change in the organism, leading to depletion of its energy reserve that results in an adverse effect on its growth and biochemical composition.

Conclusion

Experiments were conducted with a view to determining the detrimental and harmful effects of anthracene on the concentration dependent decreased in growth, photosynthetic pigments and metabolic activities of tested cyanobacteria. Indeed anthracene, an indicator of PAHs element harmful to the useful cyanobacteria. The results suggest that *Nostoc muscorum* was the most susceptible organism while the order of tolerance to each organism towards PAH can be described as *Synechocystis sp.* being more tolerant than *A. fertilissima*.

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