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Microbiology

Chronic toxic effect of Acenapthene on diverse microalgaes and cyanobacteria: *Chlorella vulgaris* Beijerinck, *Desmodesmus subspicatus* Chodat and *Scytonema sp.*

Miral S. Patel*[1], J.I. Nirmal Kumar[2] and K.K.Tiwari[1]

[1]Sophisticated Instrumentation Center for Applied Research and Testing (SICART), Vallabh Vidya Nagar, Gujarat, India- 388 120.
[2]P.G. Department of environmental Science and Technology, Institute of Science and Technology for Advanced Studies and Research (ISTAR), Vallabh Vidya Nagar, Gujarat, India- 388 120.

**Corresponding author: Miral Patel; istares2005@yahoo.com*

Abstract

There is an increasing need to describe the growth characteristics of algae exposed to polycyclic aromatic hydrocarbons (PAHs) because of occurrence of PAHs in lakes is known to deleteriously affect the growth of microorganisms. This study explored the chronic effect of different doses of three ring structure polycyclic hydrocarbon Acenapthene on the growth of two microalgal species and one cyanobacterial species. *C. vulgaris*, *D. subspicatus* and *Scytonema sp.* cultivated in the medium with different concentration of PAH and its affect was investigated during increasing 4, 8, 12 and 16 Days of exposure. The growth of *Chlorella vulgaris*, *Desmodesmus subspicatus* and *Scytonema sp*. was adversely affected by Acenapthene. The results indicated that the increased concentration of Acenapthene negatively impacted on chlorophyll content, carotenoids, phycobilliproteins, carbohydrate, amino acids, proteins, nitrate reductase, succinate dehydrogenase and glutamate synthetase except phenol . However, the raise in Phenol content was observed during the incubation period. Moreover, a high significance correlation (F>0.05) existed between different metabolites, pigments and enzymes which was statistically confirmed by Two Way Analysis of variance (ANOVA).

Highlights

- In the present study an attempt has been made to determine the chronic effects of various concentrations of PAH acenapthene on pigments like total chlorophyll, carotenoids, phycocyanin, allophycocyanin, and phycoerythrene, as well as on the biochemical compounds, C/N ratio and nitrate reductase, succinate dehydrogenase and glutamate synthetase of the two important microalgal species C. vulgaris and D. subspicatus and a cynobacterialspeciesScytonema.
- It was proved that acenapthene found more toxicity to D. subspicatus>C. vulgarsi>Scytonema sp. and revealed thatincreased concentrations of acenapthene doses subsequently decline different metabolic, enzymatic activities and pigments of species of microalgae and cynobaceteria.

Keywords: Acenapthene, microalgae, photosynthetic pigments, biochemical compounds, C/N ratio, enzymatic activities.

Aquatic ecosystems are affected by a number of toxic compounds including polycyclic aromatic hydrocarbons (PAHs). These lipophilic organic pollutants represent a group of several hundred individual compounds containing at least two condensed rings (Wilcke *et al.*, 2002). Many PAHs and their derivatives, especially epoxides, are highly toxic, mutagenic and/or carcinogenic to microorganisms as well as to higher living systems including humans (Samanta *et al.,* 2002). Contamination of polycyclic aromatic hydrocarbons (PAHs) in the environment has attracted increasing public and academic concern because some of them are known or suspected mutagens or carcinogens. PAHs are produced into the environment through natural and anthropogenic sources.

(Azuma *et al.* 1996, Wilcke *et al.* 2000 and Krauss *et al.* 2005).

Production from anthropogenic activities such as coking of coal, distillation of wood, operation of the gas works and oil refineries, runoff from asphalt pavements, vehicular emission, petroleum spills, and the incomplete combustion of fossil fuels and organic matter (Canet *et al.,* 2001). The accumulation of hydrocarbons in the seas and oceans has become a source of considerable environmental concern, especially in recent decades. In the last century, human activities have resulted in substantial additional hydrocarbon inputs to the oceans (Shaw, 1981). The growing demand for oil has been accompanied by an increase in oil pollution due to spillage in both freshwater and marine ecosystems. When an oil spill occurs in the sea, the oil spreads on the water surface and drifts by wind and currents, the low-boiling fraction evaporates, and the low-boiling aromatic fraction dissolves easily in the water (Gunkel *et al.,* 1980).

The non-target effects of organic pollutants on autotrophic microalgae and cyanobacteria have been reviewed by many researchers (Ramakrishnan *et al.,* 2011 and Venkateswarlu, 1993). Recently, the phycoremediation by microalgae has been attempted for various pollutants (Mallick, 2002, Mehta *et al.,* 2005 and Olguín, 2003). The data about the effect of PAHs on different physiological and biochemical aspects of photosynthetic activity in microalgae are still insufficient in spite of the fact that photosynthetic parameters are recognized as reliable indicators of many biotic stresses (Huang *et al.,* 2010 and Mallakin *et al.,* 2002). Recently several methods have been developed for measuring of PAHs impact on microalgae based on evaluation of its primary metabolites, photosynthesis pigments and enzymatic activities. Miral Patel *et al.,* 2014; 2014) had also studied the effect of PAHs on several biological organisms treated with different PAHs. An attempt has, therefore, been made in the present study to determine the chronic effects of Acenapthene on pigments like total chlorophyll, carotenoids, phycocyanin, allophycocyanin, and phycoerythrene, as well as on the biochemical compounds, C/N ratio and nitrate reductase, succinate dehydrogenase and glutamate synthetase of the two important microalgal species *C. vulgaris* and *D. subspicatus* and cynobacterial *species Scytonema*.

Materials and Methods

Growth Conditions and Acenapthene Treatment

Axenic cultures of *Scytonema*, *Chlorella vulgaris* Beijrinck and *Desmodesmus subspicatus* Chodat procured from the National Facility for Blue-Green Algae (IARI, New Delhi, India) were grown at 25±2ºC in BG-11 medium (Rippka *et al.,* 1979) under an illumination of 3,000 Lux light with a photoperiod of 14:10 (L/D). PAH treatment was carried out on the cultures in the logarithmic phase of growth, Acenapthene, obtained from Sigma Aldrich (Mumbai, India). Following a series of experiments to determine the LC50 dose, 2 ml of the inoculums from previously grown logarithmic cultures was introduced into a freshly prepared autoclaved modified BG-11 medium for *D. subspicatus,* BG-11 medium for *Scytonema sp.* and Zarrouk's medium (Zarrouk, 1996) for *C. vulgaris* in which Acenapthene had been added at final concentrations of 0 (control), 0.5, 1.0, 5.0, 10, 20, 40, 60 and 80 ppm to make up a total volume of 20 ml. The LC50 can be defined as the standard measure of toxicity that leads to 50% reduction in the sample population of a specific test organism in a specified period of exposure to a compound. The final LC50 value determined was given in Table 1. To prevent PAH degradation, stock solutions were prepared just prior to each experiment by dissolving the Acenapthene in HPLC grade Acetone. Each experiment was conducted in triplicate and all spectrophotometer reading was taken in UV-VIS-NIR spectrophotometer, Lambda-19, Perkin Elmer.

Table 1: On the basis of a series of experiments for LC50, the effective doses were resulted.

Species	Lower LC50	LC50	Higher LC50
C. vulgaris	1.25 ppm	2.5 ppm	5.0 ppm
D. subspicatus	1.5 ppm	3.0 ppm	6.0 ppm
Scytonema sp.	2.5 ppm	5.0 ppm	10 ppm

Pigment Analysis

The growth of the test organism was determined in terms of total chlorophyll. Total Chlorophyll and carotenoids were measured spectrophotometrically in cell lysates after extraction in 80% acetone (Jeffrey and Humphrey,

Table 2: Two Way Analysis of variance (ANOVA) of *C. vulgaris, D. subspicatus* and *Scytonema sp.* with reference to biochemical parameters (pigments, metabolites and enzymes) of control and three graded concentrations of Acenapthene after 16 days of incubation.

Parameters	Chlorella vulgaris			Desmodesmus subspicatus			Scytonema sp.		
	F(cal)	P(F<=F(cal))	F(0.05)	F(cal)	P(F<=F(cal))	F(0.05)	F(cal)	P(F<=F(cal))	F(0.05)
Total Chlorophyll	0.81	N.S. (P>0.05)	0.51	0.83	N.S. (P>0.05)	0.50	0.56	N.S. (P>0.05)	0.65
Carotenoids	0.11	N.S. (P>0.05)	0.95	0.01	N.S. (P>0.05)	0.99	0.03	N.S. (P>0.05)	0.98
Phycobilliproteins	2.07	* (P<=0.05)	0.05	2.39	* (P<=0.05)	0.02	2.89	* (P<=0.05)	0.01
Carbohydrate	0.70	N.S. (P>0.05)	0.57	0.48	N.S. (P>0.05)	0.70	0.31	N.S. (P>0.05)	0.81
Protein	0.12	N.S. (P>0.05)	0.95	0.19	N.S. (P>0.05)	0.90	0.003	N.S. (P>0.05)	0.99
Amino Acid	1.44	N.S. (P>0.05)	0.28	0.01	N.S. (P>0.05)	0.99	0.04	N.S. (P>0.05)	0.98
Phenol	3.23	N.S. (P>0.05)	0.06	6.04	** (P<=0.01)	0.009	3.18	N.S. (P>0.05)	0.08
Nitrate Reductase	0.15	N.S. (P>0.05)	0.93	0.03	N.S. (P>0.05)	0.99	0.42	N.S. (P>0.05)	0.73
Glutamine Synthetase	0.19	N.S. (P>0.05)	0.90	0.63	N.S. (P>0.05)	0.61	0.06	N.S. (P>0.05)	0.97
Succinate Dehydrogenase	0.56	N.S. (P>0.05)	0.65	1.34	N.S. (P>0.05)	0.30	0.43	N.S. (P>0.05)	0.73

1975). The cells were further suspended in 50 mM potassium phosphate buffer (pH 7.0) and the levels of phycobiliproteins, phycocyanin, allophycocyanin, and phycoerythrene were measured spectrophotometrically at 562, 615, and 652 nm, respectively, after repeated freezing and thawing (Bennett and Bogorad, 1973).

Biochemical Analysis

Biochemical studies included an estimation of the levels of carbohydrates, proteins, amino acids, and phenols. 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 biochemical analysis. The Anthrone method (Roe, 1955) was applied for total carbohydrate estimate on using glucose as a standard. Total soluble proteins were determined using bovine serum albumin as the standard (Lowry *et al.*, 1951). Amino acid content was estimated through the Ninhydrin method (Lee and Takahasi, 1996) and phenol levels were determined by the Folin-Coicalteau reagent method (Malick and Singh, 1980).

Estimation of Carbon and Nitrogen

Carbon and Nitrogen were determined using the elemental analyzer PE2400 Series II CHNS/O. (Mohammady et al. 2005).

Enzymatic Assays

Estimation of in-vivo nitrate reductase activity was made based on total nitrite formation (Sempruch, 2008). Ammonia-assimilating glutamine synthetase (GS) activity was determined through a reading of Mn2+ γ-glutamyl transferase activity (Pamiljans *et al.,* 1962). Succinate dehydrogenase (SDH), a major enzyme in the TCA cycle catalyzing the conversion of succinates to fumarates, was measured (Kun and Abood, 1949).

Statistical Analysis

Two way Analysis of variance was carried out using Ky-Plot software. Results were tested by multivariate analysis to correlate between chlorophyll-a, carotenoids, phycocyanin, allophycocyanin, phycoerythrin, nitrate reductase, glutamate synthetase, succinate dehydrogenase,

carbohydrates, proteins, amino acids, and phenols. where $P<0.05$ was considered as significant.

Results and Discussion

The toxicity is influenced by viscosity and surface tension, meteorological conditions, ecosystem, methods, other environmental conditions (Gunkel and Gassmann, 1980). The effect of crude oil and other contaminants on marine microalgae can be assayed by growth measurements (Atlas *et al.*, 1976 and Ukeles, 1965) or metabolic or photosynthetic activities (Gordon *et al.,* 1973, Soto 1975 and Kusk 1978). We report herein on the effects of Acenapthene on the metabolic response of two different microalgea and one cyanobacteria.

Photosynthetic pigments and Metabolites

Nevertheless, it must be taken into account that some crude oils have been shown to be nontoxic to algae (Coffey *et al.,* 1977), whereas others are toxic (Kauss *et al.,* 1976) to various degrees depending on the species studied and experimental conditions. Growth stimulation or inhibition depends on PAHs concentration. Toxicity was increased with higher concentrations, and longer extension of the lag phase and lower cellular density in the stationary phase occurred. However, PAHs toxicity level for microalgae may not be a reliable indication of what may happen in the natural environment.

In our study, Acenapthene treatments at various concentrations caused reduction in the total chlorophyll content of the cells, which was found significant after 4 days of treatment. The effect of Acenapthene on total chlorophyll, carotenoids and phycobiliproteins in both microalgal species is shown in (Fig. 1a). Highest treatments of Acenapthene reduced total chlorophyll in *C. vulgaris* by 31% and 81%, while in *D. subspicatus* reduced by 31% and 77% and 9% to 93% in *Scytonema sp*. respectively after 4 and 16 days. The results were highly indicative of their inhibitory effects on photosynthetic activities of the cells. Corresponding to total chlorophyll, carotenoids and phycobiliproteins decreased with increasing Acenapthene treatments. The reduction of 83%, 97% and 96% was recorded in carotenoids content of the respective organisms after PAH treatment after 16 days. (Fig. 1b) Fall of carotenoids of the microalga explained that the PAH not only accelerated the degradation but also blocked their pigment synthesis.

Phycobilin contents of the cells ceased significantly by 95%, 89% and 84% in presence of Acenapthene on *C. vulgaris*, *D. subspicatus* and *Scytonema sp.* after 16 days. (Fig. 1c, d, e) These water-soluble pigments were found to degrade at a faster rate than those of total chlorophyll and carotenoids. The degradation of these photosynthetic pigments (phycocyanin, allophycocyanin and phycoerythrene) could be also attributed to the PAHs-thylakoid membrane negative interaction. The findings are also in agreement with Mostafa *et al.* (2002) who suggested that drop in chlorophyll-a, carotenoid and phycobilinprotein contents might be ascribed due to the inhibition of pigment synthesis directly by the insecticide.

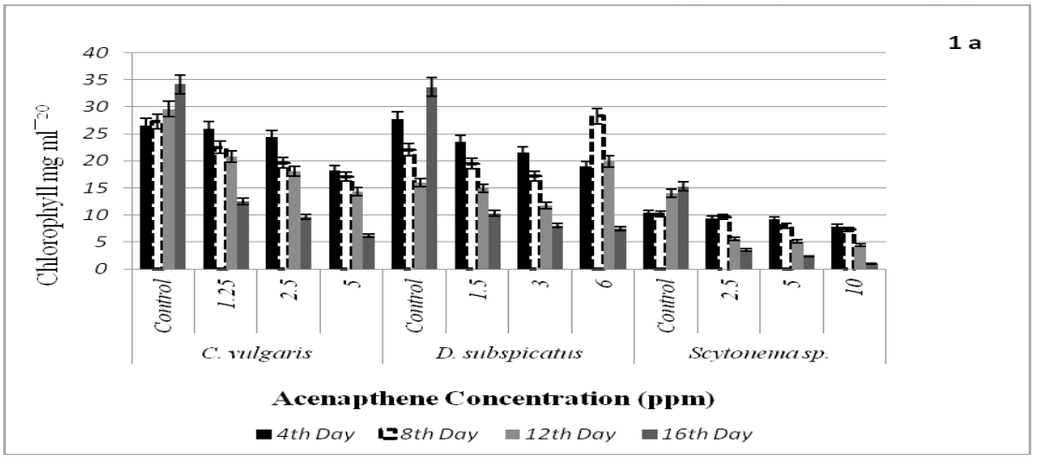

Fig. 1a: Variation in Chlorophyll content of algea treated with different doses of Acenapthene with increasing time.

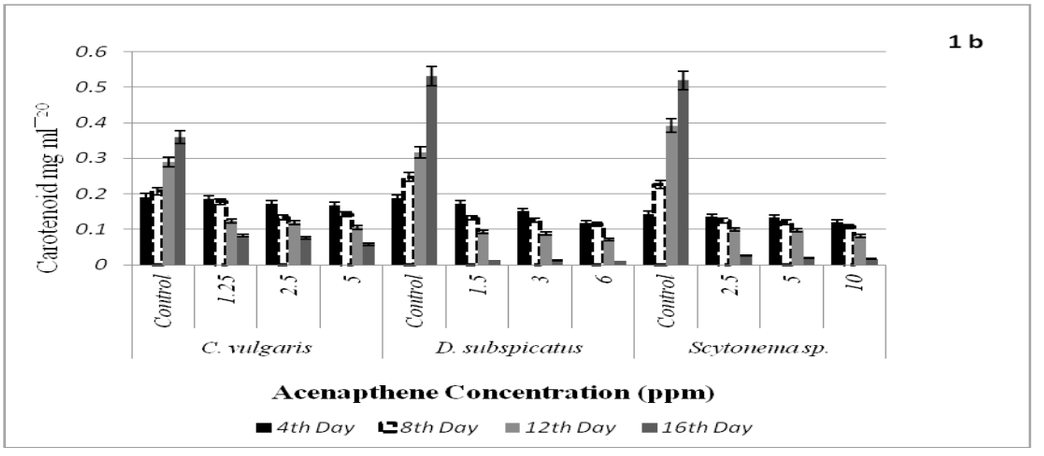

Fig. 1b: : Variation in Carotenoid content of algea treated with different doses of Acenapthene with increasing time.

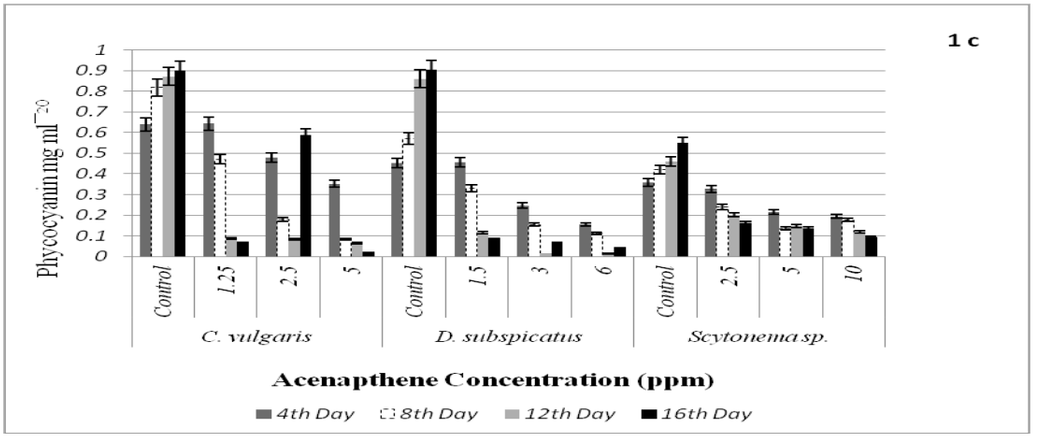

Fig 1c: Variation in Phycocyanin content of algea treated with different doses of Acenapthene with increasing time.

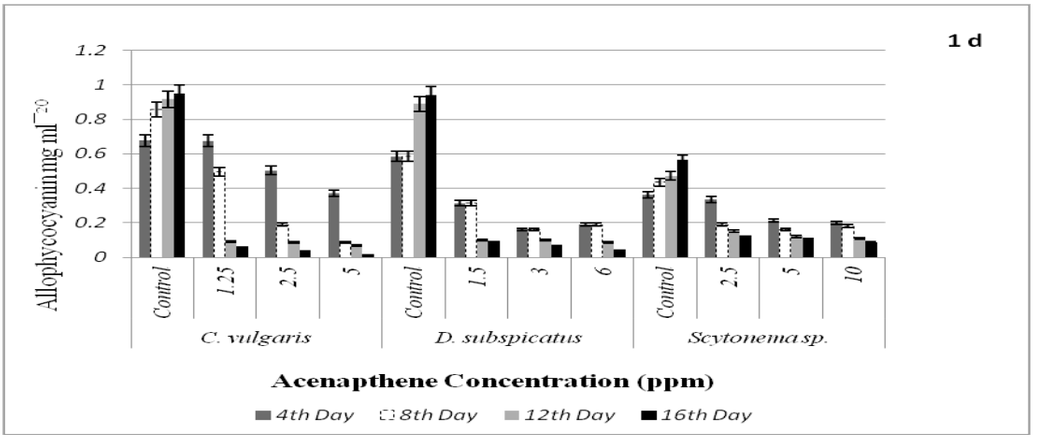

Fig 1d: Variation in Allophycocyanin content of algea treated with different doses of Acenapthene with increasing time.

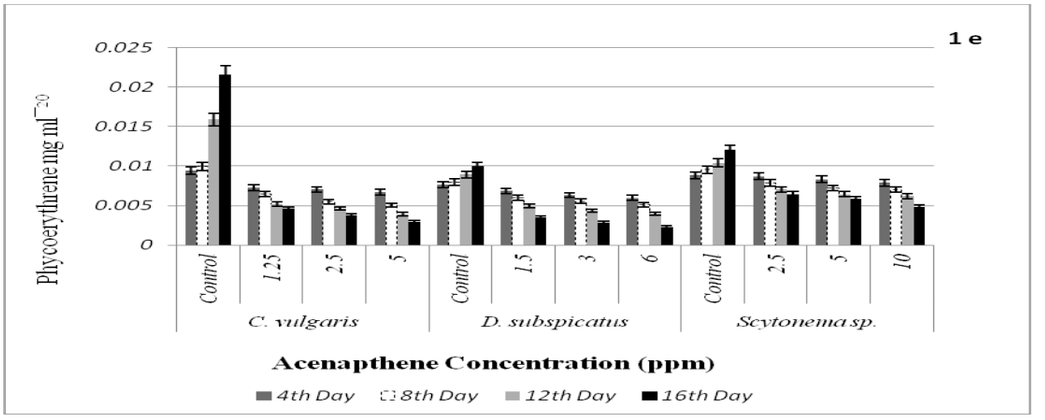

Fig 1e: Variation in Phycoerythrene content of algea treated with different doses of Acenapthene with increasing time.

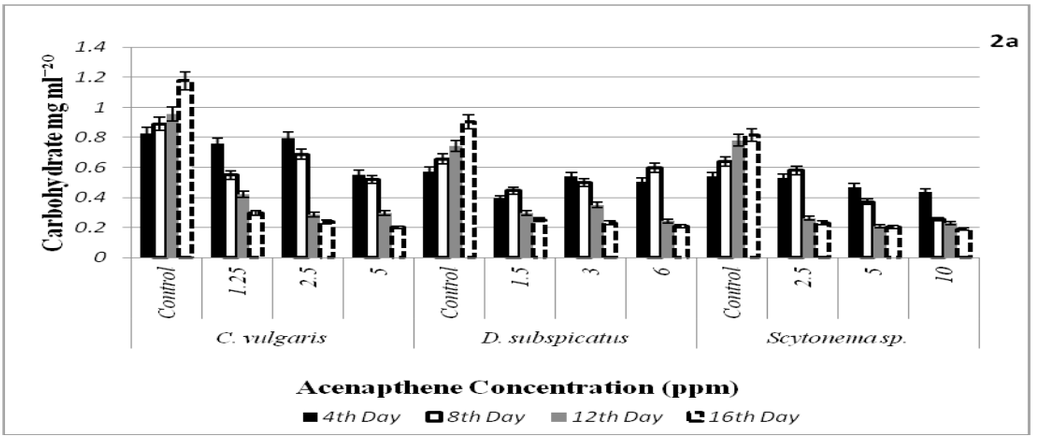

Fig 2a: Variation in Carbohydrate content of algea treated with different doses of Acenapthene with increasing time.

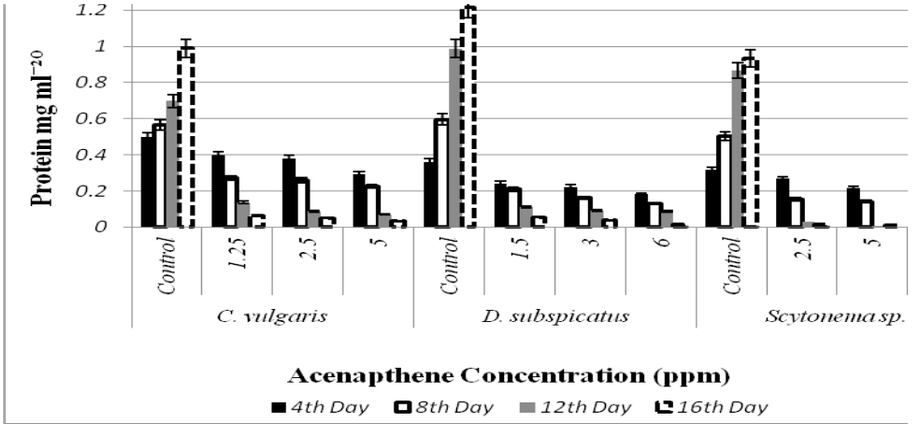

Fig 2b: Variation in Protein content of algea treated with different doses of Acenapthene with increasing time.

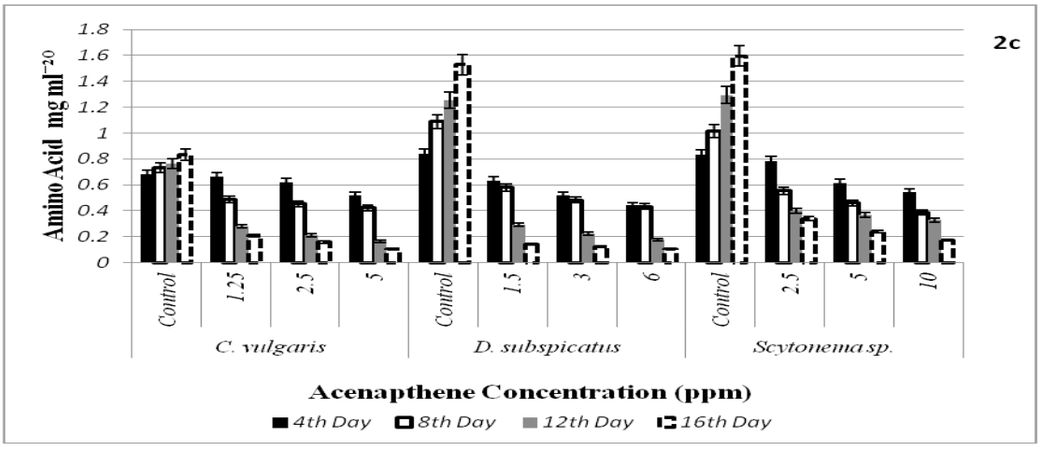

Fig 2c: Variation in Amino acid content of algea treated with different doses of Acenapthene with increasing time.

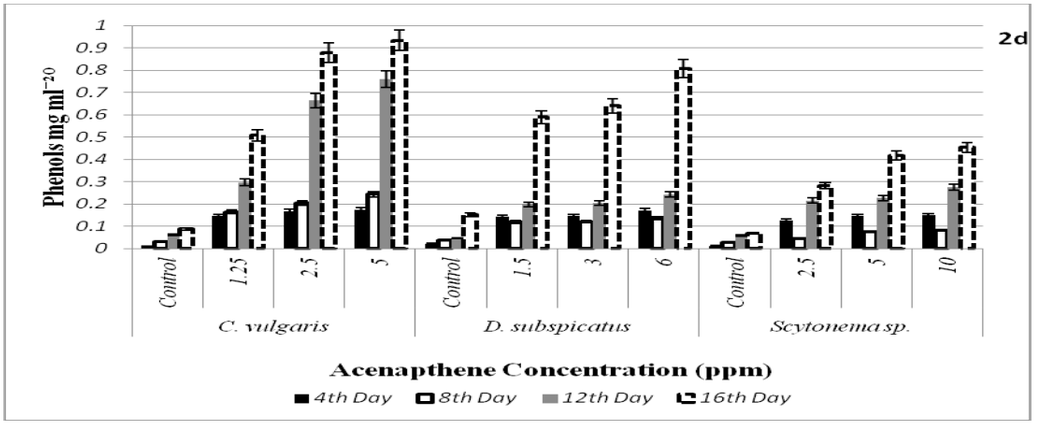

Fig 2d: Variation in Phenol content of algea treated with different doses of Acenapthene with increasing time.

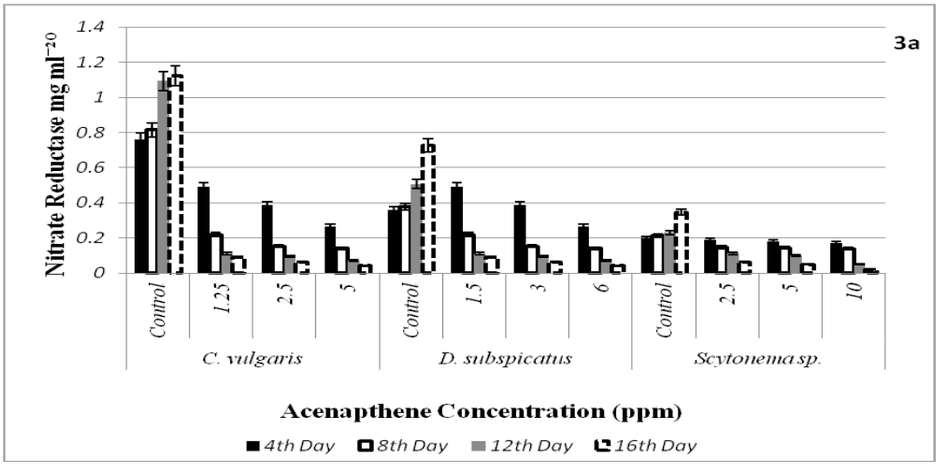

Fig 3a: Variation in Nitrate Reductase content of algea treated with different doses of Acenapthene with increasing time.

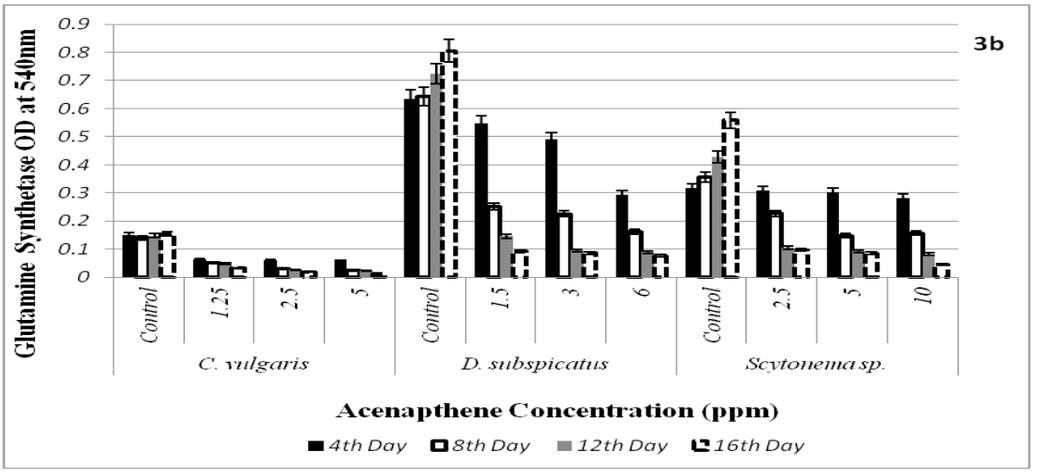

Fig 3b: Variation in Glutamate Synthetase content of algea treated with different doses of Acenapthene with increasing time.

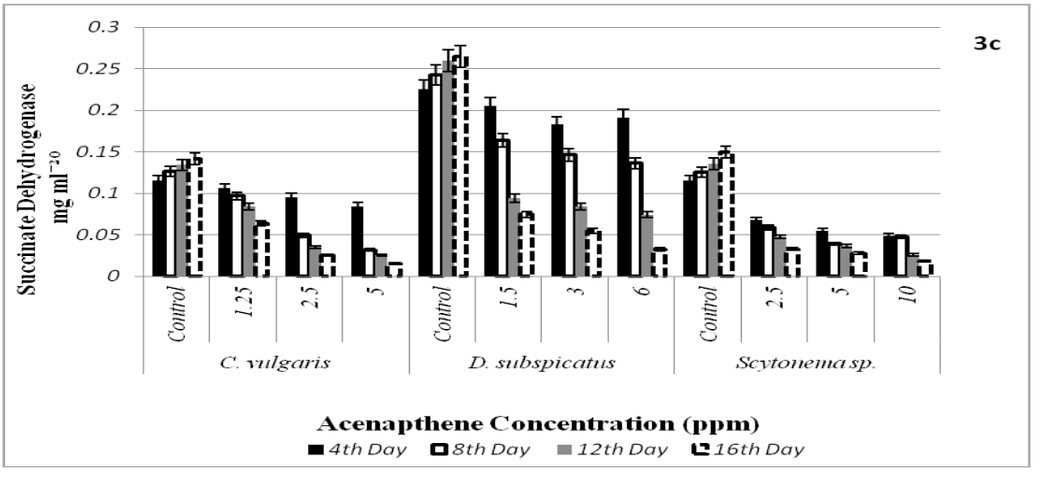

Fig 3c: Variation in Succinate Dehydrogenase content of algea treated with different doses of Acenapthene with increasing time.

Moreover, the present results are also in consonance with the deleterious effects of other fungicides on chl-a, carotenoids and phycobiliproteins of marine microalgal communities investigated by Porsbring *et al.* (2009).

Drastic reduction in carbohydrate content of *C. vulgaris* and *D. subspicatus* was observed with rise in concentrations of the Acenapthene. Total carbohydrates of *C. vulgaris* diminished by 0.29 ± 0.011 , 0.23 ± 0.01, and 0.20±0.05 mg ml^{-2o} in Acenapthene treatments with doses of 1.25, 2.5 and 5 ppm of the end of 16 days (Fig. 2a). Reduction by 0.25±0.01, 0.23±0.05 and 0.21±0.02 mg ml^{-2o} in *D. subpicatus* was registered in three different applied doses at the end of 16th day. Drop by 0.19±0.03 mg ml^{-2o} at highest concentration of Acenapthene(10ppm) was observed in *Scytonema sp*. Few studies endorsed that PAHs adversely affects the carbohydrates in algae. Kumar et al. (2008) quoted similar observations while studying with Endosulfan induced biochemical changes in nitrogen-fixing cyanobacteria like *Aulosira fertilissima, Anabaena variabilis and Nostoc muscorum*. Nirmal Kumar (1991) reported the inhibition of sugar contents of the algae by increasing doses of substituted urea herbicide N, N-dimethyl N-(4-isopopylphenyl) urea and stated that the retardation might be due to the interference of chemicals during photosynthetic process, which ultimately lapse the production of a net gain of carbohydrates.

Acenapthene stress had a pronounced effect on the production of proteins in microalgae. The protein content from all experimental flasks treated with Acenapthene was lower than that of control (fig.2b). Even though amount of protein in cultures treated with all three doses, increased up to certain time but it did not exceed control values. However PAHs at higher concentration (5 ppm) in *C. vulgaris* showed a maximum reduction of 97% in the protein content after 16th day. Protein levels in *D. subspicatus* was 0.01 mg ml^{-2o} upon Acenapthene treatments over 16 days. In *Scytonema sp.* protein content completely diminished at the end of 16 days. Kapoor *et al.* (1996) 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. Time dependent inhibition of amino acids by Acenapthene was recorded. Acenapthene reduced amino acid content by, 87%, 95% and 86% in *C. vulgaris, D. subspicatus* and *Scytonema sp.* respectively (fig 2c). Measures (1975) elaborated that changes in amino acid concentration may be due to synthesis from endogenous precursors or to inhibition of normal catabolism.

Phenols increased by 1.03±0.23 mg ml^{-2o} of *C. vulgaris,* in presence of Acenapthene treatments. Similarly in *D. subspicatus*, shot up of phenol content to 1.07±0.01 mg ml^{-2o} and 0.45±0.02 mg ml^{-2o} in *Scytonema sp*. after 16 days of PAH treatment (fig 2d). These findings also corroborated with the observations of Mallick et al. (1994) who substantiated earlier that phenols could be used as protectants to the organisms during stress or drought conditions and further stated that this could be due to the possible conversion of primary metabolites into phenols.

C/N Ratio

The C/N ratio increased slightly from 4.5±0.1 in the control *C. Vulgaris* culture to 5.14±0.15 in the concentration of 1.25 ppm Acenapthene treated culture, and then encountered somewhat to reach 3.8±0.1 in 5 ppm PAH treated cells at the end of 16th day. In *D. subspicatus* treated culture, the C/N ratio in control was 5.1±0.14 while in PAHs treated cell maximum reduction was 4.3±0.13 at the end of 16th day. In Scytonema sp., highest reduction was registered of 3.1±0.2 at the end of experimental day. The reduction of C/N ratio with increasing time and PAHs concentration may be due to toxic effect of PAHs which has significantly damaged the cells of alga . Carman et al. (1997) found that hydrocarbon contamination enhanced nitrogen availability. Other investigators Burkhardt et al. (1999) and Riebesell et al. (2000) demonstrated that the C/N ratio was influenced by a carbon enriched culture medium. In contrast, Chabbi et al. (2004) considered that C/N ratios are a consequence of the presence of decomposing plant and/or microbial (including algae) residues.

Assimilating Enzymes

Nitrate reductase (NR) was affected drastically in presence of Acenapthene in two microalgal species. NR activity of *C. vulgaris*, *D. subspicatus* and *Scytonema sp.* declined by 97%, 91% and 93% in presence of Acenapthene respectively when assayed after 16 days (fig. 3a). Adikhary *et al.* (1984) studied the effect of carbamate insecticide Sevin on the growth, survival and

nitrogen fixation of *Anabaena spp*. and *Westiellopsis prolifica* and quoted similar results. Glutamine synthetase (GS) leads to the conversion of ammonia to glutamine and also expressed a concentration dependent inhibition when treated with the PAH. The GS activities of *C. vulgaris*, *D. subspicatus* and *Scytonema sp.* suppressed by 92%, 90% and 91% (fig. 3b) after Acenapthene treatments respectively which has also been further supported by Rajendran et al. (2007) expressing a remarkable decrease in the GS activity on utilization of pesticides. Succinate dehydrogenase (SDH) enzyme is a major respiratory enzyme responsible for conversion of succinate to fumarate in the tricarboxylic acid cycle (TCA). But the treatment of this PAH (Acenapthene) lowered the activities of *C. vulgaris*, *D. subspicatus* and *Scytonema sp.* by 89%, 87% and 88% respectively after 16 days (fig. 3c). Similar inhibition of the enzyme succinate dehydrogenase activity was observed in the cultures of four Gram (+) bacteria, *Rhodococcus Sp. AK 1*, *Bacillus cereus* Frankland & Frankland, *Bacillus subtilis* (Ehrenberg) Cohn, *Nocardia asteroides* and a Gram (-) bacterium, *Rhizobium leguminosarum* when treated with the fungicide tridenmorph by Kalam *et al.,* (1995).

Statistical Analysis

ANOVA: All the parameters found to be non- significant ($P>0.05$) between the pigments, metabolites, enzymatic activities and concentrations applied (Table 2).

Conclusion

In the present investigation the microalgal cells were exposed to constant concentrations of PAHs- Acenapthene which proved more toxicity to *D. subspicatus*>*C. vulgarsi*>*Scytonema sp*. It has been revealed that increased concentrations of Acenapthene doses subsequently decline different metabolites, enzyme activities and other pigments of microalgae and cynobaceteria studied.

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