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Effect of herbicides on two species of fresh water cyanobacteria

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Abstract
Two species of cyanobacteria, such as *Anabaena variabilis* and *Hapalosiphon* sp. were collected from the various agro-climatic regions of Warangal district of Telangana state. Purified isolated culture were inoculated in 500ml Erlenmeyer flask containing 100 ml BG-11 N+ media. The cultures kept under optimum conditions and routinely sub-cultured. The impact of herbicides such as 2,4-D, Excel Mera were studied two filamentous cyanobacteria with reference to Chlorophyll-a, Carotenoid, Phycocyanin contents. In *Anabaena variabilis* the Chlorophyll-a revealed that the lower concentration of 2,4-D (6ppm) on 4th day has shown some increase when compare with other concentrations (12,18,24ppm) and the *Hapalosiphon* species at 5ppm concentration on 8th day expressed with little higher than the other concentrational under study.

Keywords: cyanobacteria, *Anabaena variabilis*, *Hapalosiphon* sps, herbicides, 2,4-D, excel mera, chlorophyll-a, carotenoid, phycocyanin

Introduction
Singh *et al.* (1979) [12] found that recommended dose of herbicide- Butachlor at the rate of 100-200 µg/ml toxic to *Anabaena doliolum*, *Nostoc muscorum* and *Aphanothece stagnina.* Pachpande and David (1980) [8] studied the effect of some common herbicides like 2-4 D, Malic Hydrozide etc. on the growth and development of soil algae like strains of *Chlorococcum infusionum.* Gangawane and Saler (1979) [2] investigated the effect of fungicide Brassicol, Methyl Benzimidazole Carbamate (MBC), Difolatum and Hexacap on *Westiellopsis*, *Autosira*, *Nostoc*, *Tolypothrix* and *Calothrix.* Anand and Veerappan (1980) [1] studied the effect of pesticides-Demicron 100, Ekalux 25, fungicides Blitox and Dithane on three blue-green algae viz. *Anabaena variabilis*, *Cylindrospermum muscicola* and *Nostoc entophytum.* Goyal (1982) noted that Endosulfon induced reduction in growth of *Anabaena iyengarii*, *Hapalosiphon intricatus* and *Calothrix bharadwajae.* Sahu *et al.* (1992) [10] reported the Endosulfon toxicity to several cyanobacteria. Benthiocar has been very widely used in rice cultivation because of its selective effect and persistence. Rajendraprakash *et al.* (1990) [9] have shown that Benthiocarb reduces porphyrin and chlorophyll biosynthesis in rice and barnyard grass; Mahapatra *et al.* (1992) [5] studied the effect of Demithoate on *Anabaena doliolum* and found highly toxic to the algae even at very low concentration. Pabbi and Vaishya (1992) [7] studied the effect of two insecticides BHC and Carbaryl in different concentration on the growth of two strains of *Anabaena variabilis* and they found that higher concentrations of both the insecticides were lethal for both the strains. Sharma and Gaur (1981) [11] studied the effect of Lindane on *Anabaena doliolum*, *Aulosira fertilissima* and *Nostoc* sp. from paddy fields and found that the level of tolerance of these organisms to Lindane was 9, 15 and 10 ppm respectively. Effect of 2,4-D was tested on few nitrogen fixing cyanobacteria, and it is reported that concentration of 200 kg per hector application have neither stimulatory nor inhibitory effect on *Aulosira ferlilissima* (Venkataraman and Rajyalaxmi, 1971) [15]. Kapoor and Sarma (1980) [6] found the same result on *Anabaena doliolum* but Tiwari *et al* (1981) [14] reported different results in the case of some *Nostoc* sps and *Anabaena* sps as 2000 micro gm per ml or above concentration. Nath and Sarma (2010) [6] found *Lyngbya limnetica* and *Oscillaoria perrnata* as more resistant blue green algal species against 2,4-D. Effect of Malathion was studied by Subramanium *et al* (1994) on 10 strains of *Aulosira fertlissima* ARM 68 and *Nostoc muscorum* ARM 221 and found that in absence of inorganic phosphate in the medium phosphate starvation causd increased activity of alkaline phosphate while the pesticide induced acid phosphate activity. According to Vyas (1988) [16] fungicides are a potentially serious threat on the overall productivity of soil and interfere with the life processes of cyanobacteria.

Material and Methods
1. Organism and growth conditions
The experimental samples (Microorganisms) were collected from paddy fields of Warangal district, T.S, India, and they were isolated and was cultured in BG-II medium (Rippka : 1979) and kept at 26± 2^0 C temperature in the uniaxenic cultures of *A. variabilis* and *Hapalosiphon* sp., were maintained from paddy fields of Warangal district T.S, India.
Lc $_{50}$ values of the selected microorganisms for 2, 4-D (Dicholoro phenoxy acetic acid) and Excel mera were procured from Bayer crop science, Mumbai. The Lc $_{50}$ values of the organisms for 2, 4-D and Exel mera were determined in terms of qualitative estimations of chlorophyll-a, accordingly, various concentrations of the pesticides were used in all experiments (Table-1). Sterile cultures and conditions are maintained throughout the experimental period. Stock solution of both the pesticides were prepare in sterilized double -

distilled water and added aseptically to the culture medium to the final concentrations indicated for the each treatment.

Purified isolated axenic cultures, which was collected from paddy fields of Warangal District T.S. India, were grow in nitrogen free BG-II medium and kept under exposed illumination light of 4000-5000 lux intensity region of 16h light/8h dark cycle at 26± 2^0 C for 28 days.

The two test samples used for the present study were *Anabaena variabilis, Hapalosiphon* sps, obtained from paddy fields of Warangal district, were conducted Media selection studies have shown that BG-11 medium at P^H 7.5 was the best suited for these species and kept in the culture room under exposed illumination light of 4000-5000lux intensity regime of 16h light and8 h dark cycle at 26±2º for 28 days.

2. Herbicides and chemical requirements

The herbicides 2,4-D (Dichloro phenoxy acetic acid), Excel Mera 71(Ammonium salt of Glyphosate) chemical combination were obtained from the Bayer (India) limited, Mumbai, India.

3. Work Plan

Various concentrations of 2,4-D and excel Mera for *Anabaena variabilis* (LC_{50} 20ppm) 6,12,18,24 and *Hapalosiphon* sp (LC_{50} 18ppm) 5,10,15,20. Various concentrations of Excel Mera 71 *Anabaena variabilis* LC_{50} 10ppm 3,6,9,12 *Hapalosiphon* sp LC_{50} 30ppm 9,18,27,36. The stock solution herbicides were prepared aseptically in sterilized basal medium concentrations of 2,4-D, Excel Mera were mixed at room temperature in respective 100ml conical flask. Each flask contains different concentrations of the test herbicides were inoculated with *Anabaena variabilis Hapalosiphon* sp a set of four flask were used for each concentration and also one set as control, various morphological characteristics were monitored every 4^{th} day interval up to 12 days. The study includes chlorophyll-a, carotenoids, phycocyanin.

4. Estimation of chlorophyll (Moran, 1982)

Chlorophyll-a can be extracted using Methanol as an extractant (Mac Kinney, 1941). The algal growth from the medium is harvested by centrifuging the known volume (10 ml) at 6000 rpm for 10 minutes. The algal pellet is washed twice with distilled water and suspended in same volume of 95% methanol. The tubes containing the suspension are kept on a water bath at 60^0C for 30 min to minimize the evaporation of methanol, glass balls are used to cover the mouths of the tubes. Intermittent shaking of the tubes ensures complete extraction of the pigment. The tubes are then removed from the water bath, allowed to cool to room temperature and the contents are centrifuged gain to remove the cell debris. Clear supernatant containing the pigment is transferred to a volumetric flask and volume is made up to 10 ml. by adding Methanol. Optical density is measured at 650 and 665 nm, using 95% Methanol as blank. The chlorophyll-a content is calculated using the following formula:

5. Estimation of Carotenoids (Chamovitz *et al*., 1993)

Carotenoids are extracted by using the centrifuge, the algal sample at 3000 rpm for 10 min to discard the supernatant. Grind the pellet in a pestle and mortar in presence at Acetone. Centrifuge, save the supernatant and repeat the grinding twice. Pool the supernatants from each operation and note the volume. This crude extract can be used estimate the total carotenoids if chlorophyll content in the sample is expected to be low. Absorbance of the crude extract is read at 450 nm using pure acetone as blank. Total carotenoids are calculated using the following formula.

C = D x V x f/2500

Where,

C = Total carotenoids in mg/ml

D = Absorbance at 450 mm

V = Volume of the extract

f = Dilution factor

(Assuming that average extinction coefficient of pigments is 2500).

5. Estimation of Phycocyanin (Bennet and Bogorad, 1971)

Most commonly used method for the extraction of phycocyanin is repeated freezing and thawing of the sample in 0.05M phosphate buffer containing equal volumes of 0.1M solution of K_2HPO_4 and KH_2PO_4. The finally thawed algal suspension if ground in a pestle and mortar using sand as abrasive. The cells can also be disrupted in a blender. The suspension containing the ruptured cells is centrifuged and the quantity of Phycocyanin (PC).

$$\text{Phycocyanin (PC)} = \frac{A_{615} - 0.474\ (A_{652})}{5.34}$$

Results

During the present investigation, the effect of Herbicides, such as 2.4-D, Excel Mera was studied on two filamentous cyanobacteria namely, *Anabaena variabilis, Hapalosiphon sp.*

Chlorophyll-a

The chlorophyll-a content of cyanobacterial species such as *Anabaena variabilis, Hapalosiphon* sp. were studied. Among the two isolates *Anabaena variabilis* have expressed maximum chlorophyll content on 12^{th} day with 0.9 µg/ml. with the comparison of *Hapalosiphon* sp. (8.6 µg/ml).

The extent sensitivity slightly differed in four test organisms. Figures 1, 2. shows the influence of Herbicides (2,4-D and Excel Mera) on chlorophyll-a. The *A. variabilis* and maximum chlorophyll-a content on the 12^{th} day (0.89 µg/m) at 6ppm concentration of 2, 4-D followed by *Hapalosiphon* sp. was with 0.81 µg/ml. in the 5ppm concentration of 2, 4-D, *N. muscorum* was 0.62 µg/ml in the concentration of 4ppm on 4^{th} day of Excel Mera.

Relatively higher concentration 24ppm of 2,4-D proved lethal for *A. variabilis*, similarly 12ppm of Excel Mera have shown maximum fragmentation with dead cell.

The concentration of 5ppm on 12^{th} day (2, 4-D), 9 ppm on 4^{th} day (Excel Mera) have expressed good growth in terms of chlorophyll-a content in *Hapalosiphon* sp.. The Figures: 1, 2, indicates that Herbicides with various concentrations used for the chosen cyanobacteria have not exhibited good results in *Hapalosiphon* sp.. The Excel Mera at 4 ppm concentration on 12^{th} day have expressed high content of chlorophyll-a (0.62 µg/ml) when compare to other Herbicides under study. Thus,

the present study indicates that the various concentrations of Herbicides and Fungicides used were not having stimulating factor on the growth of the algae with reference to chlorophyll-a.

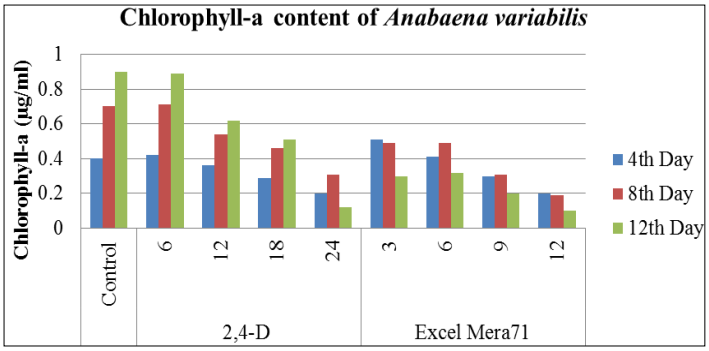

Fig 1

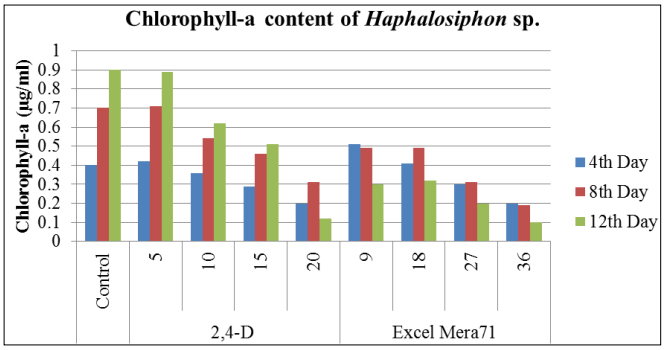

Fig 2

Carotenoids

Carotenoids could be on important biological indicators for monitoring the effect of Herbicides on the organism's survival pattern. Therefore, the Concentration of carotenoids in four organisms was measured by exposing them to varying concentrations of the Herbicides with different durations (Figures: 5, 6).

In control samples of test organisms, *A. variabilis* have shown 0.5 µg/ml, 0.8µg/ml. and 1.1 µg/ml of carotenoid content at various durations (4th, 8th & 12th days). In the *Hapalosiphon* sp. the carotenoid content varied from 0.6 µg/ml, 0.8 µg/ml and 1.2 µg/ml. The results indicated in *A. variabilis* carotenoid content at 6ppm concentration of 2,4-D on 12th day with 0.85 µg/ml.

In all the herbicides the carotenoid content in even *A. variabilis* at lower concentration (6ppm, 3ppm, 4ppm and 2ppm) on 12th day have exhibited with 0.85µg/ml, 0.65 µg/ml, 0.82 µg/ml and 0.02 µg/ml under investigation. At higher concentrations, the carotenoid content was decreased gradually as the concentration of 2, 4-D increased. The carotenoid content in *Hapalosiphon* sp. at 5ppm concentration was found at various durations (4th, 8th and 12th day) of the Herbicides as 2, 4-D (0.6, 0.7 and 1.1 mg/ml) Excel Mera at 9ppm concentration on 12th day exhibited with 1.1µg/ml. However, the chosen Herbicides (2, 4-D and Excel Mera) at different concentrations such as 20ppm, 36ppm inhibited the carotenoid content gradually as the concentration increased.

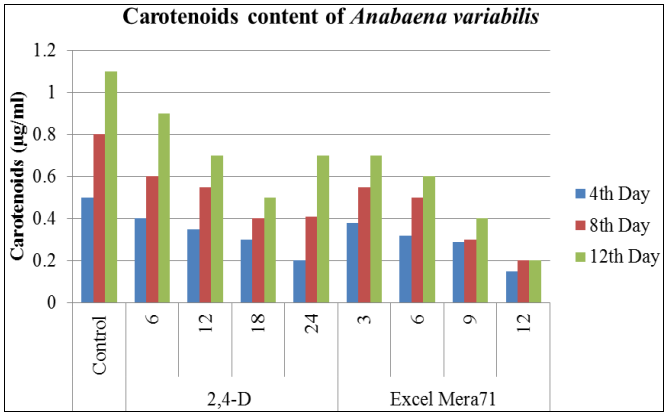

Fig 3

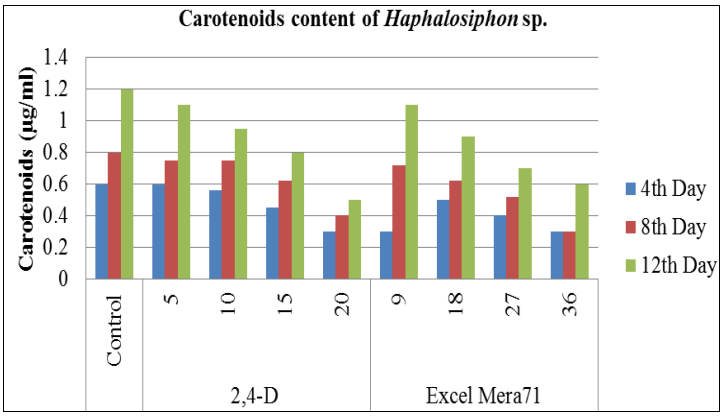

Fig 4

Phycocyanin

The component of phycobilin such as phycocyanin (PC) were studied by treating the selected algae with Herbicides. The phycocyanin content in the control sample of *A. variabilis* shown with 0.6 0.8 and 1.1 µg/ml. on 4th, 8th and 12th day observation. The highest content of phycocyanin was observed on 12th day in *Hapalosiphon* sp.. (1.4 µg/ml).

The 2,4-D treated cultures of *A. variabilis* at 6ppm and 12ppm concentrations shown little high content of phycocyanin in all durations (4th, 8th and 12th day) as compared to control sample. Different concentrations (3ppm, 6ppm 9ppm and 12ppm) of Excel Mera, treated cultures were also found with high content of phycocyanin at 3ppm concentration in all duration. At higher concentration (24ppm) of 2, 4-D has exhibited specially significant reduction and it was followed by Excel Mera (12ppm).

In the present investigation the results of phycocyanin clearly shows that 2, 4-D at lower concentrations (6ppm and 12 ppm) expressed higher content of phycocyanin than the control. The results of the *Hapalosiphon* sps at 5ppm on 4th day and 8th day (0.82 µg/ml, 0.84 µg/ml) and at 10ppm on 4th day (0.9 µg/ml) was found with high content of phyocyanin in 2, 4-D treated cultures. All other Herbicide (Excel Mera) at different concentration and durations expressed with lower content of

phycocyanin than the control samples. At higher concentration except in 2, 4-D treated cultures phycocyanin content was inhibited to minimum level in all other Herbicide treated cultures. (Figures: 9&10).

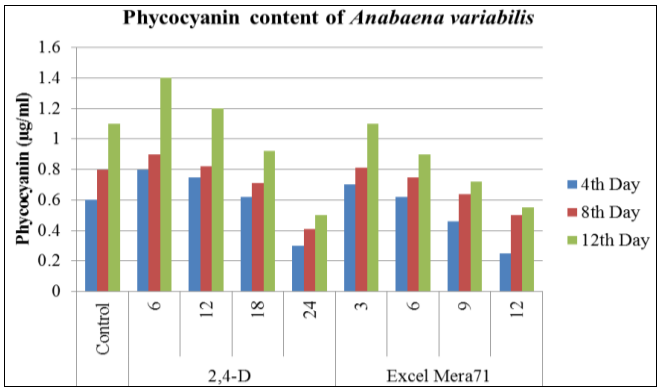

Fig 5

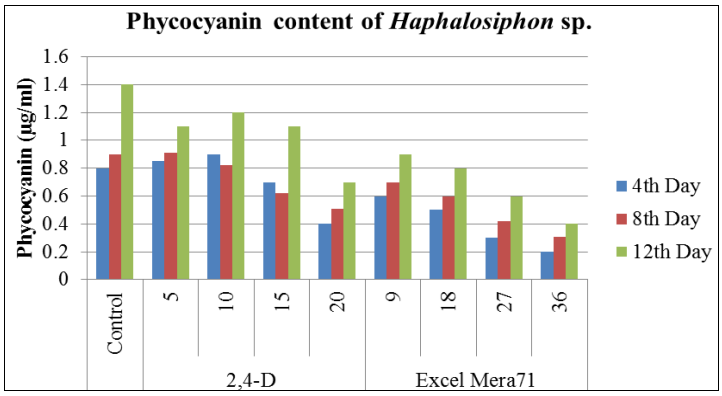

Fig 6

Discussions

In the present investigation efforts have been made to assess the effect of Herbicides on the growth, Chlorophyll-a, Carotenoid, Phycocyanin. The extent of sensitivity slightly differed in two test organisms. Relatively higher concentrations of Herbicides proved lethal for *Anabaena variabilis*, *Hapalosiphon* sp. except in 2,4- D lower concentration (6 ppm), the other Herbicide such as Excel Mera was observed with gradual decrease of chlorophyll-a even at lower concentrations. Similarly, for phycobiliprotein content and carotenoid contents were also expressed with declining as concentrations increases in the chosen Herbicides. The 2,4- D, Excel Mera treated organisms at higher concentrations with various durations were found to be toxic and the level of pesticides used strongly affected the cyanobacterial and also biochemical metabolites. The possible reason for such behavior of the test algae may be the differential permeability of the pesticides across the cell membrane. Relatively higher tolerance of *A. variabilis* and *Haphalosiphon* sp. Nearly identical pattern of toxic effects, at higher concentrations (4.0µg/ml. and 5.0µg/ml.) of the pesticides indicates involvement of some common step(s) in their metabolic pathway. The possible utilization phycocyanin by algal cells under N-stress, caused by the pesticides, results in reduction of protein level. These phytotoxic chemicals move into the cell affecting the electron transport and enzymatic activities thus resulting in the destruction of metabolic process (Lal and Saxena, 1980). Saxena *et al.* (1997) and Anand and Subramanian also worked with pesticides on cyanobacteria and reported that lower concentrations of these chemicals enhanced chlorophyll-a indicating better growth while at higher concentrations were inhibitory. In the present investigations, chlorophyll, carotenoid and phycobiliprotein contents were significantly reduced in presence of the pesticides.

Conclusion

Two species of nitrogen fixing cynobacteria viz., anabeana variablis and Haplosiphon sp were collected from paddy fields Warangal District (17.96 89^0 N, 79.59 41^0).The effect of 2, 4 - D, Ammonium salts of glyphosate was studied on Chlorophyll content, Carotenoids and Phycocynin pigments.

It is observed that *A.variables* has expressed maximum Chlorophyll content on 12^{th} day with 0.9 µg/ml in comparison to hapalosiphan sps. (0.8 µg/ml) in 2, 4-D Excel Mera treatments. Higher concentration proved lethal.

In all the herbicides the caroteniod content in even *A. variabilis* at lower concentration (6ppm, 3ppm, 4ppm and 2ppm) on 12^{th} day have exhibited with 0.85µg/ml, 0.65 µg/ml, 0.82 µg/ml and 0.02 µg/ml under investigation. At higher concentrations, the carotenoid content was decreased gradually as the concentration of 2,4-D increased. The carotenoid content in *Hapalosiphon* sp. at 5ppm concentration was found at various durations (4^{th}, 8^{th} and 12^{th} day) of the Herbicides as 2, 4-D (0.6, 0.7 and 1.1 mg/ml) Excel Mera at 9ppm concentration on 12^{th} day exhibited with 1.1µg/ml. However, the chosen Herbicides (2, 4-D and Excel Mera) at different concentrations such as 20ppm, 36ppm inhibited the caroteniod content gradually as the concentration increased.

The 2,4-D treated cultures of *A. variabilis* at 6ppm and 12ppm concentrations shown little high content of phycocyanin in all durations (4^{th}, 8^{th} and 12^{th} day) as compared to control sample. Different concentrations (3ppm, 6ppm 9ppm and 12ppm) of Excel Mera, treated cultures were also found with high content of phycocyanin at 3ppm concentration in all duration. At higher concentration (24ppm) of 2, 4-D has exhibited especially significant reduction and it was followed by Excel Mera (12ppm).

In the present investigations, chlorophyll, carotenoid and phycobiliprotein contents were significantly reduced in presence of the pesticides.

Table 1

S. No	Herbicide name	Organism selected	Determination of LC_{50} values	Based on LC_{50} (ppm) values treatment was made
1	2,4-D	Anabaena variabilis Hapalosiphon sp.	20 18	6, 12, 18, 24 5, 10, 15, 20
2	Exel Mera71	Anabaena variabilis Hapalosiphon sp.	10 30	3, 6, 9, 12 9, 18, 27, 36

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