# PHOTOSYNTHETIC AND BIOCHEMICAL INVESTIGATION OF *ANABAENA ORYZAE* IN RESPONSE TO AN INSECTICIDE-ENDOSULFAN

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***Abstract*: A study on the heterocysts nitrogen fixing Cyanobacterium, *Anabaena oryzae* was carried out to investigate the effect of an organo chlorine insecticide hexachloro-hexahydro-methano-benzodioxatheipine-oxide, (called as Endosulfan) at different concentration of 4,8 and 12 µg ml$^{-1}$ on the photosynthetic pigments Chl-a, Carotenoids and phycobiliproteins-phycocyanin, allophycocyanin and phycoerythrin. The insecticide Endosulfan showed to be deleteriously affecting the activities in the cyanobacterium. As early as the 4$^{th}$ day, Chl-a and carotenoids reduced by 38% and 20% respectively. The phycobilioproteins declined by 60%, 64%, and 28% with respect to phycocyanin, Allophycocyanin and phycoerythrin. Moreover, endosulfan adversely depleted the cellular activities, leading to a marked decrease in the carbohydrates, proteins and phenols. Despite of deleterious effect of Endosulfan on the Cyanobacterium, *Anabaena oryzae*. A unique regenerating ability in presence of the insecticide was observed by the end of 12 days in the lower doses of insecticides.**

***Keywords*: Cyanobacteria, *Anabaena oryzae*, Endosulfan and Insecticide.**

**INTRODUCTION:-** Cyanobacteria are the pioneer oxygenic phototrophs on earth whose distribution around the world is surpassed only by bacteria. Fossil evidence point to their presence in geographically diverse regions during the Precambrian (2 to more than 3.5 billion years ago).

They are a large and morphologically diverse group of phototrophic prokaryotes, which occur in almost every habit on the earth. Algae constitute a dynamic component of the soil. Which add and increase the availability of the crop nutrients. They help in involving the solid structure and amend the chemical nature of the soil.

In agriculture practices throughout the industrial world and to a lesser extent in the developing countries. Pesticides are an important factor in maintain high agriculture productivity (**Day**, 1987). But the emphasis on chemical control of plant insects and diseases has caused serious imbalance in the agro-ecosystem. The increasing use of pesticide in agriculture demands investigation to examine the effect of pesticides on the non-target soil microorganisms including nitrogen fixing cyanobacteria (**Singh *et al.*, 2018**). Cyanobacteria have been applied in rice field as a biofertilizer for better yield of paddy (**Relwani, 1963, Singh *et al.*, 2014**). Weedicides, fungicides and insecticides used for plant protection in rice fields affect adversely on the cyanobacteria population (**Anand, 1980; Stratton, 1987**; **Kolte** and **Goyal, 1990**). Some problems like non-target effects of chemicals (**Hill & Weight, 1978**) are being constantly experienced in recent year; the pesticide effect on cyanobacteria has gained greater attention than eukaryotic algae. This shift in focus appears to have been promoted by the acknowledgement of the important role of many of the cyanobacteria as nitrogen fixers in soil (**Da Silva *et al.*, 1975**, **Bold** and **Wynne, 1978**). Many of these studies have been conducted in India (**Ahmed** and **Venkataraman, 1973**; **Sardeshpande** and **Goyal, 1982**; **Kaushik** and **Venkataraman, 1983, Singh *et al.*, 2016**).

Endosulfan is most popular amongst the organochlorine insecticides. It is being extensively used in crops field due to its broad spectrum of activity and relatively low cost. Applications rate of 35 EC endosulfan is 560 ml in 100 liters of water per acre in rice field (ICAR). Depending on the type of crop and the area in which it is grown, application rates usually range between 0.45 kg/ha and 1.4kg/ha, but both smaller and larger doses have occasionally been used (**Hoechst, 1977**).

**MATERIAL AND METHODS:**

For the present study, the blue-green algae, *Anabaena oryzae*, was selected. The algae under investigation were isolated from the paddy fields of Warangal district, Telangana. Initially studies was conducted to select suitable medium, for the growth of these algae. Media selection studies have shown that Fogg's medium (**Fogg,** 1949) at pH 7 was the best suited for *Anabaena oryzae* (Fritsch), under investigation. Therefore in the present studies Fogg's medium served as the basal medium throughout the study of the respective algae. The clonal cultures of these algae was raised from single filament. The axenic cultures was obtained by repeated sub-culturing (**Venkataraman**, 1969). All the experiments was conducted in aseptic conditions by keeping the cultures in culture chamber at 24±2$^0$C temperature **(Fig-1)**.

**Table:-1. Showing Fogg's medium.**

| Fogg's medium (Fogg, 1949) | | |
|---|---|---|
| **Composition of Media:** | | |
| **S. No.** | **Name of the Compound** | **g/l** |
| 1 | Potassium dihydrogen phosphate | 0.2 |
| 2 | Magnesium sulphate | 0.2 |
| 3 | Calcium chloride | 0.1 |
| 4 | $A_5$ micronutrient solution | 1.0 |
| 5 | Fe-EDTA stock solution | 1.0 |
| **$A_5$ Micronutrient solution** | | |
| **S. No.** | **Name of the Compound** | **g/l** |
| 1 | Boric acid | 2.86 |
| 2 | Manganese chloride | 1.81 |
| 3 | Zinc sulphate | 0.222 |
| 4 | Sodium molybdate | 0.177 |
| 5 | Copper sulphate | 0.079 |

**Preparation of Fe-EDTA solution**

Dissolve 26.1 g of (EDTA) ethylene diamine tetra-acetic acid (Disodium salt) in 268 ml of 1 N Potassium hydroxide solution and add 24.9 g of Ferrous sulphate. Make up the volume to 1 liter. Aerate the solution overnight to produce a stable complex marked by the change in colour to dark brown. Make up the volume again to 1 liter. Add 1 ml. of this stock solution to 1 liter medium to give 5 ppm of iron.

The culture medium and glassware were sterilized in an autoclave at the temperature of $121^0$C and pressure of 151b/inch$^2$ for 30 minutes.

Selected algae were grown in 250 ml. Erlenmeyer flasks containing 100 ml of culture medium were agitated twice a day in order to maintain them in actively growing stage. Sub culturing was done after every 15 days of inoculation.

***Anabaena oryzae*** Fritsch

Thallus soft, green gelatinous, membranous, trichomes, short, straight, densely aggregated, generally parallel cells 2.5-3 µ broad more or less barrel shaped, 1½-2 times as long as broad, heterocysts terminal and intercalary, broader than the vegetative cells, 3-3.5 µ broad, terminal ones conical and twice longer than broad intercalary ones, single or 2-3 series, generally barrel-shaped sometimes. Spherical, single spores rarely single next to the terminal heterocyst, commonly away from the intercalary heterocysts, single or 2-7 in series, subspherical or short ellipsoidal, 5x5, 6.5x7, 5x5.5, 5x6.5 µ exospore yellowish brown (**Plate – 3A**).

**Estimation of Chlorophyll**

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 $60^0$C 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:

**Estimation of Carotenoids**

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.

$$C = D \times V \times f/2500$$

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).

**Estimation of Phycobiliprotein**

Most commonly used method for the extraction of phycobiliproteins 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 grind 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), phycoerythrin (PE) and allophycocyanin (APC) in mg/ml is calculated from the absorbance read at 562, 615 and 652 nm respectively, using the equations derived from the extinction coefficients of purified phycobiliproteins (**Bennet and Bogorad,** 1971).

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

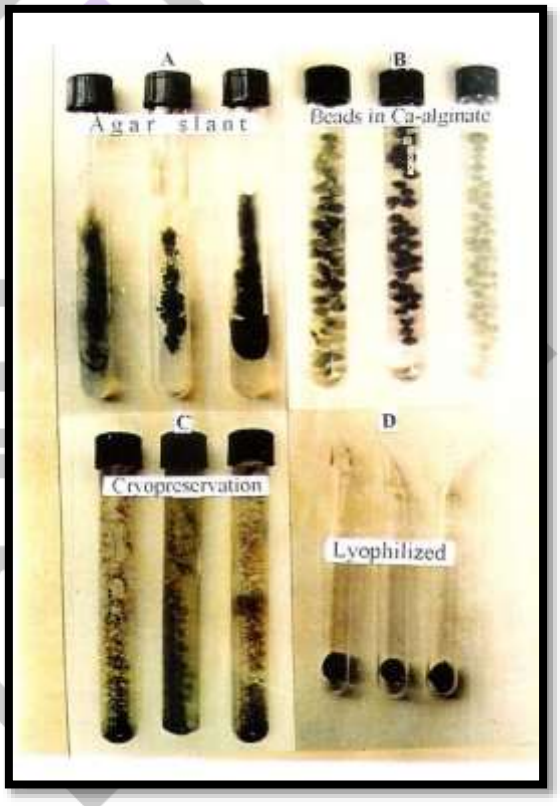
$$\text{Phycoerythrin (PE)} = \frac{A_{562} - 2.41\,(PC) - 0.849\,(APC)}{9.62}$$

$$\text{Allophycocyanin (APC)} = \frac{A_{652} - 0.208\,(A_{615})}{5.09}$$

**RESULTS AND DISCUSSION:**

**Fig-1** Showing the Cabin of culture plates.

**Fig-2:** Showing the methods of preservation.




A. Agar slant B. Beads in Ca-alginate C. Cryopreservation D. Lyophilised

**Table-2:-** Showing the concentration of pigments at 4 days of treatment in *Anabaena oryzae*

| S. No. | Duration of intervals (Days) | Concentration | Chl-a | Carotenoids | Phycocyanin | Allophycocyanin | Phycoerythrin |
|---|---|---|---|---|---|---|---|
| 1 | 4 day | 0.4 µg/ml | 0.3 g/ml | 0.5 µg/ml | 0.8 µg/ml | 0.4 µg/ml | 0.4 µg/ml |
| 2 | 8 day | 0.8 µg/ml | 0.6 g/ml | 1.0 µg/ml | 1.2 µg/ml | 0.65 µg/ml | 0.8 µg/ml |
| 3 | 12 day | 1.2 µg/ml | 0.95g/ml | 1.5 µg/ml | 1.5 µg/ml | 1.0 µg/ml | 1.2 µg/ml |

**Table-3:- Showing the concentration of pigments at 4 days of treatment in *Nostoc muscorum.***

| S. No. | Duration of intervals (Days) | Concentration | Chl-a | Carotenoids | Phycocyanin | Allophycocyanin | Phycoerythrin |
|---|---|---|---|---|---|---|---|
| 1 | 4 day | 0.4 µg/ml | 0.5 µg/ml | 0.6 µg/ml | 0.034 µg/ml | 0.018 µg/ml | 0.017 µg/ml |
| 2 | 8 day | 0.8 µg/ml | 2.0µg/ml | 0.9 µg/ml | 0.040 µg/ml | 0.020 µg/ml | 0.09 µg/ml µg/ml |
| 3 | 12 day | 1.2 µg/ml | 5.0µg/ml | 1.5 µg/ml | 0.050 µg/ml | 0.022 µg/ml | 0.022 µg/ml |

During the present investigation, the effect of pesticide Endosulfan was studied on two filamentous cyanobacteria namely *Anabaena oryzae* and *Nostoc muscorum.* Results and observations were recorded mainly with reference to changes in chlorophyll-a, carotenoid content, phycocyanin, phycoerythrin, allophycocyanin, once every four days for a period of 12 days.

**Chlorophyll-a:**

Among the two isolates *Anabaena oryzae* has maximum chlorophyll-a on the 12$^{th}$ day (0.95 µg/ml.) as compared to *Nostoc muscurom,* chlorophyll-a on the 12$^{th}$ day (0.8 µg/ml.) in control sample.

The extent of sensitivity slightly differed in both the test organisms. **Figures-1&2** shows the influence of pesticide (Endosulfan) on chlorophylla-a. *A. oryzae* has maximum chlorophyll-a content on the 12$^{th}$ day (1.2 µg/ml.) followed by *Nostoc muscorum* (5.0 µg/ml.). Among these, growth of *A. oryzae* was better than *Nostoc muscorum.* Endosulfan have shown maximum damage to *Nostoc muscorum* chlorophyll-a at 5.0 µg/ml. concentration (12$^{th}$ day) under investigation.

Relatively higher concentrations (5.0 µg/ml.), proved lethal for *A. oryzae* whereas extensive fragmentation. Supported the maximum growth in terms of chlorophyll-a content which has almost equaled to the control value in both test algae.

**Carotenoids:**

Carotenoids could be an important biological indicators for monitoring the effects of pesticides on the organism's health. Therefore, the concentration of carotenoids in both the organisms was measured by exposing them to varying concentrations of the Endosulfan.

In control samples of test organisms, *A. oryzae* have shown 0.5 µg/ml., 1.0 µg/ml. and 1.5 µg/ml. carotenoid content of various durations (4$^{th}$, 8$^{th}$ & 12$^{th}$ day). The results indicated in *A oryzae* carotenoid content at 0.5 µg/ml. concentration of all pesticides used was almost similar with little differences to the control samples in all durations (**Figure-4**). In all the pesticides the carotenoid content in *A. oryzae* at 0.5 µg/ml. concentration was observed at various durations, and the results are found as Endosulfan (0.6 µg/ml., 0.9 µg/ml. and 1.5 µg/ml.) in Nostoc muscorum respectively. At higher concentrations (1,2,4 and 5 µg/ml.),

The pesticide Endosulfan inhibited the carotenoid contents in *N.muscorum* at all the concentrations (**Figure-4**). In *N.muscorum,* the inhibition was at its maximum on 12$^{th}$ day, Similar to Endosulfan and Malathion, Butachlor at low concentration (0.5 µg/ml.) shown not much difference in carotenoid content as compared to control samples in all durations of the two test organisms. At high concentrations both algae under study have shown maximum decrease in carotenoid contents. In *A. oryzae* and *N.muscorum.* Carotenoid decreased with increasing concentrations of the pesticides and showed a trend almost identical to chlorophyll-a.

**Phycocyanin:**

The components of Phycobilin such as Phycocyanin (PC), Phycoerythrin (PE) and Allophycocyanin (APC) were studied by treating the selected algae with various pesticides. The Phycocyanin contents in the control samples *A. oryzae* shown 0.8, 1.2

and 1.5 µg/ml. and in *N.muscorum*, 0.034, 0.040 and 0.050 µg/ml. at 4th, 8th and 12th day observations. The higher content of phycocyanin was observed on 12th day in both the algae (1.5 µg/ml. and 0.050 µg/ml.) under study (**Figures-7 & 8**).

The results indicated that *N.muscorum* was grown in 0.5, 0.1, 2.0, 4.0 and 5.0 µg/ml. concentrations of pesticides, the phycocyanin content was inhibited gradually as the concentrations are increasing. At higher concentrations (5.0 µg/ml.), the treated cultures phycocyanin content was inhibited to Endosulfan (0.008, 0.006 and 0.002 µg/ml.**),** as compared to control samples.

**Phycoerythrin:**

The phycoerythrin content of *A. oryzae* was observed at various durations (4th, 8th and 12th day) in control samples shown 0.4, 0.8, 1.2 µg/ml. In this algal member under study, it is clearly shown that phycoerythrin decreased with increasing concentrations of the pesticides and showed a trend identical to phycocyanin (**Figures-10 & 11**). In 0.5 µg/ml. concentration of endosulfon treated cultures at 4th, 8th and 12th day duration have shown slight increase in the production of phycoerythrin in *N.muscorum* (0.017, 0.19 and 0.022 µg/ml.), respectively.

**Allophycocyanin:**

In control samples of test organisms, *A. oryzae* has shown 0.4, 0.65, 1.0 µg/ml. and *Nostoc muscorum* has shown 0.018, 0.020, 0.022 µg/ml., allophycocyanin content at various durations (4th, 8th and 12th day), respectively. The results shown in **Figures-13 & 14** clearly indicate that endosulfon inhibited the content of allophycocyanin significantly as concentration increased and a showed trend identical to phycocyanin in this algae.

**Table-4:** Effect of Endosulfan on the formation of heterocyst.

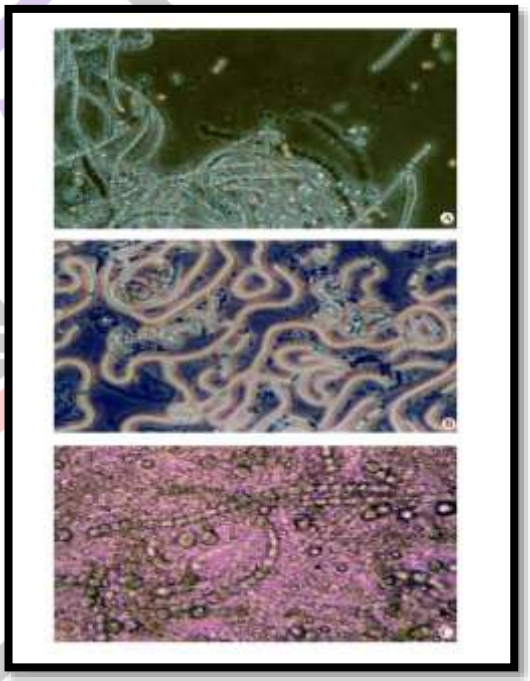
| | |
|---|---|
| A. *Anabaena oryzae* (Fritsch) Filament with high number of Heterocysts at 0.5 µg/ml concentration of Butachlor X 250<br>B. Terminal heterocysts in the filaments at 1.0 µg/ml concentration of Endosulfan X 250.<br>C. Diad cells, breakage of filaments, chlorosis and cell lysis at 5.0 µg/ml concentration of Thimet X 250. |  |

**Table-5: Effects of endosulfan on *Nostoc muscorum* Ag. Ex Born. Et Flah**

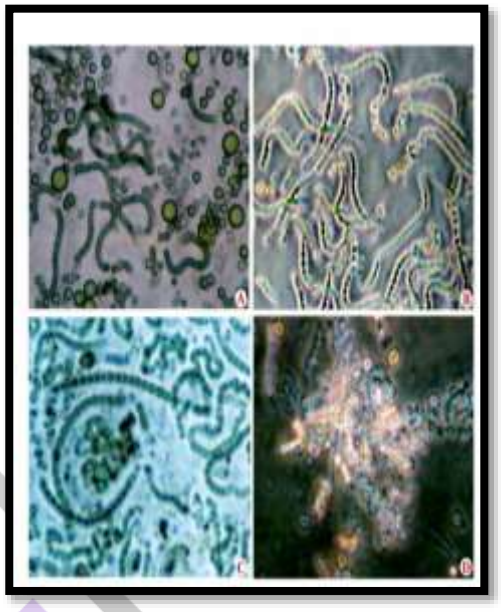
| | |
|---|---|
| a. Less number of heterocysts at 1.0 µg/ml concentration X 250.<br>b. Diad and Tetrad cells with granulation at 2.0 µg/ml concentration X 250.<br>c. Filaments breakage at 4.0 µg/ml concentration X 250.<br>d. Fragmentation and cell lysis at 5.0 µg/ml concentration X 250. |  |

**Fig.3** Showing effect of Endosulphon on protein content of Anabaena oryzae (Fritsch).

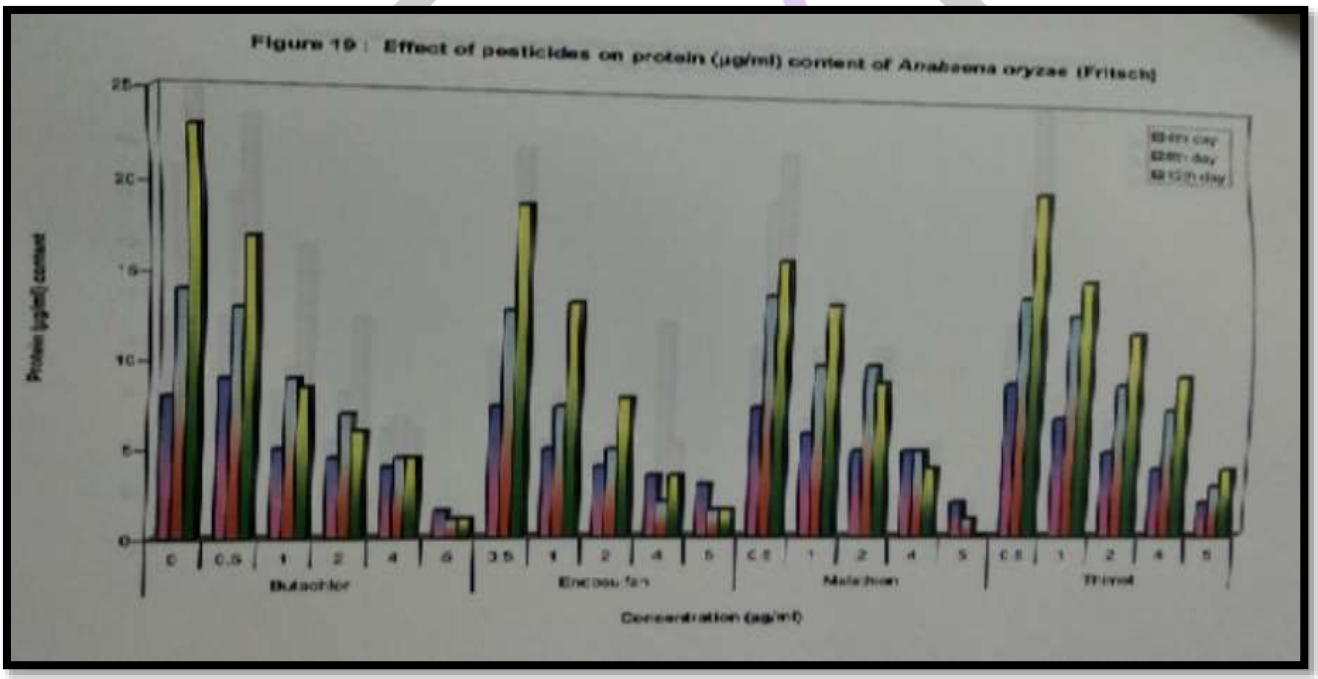


**Fig.4** Showing effect of Endosulphon on Carbohydrates content of Anabaena oryzae (Fritsch).

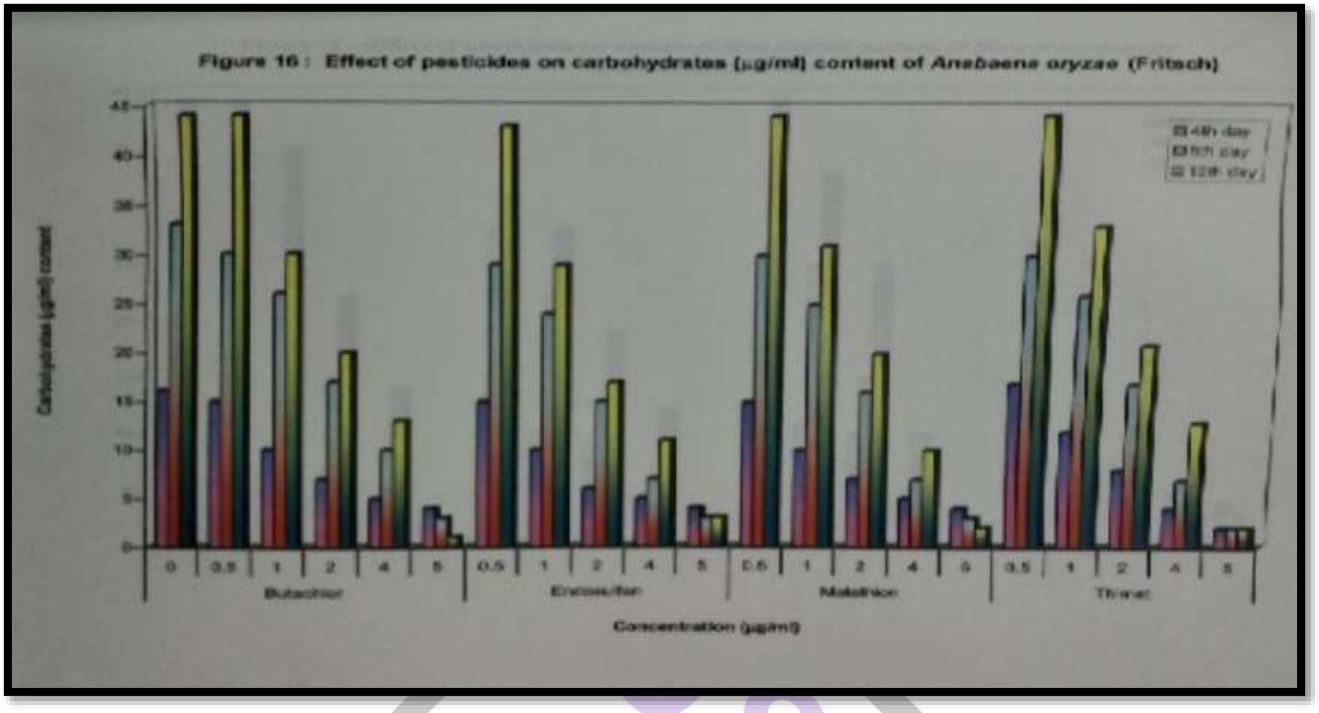



**Fig.5** Showing effect of Endosulphon on Allophycocyanin content of Anabaena oryzae (Fritsch).

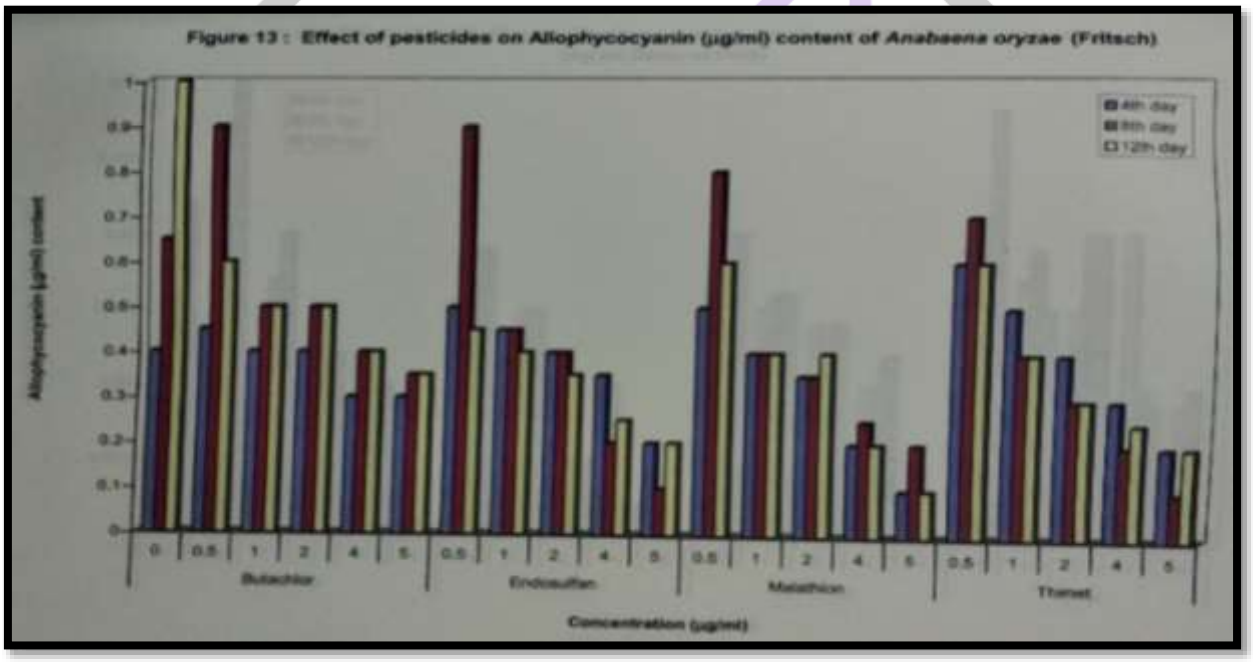


**Fig.6** Showing effect of Endosulphon on Phycoerthtrin content of Anabaena oryzae (Fritsch).

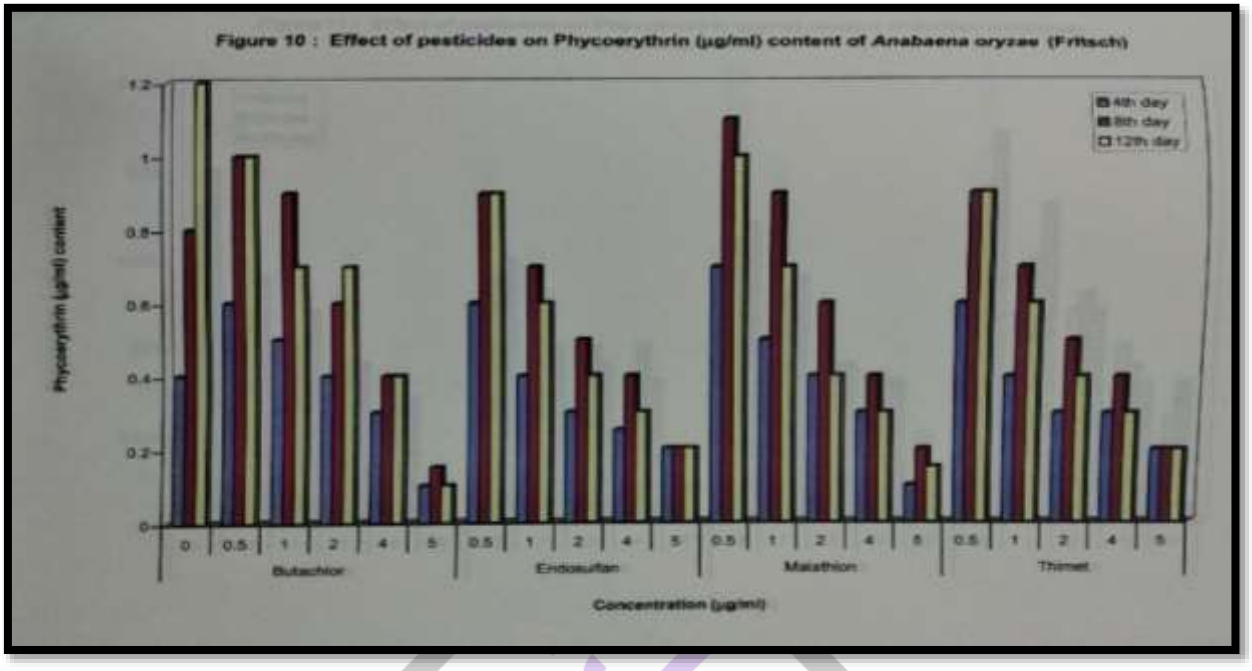



**Fig.7** Showing effect of Endosulphon on Phycoyanin content of Anabaena oryzae (Fritsch).

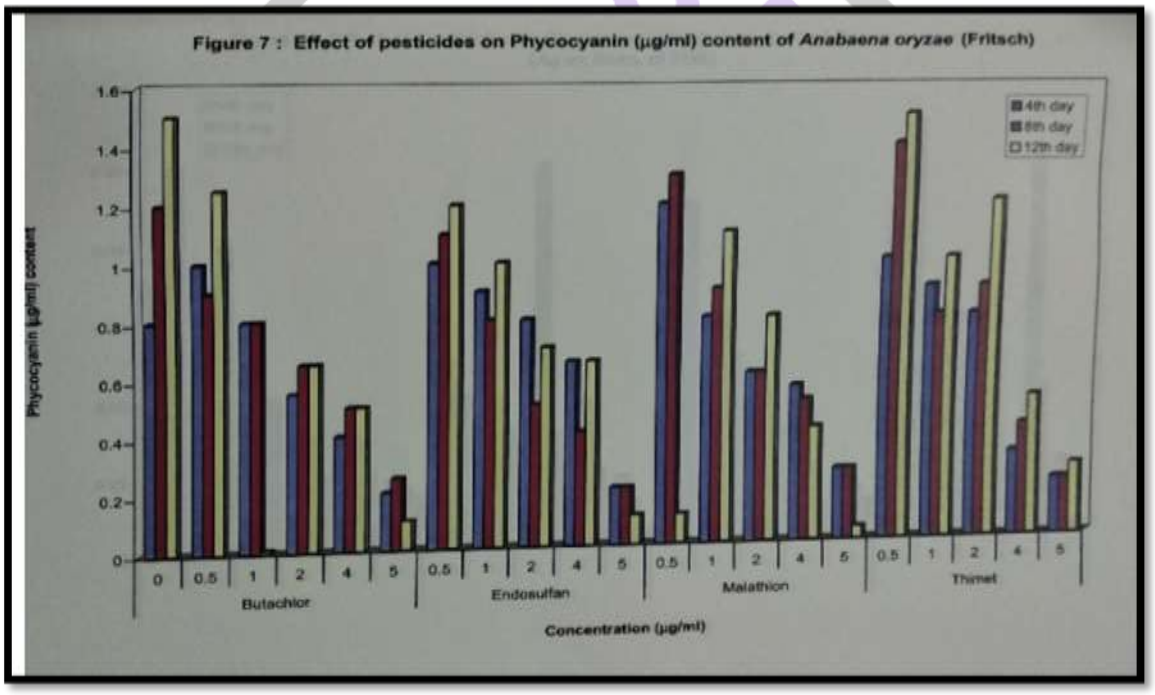


**Fig. 8 Showing effect of Endosulphon on protein content of *Anabaena oryzae* (Fritsch).**

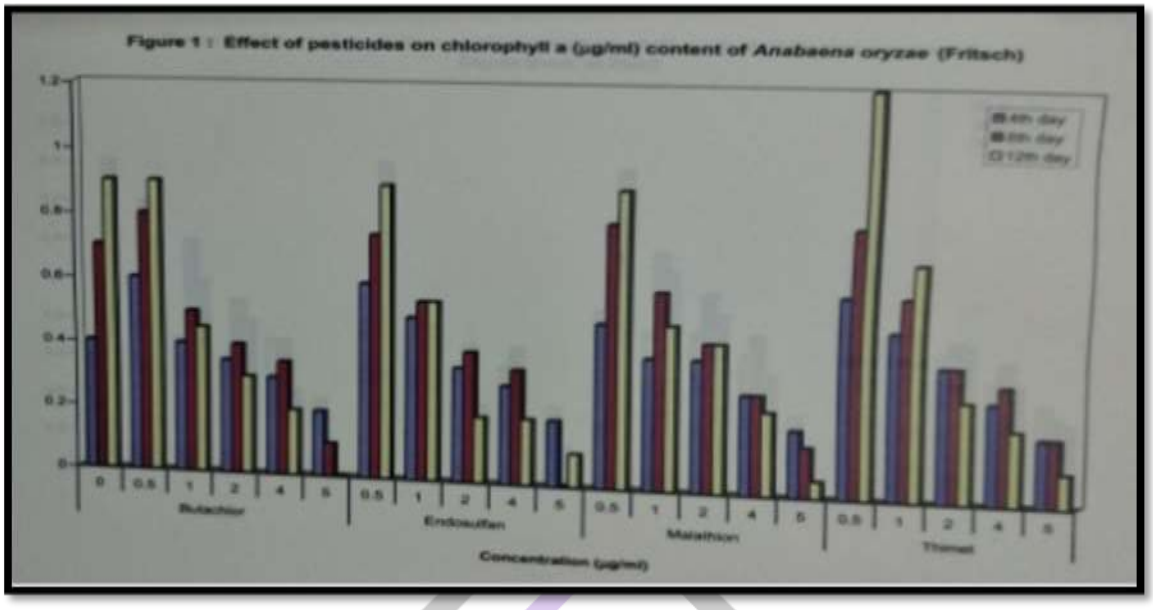
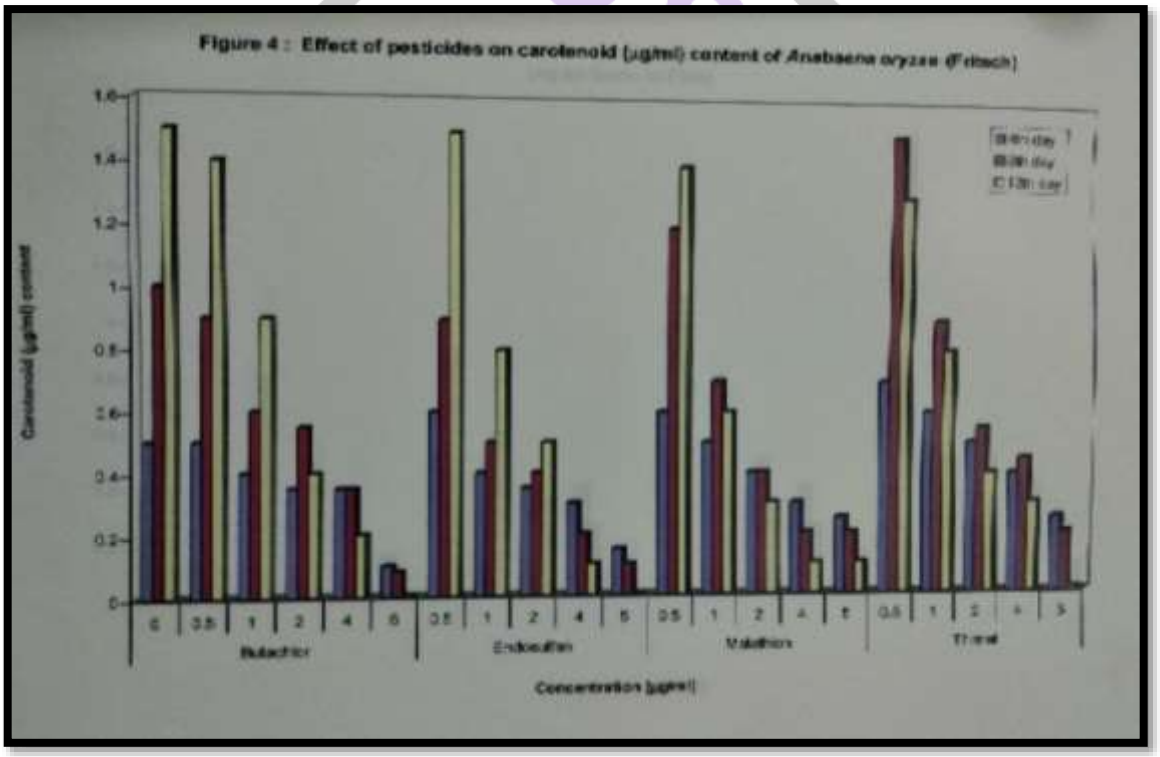



**Fig.9 Showing effect of Endosulphon on protein content of Anabaena oryzae (Fritsch).**

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