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Differential Sensitivity of Nitrogen-Fixing, Filamentous Cyanobacterial Species to an Organochlorine Insecticide - 6, 7, 8, 9, 10, 10-Hexachloro-1, 5, 5a, 6, 9, 9a-Hexahydro-6, 9-Methano-2, 4, 3-Benzodioxathiepine-3-Oxide

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***Abstract*—**Application of pesticides in the paddy fields has deleterious effects on non-target organisms including cyanobacteria which are photosynthesizing and nitrogen fixing micro-organisms contributing significantly towards soil fertility and crop yield. Pesticide contamination in the paddy fields has manifested into a serious global environmental concern. To study the effect of one such pesticide, three cyanobacterial strains; *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica* were selected for their stress responses to an Organochlorine insecticide - 6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepine-3-oxide, with reference to their photosynthesic pigments-chlorophyll-a and carotenoids as well as accessory pigments-phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin), stress induced biochemical metabolites like carbohydrates, proteins, amino acids, phenols and enzymes-nitrate reductase, glutamine synthetase and succinate dehydrogenase. All the three cyanobacterial strains were adversely affected by the insecticide doses and inhibition was dose dependent. Reduction in photosynthetic and accessory pigments, metabolites, nitrogen fixing and respiratory enzymes of the test organisms were accompanied with an initial increase in their total protein at lower Organochlorine doses. On the other hand, increased amount of phenols in all the insecticide treated concentrations was indicative of stressed activities of the organisms.

***Keywords*—**biochemical metabolites, endosulfan, enzymes, pigments

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I. Introduction

THE inherent nitrogen fixing capacity of indigenous cyanobacteria is one of the most important factors aiding in the process of biological nitrogen fixation in rice field ecosystems [1]. Due to their distinctiveness of atmospheric nitrogen fixation, these organisms form an excellent material for investigation by ecologists, physiologists, biochemists and molecular biologists. Moreover, pesticides are mainly synthetic organic compounds that are introduced into the environment to control selected pests [2]. Although pesticides are indispensable to the modern agricultural practice, the biological use of these pesticides over the years have resulted in problems caused by their interactions with the biological systems in the environment and have deleterious effects on algae by influencing soil algal growth, photosynthesis, nitrogen fixation, biochemical composition, and metabolic activities [3].

In modern days, increasing attention is given to the possible effects of pesticides on algae which play an important role in productivity and gas exchange with the biosphere [4]. It is evident that many pesticides at the recommended field application have had none or accelerating effect on growth of cyanobacteria but may affect various physiological processes like growth, pigmentation, respiration, nitrogen assimilation, carbon assimilation of cyanobacteria [5].

Metabolic responses of *Nostoc muscorum* and *Anabaena sp.310*, to herbicide N-(4-isopropylphenyl)-N, N-dimethyl urea (isoproturon) was carried out [6]. It was noticed that isoproturon inhibited many of the metabolites like growth, chlorophyll, sugar and nitrogen contents. Metabolic observations of *Nostoc muscorum* to an herbicide-fluchloralin were also studied [7]. Moreover, the inhibitory effect of Phenoxy substituted herbicide 2, 4-D on three species of cyanobacteria was also reported [8]. However, the effect of a pesticide varies from one compound to another compound on one organism to another organism. Therefore, in this perception, an attempt has been carried out to investigate differential sensitivity of Nitrogen-fixing, Filamentous

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Cyanobacterial species *Anabaena fertilissima* C.B. Rao, *Aulosira fertilissima* Ghose and *Westiellopsis prolifica* Janet to an Organochlorine Insecticide - 6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepine-3-oxide, commonly called Endosulfan, an Organochlorine insecticide, at various concentrations by assessing the pigments like Chlorophyll-a, Phycobiliproteins-Phycocyanin, Allophycocyanin, Phycoerythrin, metabolites such as Carbohydrates, Protein, Amino acids, Phenolic compounds as well as Nitrate reductase, Glutamine synthetase and Succinate dehydrogenase enzyme activities for every four days up to sixteen days.

II. Materials And Methods

Axenic cultures of *Anabaena fertilissima* Rao, *Aulosira fertilissima* Ghose and *Westiellopsis prolifica* Janet were obtained from National Facility for Blue-Green Algae, IARI, New Delhi (TABLE I). The cultures were grown photoautotrophically in nitrogen free BG_{11} medium within controlled temperatures (25±2°C) under 3000 lux light with a photoperiod of 14: 10 h [9]. Exponentially grown cyanobacterial cells were used throughout the experiment and organisms were subjected to various selected concentrations of the Organochlorine insecticide 6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepine-3-oxide based upon a set of experiments for determination of LC_{50}. Endocel (35% EC, Endosulfan manufactured by Excel Crop Care Ltd, Gujarat, India) was used for the present study. Samples were taken after every four days up to sixteen days for the determination of photosynthetic pigments (Chlorophyll-a, Carotenoids, Phycocyanin, Allophycocyanin and Phycoerythrin), metabolites (Carbohydrates, Proteins, Amino acids, Phenols) and nitrogen assimilating and respiratory enzymes (Nitrate Reductase, Glutamine Synthetase and Succinate dehydrogenase).

TABLE I
CYANOBACTERIAL SPECIES UNDER STUDY AND THEIR TAXONOMIC CLASSIFICATION

	Anabaena fertilissima C.B. Rao	***Aulosira fertilissima* Ghose**	***Westiellopsis prolifica* Janet**
Empire	Prokaryota	Prokaryota	Prokaryota
Kingdom	Bacteria	Bacteria	Bacteria
Phylum	Cyanobacteria	Cyanobacteria	Cyanobacteria
Order	Nostocales	Nostocales	Stigonematales
Family	Nostocacceae	Nostocacceae	Stigonimataceae
Genus	Anabaena	Aulosira	Westiellopsis
Scientific name	*Anabaena fertilissima* C.B. Rao	*Aulosira fertilissima* Ghose	*Westiellopsis prolifica* Janet

A. Measurement of Photosynthetic Pigments

Chlorophyll-a and Carotenoids were extracted in 80% acetone and determinedspectrophtometrically [10, 11]. Phycobyiliproteins were extracted in 50mM potassium phosphate buffer (pH 7.0) and estimated after repeated freezing and thawing [12].

B. Biochemical Analysis

The metabolites of treated cyanobacterial cultures were extracted in 80% ethanol and assayed for carbohydrates, proteins, amino acids and phenols. The anthrone method was applied for total carbohydrate [13], while total protein was measured using folin coicalteau reagent [14], total amino acids content was determined by the ninhydrin method [15] and phenols were estimated [16].

C. Enzyme Extraction and Assay

Nitrate reductase (NR) enzyme activity was measured in cysteine buffer (pH 8.8) [17]. Glutamine synthetase (GS) enzyme was extracted in 50mM ice-cold Tris HCl buffer (pH 7.5) and determined by the Mn^{2+} γ-glutamyl transferase activity with a slight modification of the method [18]. Succinate dehydrogenase (SDH) enzyme assay was estimated using Triphenyl Tetrazolium Chloride (TTC) [19].

D. Statistics

Results were tested by multivariate analysis to estimate the correlation between chlorophyll-a, carotenoids, phycocyanin, allophycocyanin, phycoerythrin, carbohydrates, proteins, amino acids, phenols, nitrate reductase, succinate dehydrogenase and glutamine synthetase where $P<0.05$ was considered as significant.

III. Results and Discussion

LC_{50} of *An.fertilissima*, *Aul.fertilissima* and *W.prolifica* for endosulfan was found to be 6, 30 and 20 μg ml^{-1} respectively after exposure to the insecticide. Based upon the LC_{50} doses, three different concentrations for each organism were selected for the study as represented in TABLE II.

TABLE II
SELECTED INSECTICIDE TREATMENTS BASED UPON DETERMINATION OF LC_{50} ON DIFFERENT CYANOBACTERIAL SPECIES

Endosulfan treatments for *An. Fertilissima*	Endosulfan treatments for *Aul. fertilissima*	Endosulfan treatments for *W. prolifica*
3 μg ml^{-1}	15 μg ml^{-1}	10 μg ml^{-1}
6 μg ml^{-1}	30 μg ml^{-1}	20 μg ml^{-1}
12 μg ml^{-1}	60 μg ml^{-1}	40 μg ml^{-1}

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A. Photosynthetic Pigments

Pigment response of all the three organisms to various concentrations of the Organochlorine insecticide has been represented in Fig. 1(a-o). Low concentration (3µg ml^{-1}) of Organochlorine insecticide treated *An.fertilissima* reduced chlorophyll-a, carotenoid and phycocyanin, allophycocyanin and phycoerythrin contents by 38, 21 and 16, 19 and 14%, respectively whereas 12 µg ml^{-1} of endosulfan sharply lowered chlorophyll-a, carotenoid and phycocyanin, allophycocyanin and phycoerythrin contents by 59, 50, 60, 65 and 29 %, respectively after 4 days of treatment. Similar observations were made while studying on the growth, photosynthesis, active oxygen species and antioxidants responses of paddy field cyanobacterium *Plectonema boryanum* to Endosulfan stress [20]. Photosynthetic and accessory pigment contents of *Aul.fertilissima* and *W.prolifica* decreased continuously with increasing Endosulfan concentrations and exposure (days). The reduction was significant at the highest Endosulfan concentration i.e. 60 µg ml^{-1} and 40 µg ml^{-1} in *Aul.fertilissima* and *W.prolifica,* respectively. The pigments such as chlorophyll-a, carotenoids, phycocyanin, allophycocyanin and phycoerythrin reduced by 94, 90, 30, 75 and 65% respectively by 16 days in *Aul.fertilissima*. Similarly, the percentage reduction of chlorophyll-a, carotenoids, phycocyanin, allophycocyanin and phycoerythrin were registered by 95, 85, 92, 81 and 86% respectively in *W. prolifica* after 16 days treatment. It was suggested that decrease in chlorophyll-a, carotenoid and phycobiliprotein contents might be ascribed 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 [21].

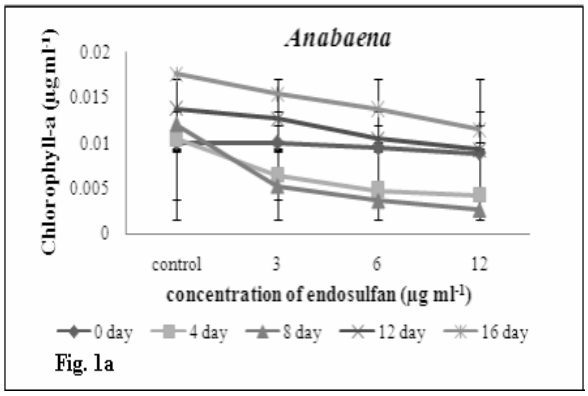


Fig. 1(a) Concentration (µgml^{-1}) of Chl-a in *An.fertilissima* at different doses of Endosulfan

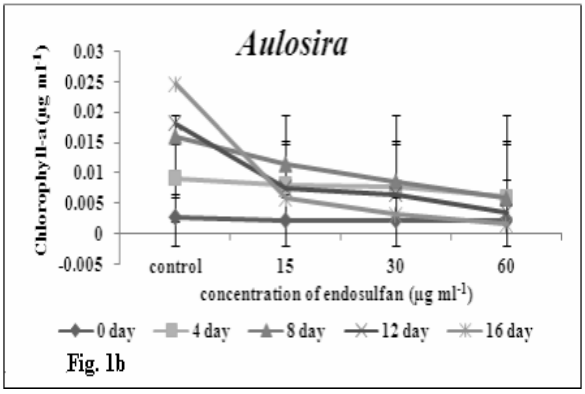


Fig. 1(b) Concentration (µgml^{-1}) of Chl-a in *Aul.fertilissima* at different doses of Endosulfan.

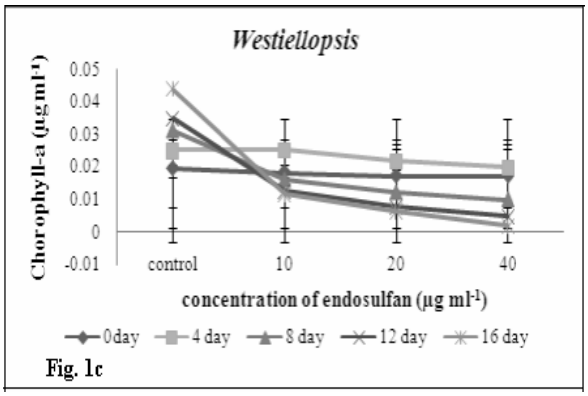


Fig. 1(c) Concentration (µgml^{-1}) of Chl-a in *W.prolifica* at different doses of Endosulfan

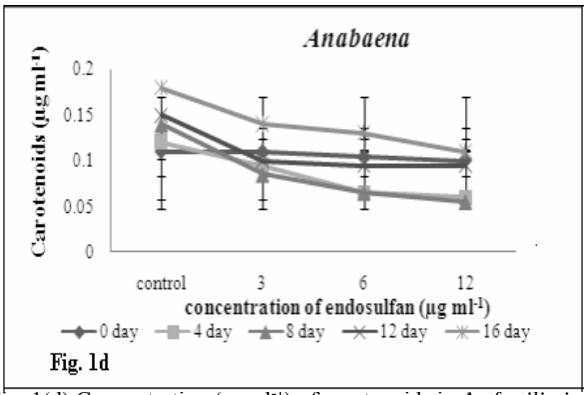


Fig. 1(d) Concentration (µgml^{-1}) of carotenoids in *An.fertilissima* at different doses of Endosulfan

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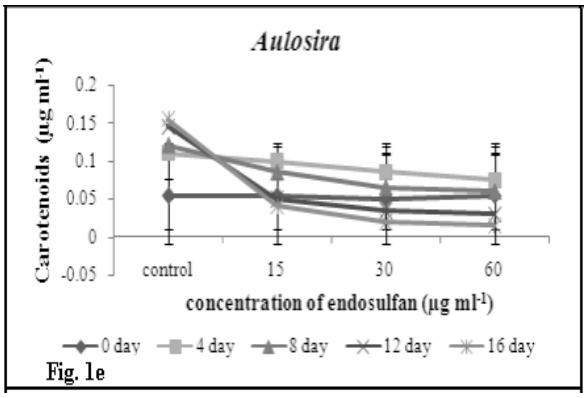


Fig. 1(e) Concentration (µgml^{-1}) of carotenoids in *Aul.fertilissima* at different doses of Endosulfan

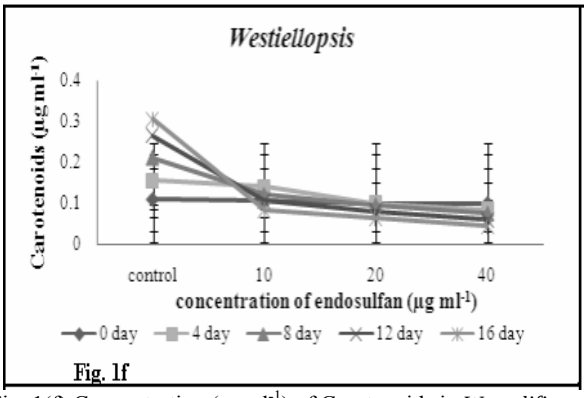


Fig. 1(f) Concentration (µgml^{-1}) of Carotenoids in *W.prolifica* at different doses of Endosulfan

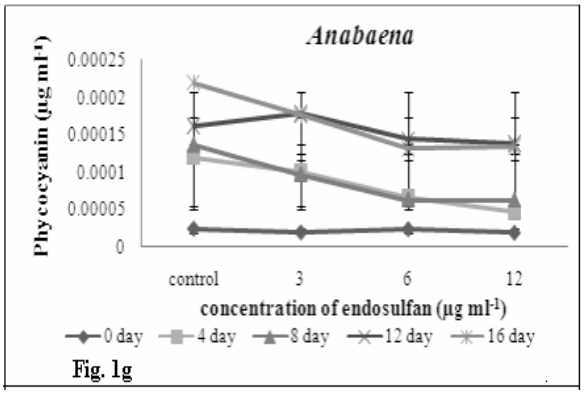


Fig. 1(g) Concentration (µgml^{-1}) of Phycocyanin in *An.fertilissima* at different doses of Endosulfan

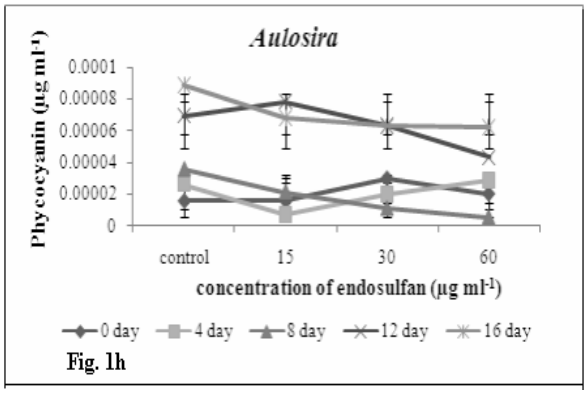


Fig. 1(h) Concentration (µgml^{-1}) of Phycocyanin in Aul.*fertilissima* at different doses of Endosulfan

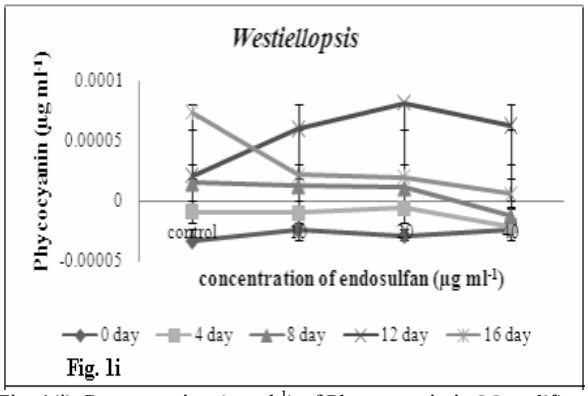


Fig. 1(i) Concentration (µgml^{-1}) of Phycocyanin in *W.prolifica* at different doses of Endosulfan

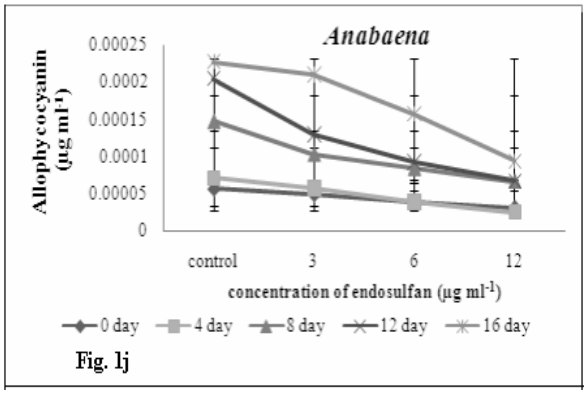


Fig. 1(j) Concentration (µgml^{-1}) of Allophycocyanin in *An.fertilissima* at different doses of Endosulfan

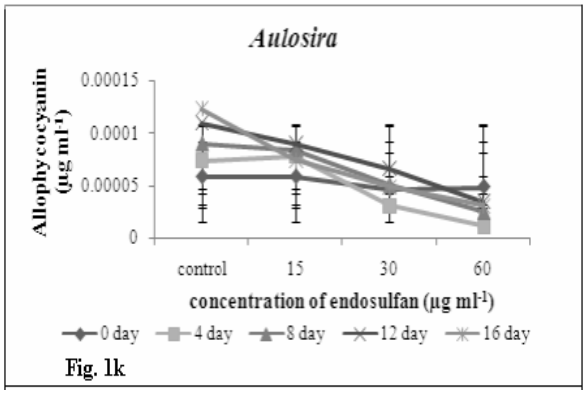


Fig. 1(k) Concentration (µgml^{-1}) of Allophycocyanin in *Aul.fertilissima* at different doses of Endosulfan

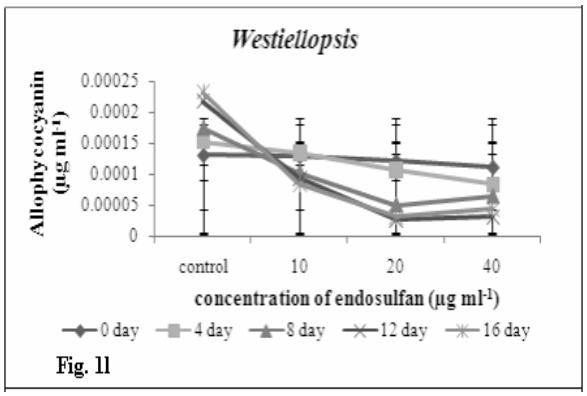


Fig. 1(l) Concentration (µgml^{-1}) of Allophycocyanin in *W.prolifica* at different doses of Endosulfan

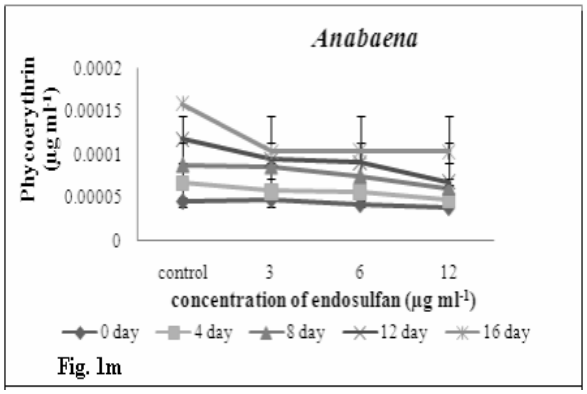


Fig. 1(m) Concentration (µgml^{-1}) of Phycoerythrin in *An.fertilissima* at different doses of Endosulfan

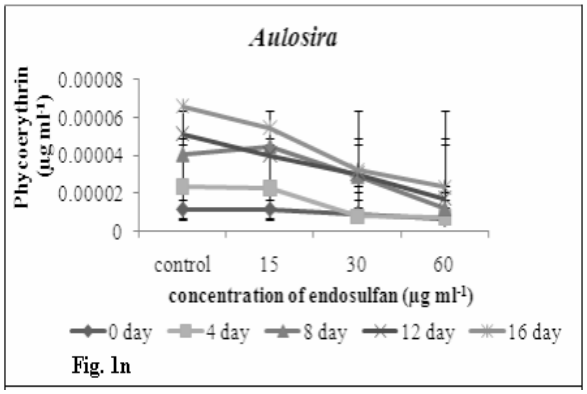


Fig. 1(n) Concentration (µgml^{-1}) of Phycoerythrin in *Aul.fertilissima* at different doses of Endosulfan

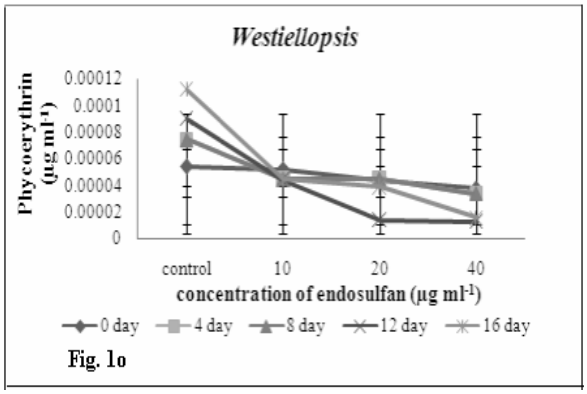


Fig. 1(o) Concentration (µgml^{-1}) of Phycoerythrin-a in *W.prolifica* at different doses of Endosulfan

B. Biochemical Metabolites

Carbohydrates are polyhydroxy aldehydes or ketones or substances that yield such components on hydrolysis. The retardation of carbohydrate content might be due to the interference of chemicals with the photosynthesis process [22]. Significant reduction in carbohydrates of *An.fertilissima*, *Aul.fertilissima* and *W.prolifica* was observed with increasing concentrations of the Organochlorine insecticide (Fig. 2a-c). Upon raising the concentration of insecticide the carbohydrate content diminished by 42, 97and 97% respectively after 16 days, depicting a concentration-dependent inhibition of growth. Similar observations were quoted while studying on Endosulfan induced biochemical changes in nitrogen-fixing cyanobacteria (*Aulosira fertilissima, Anabaena variabilis and Nostoc muscorum*) [23].

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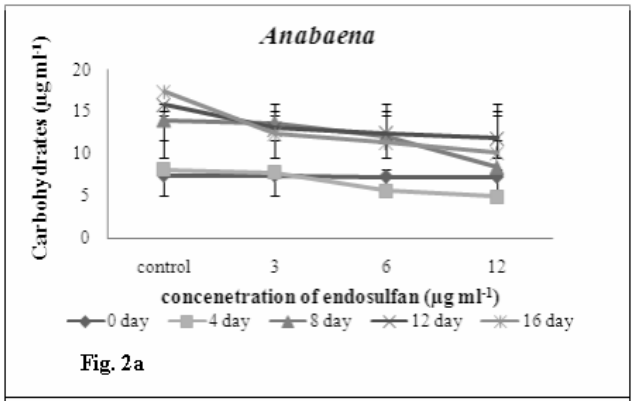


Fig. 2 (a) Concentration (µgml^{-1}) of Carbohydrates in *An.fertilissima* at different doses of Endosulfan

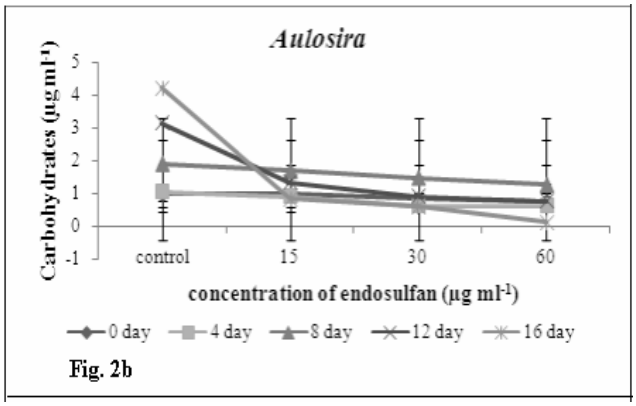


Fig. 2 (b) Concentration (µgml^{-1}) of Carbohydrates in, *Aul.fertilissima* at different doses of Endosulfan

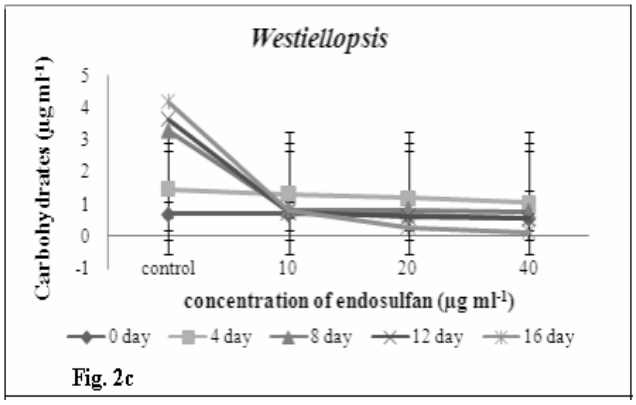


Fig. 2 (c) Concentration (µgml^{-1}) of Carbohydrates in *W.prolifica* at different doses of Endosulfan

Proteins can be defined as macromolecules composed of one or more polypeptide chains each with a characteristic sequence of amino acids linked by peptide bonds which are are usually deposited in the cytoplasm after being synthesized. Endosulfan suppressed the total protein content of all three organisms in comparison to control values (Fig. 2d-f). After 16 days of treatment, 3, 6 and 12 µg ml^{-1} of Endosulfan diminished the protein content of cyanobacterium *An.fertilissima* by 26, 52 and 85%, respectively which the results were confirmed with the findings.[24] Although, initial protein levels rose during 4 and 8 days was recorded in the other two test species *Aul.fertilissima* and *W.prolifica*, respectively in response to lower concentrations of the insecticide, which was fallen consistently by 89% and 90% at 60 µg ml^{-1} and 40 µg ml^{-1} of the insecticide after 16 days. Similar observations were recorded during the investigation of the toxic response of three species of cyanobacteria to substituted Phenoxy herbicide 2, 4-D also recorded similar observations [8].

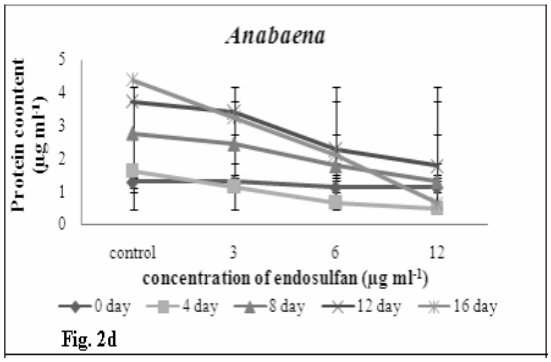


Fig. 2 (d) Concentration (µgml^{-1}) of Proteins in *An.fertilissima* at different doses of Endosulfan

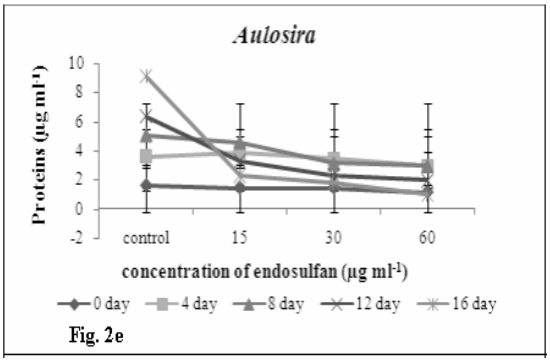


Fig. 2 (e) Concentration (µgml^{-1}) of Proteins in *Aul.fertilissima* at different doses of Endosulfan

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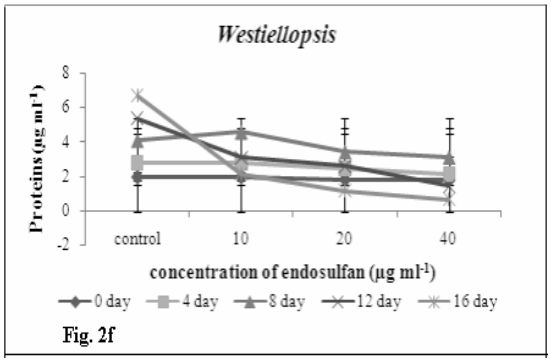


Fig. 2 (f) Concentration (µgml^{-1}) of Protiens in *W.prolifica* at different doses of Endosulfan

A consistent decrease in the Amino acids content of the selected organisms was registered in response to the treated concentrations of the insecticide (Fig. 2g-i). Reduction in Amino acids of *An.fertilissima*, *Aul.fertilissima* and *W.prolifica* with respect to highest concentrations of insecticide was recorded by 83, 80 and 90% respectively after 16 days. Changes in amino acid concentration may be due to synthesis from endogenous precursors or to inhibition of normal catabolism [25].

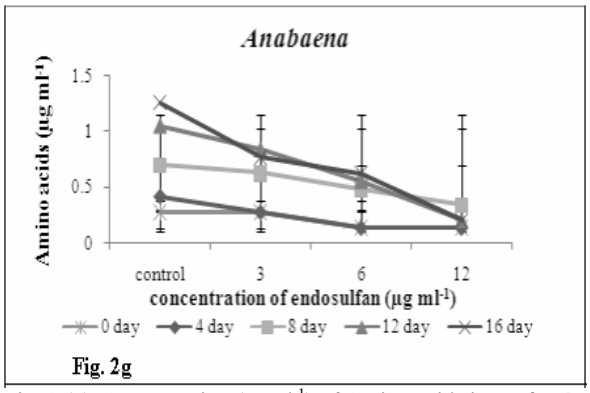


Fig. 2 (g) Concentration (µgml^{-1}) of Amino acids in *An.fertilissima* at different doses of Endosulfan

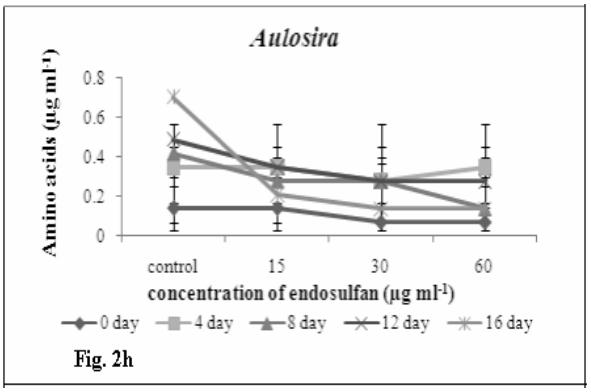


Fig. 2 (h) Concentration (µgml^{-1}) of Amino acids in *Aul.fertilissima* at different doses of Endosulfan

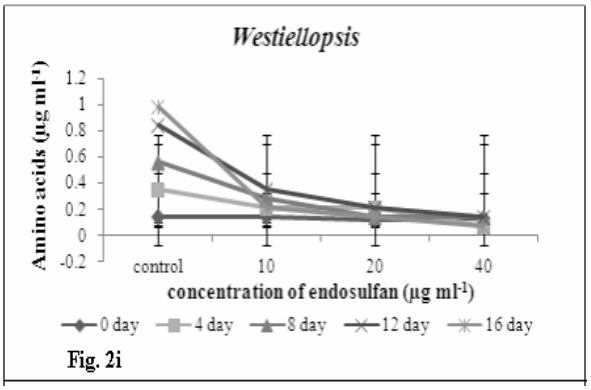


Fig. 2 (i) Concentration (µgml^{-1}) of Amino acids in *W.prolifica* at different doses of Endosulfan

Phenols are aromatic molecules which consist of a hydroxide group, widespread in photosynthetic organisms, which are produced during the stress conditions. Phenol content in respective to treated cells of *An.fertilissima* as compared to the control enhanced until 8th day (Fig. 2j). However, from 12th day onwards, phenol level reduced with respect to higher concentrations of the insecticide as well as exposure periods. Phenol content of *An.fertilissima* was registered to decrease by 92% when treated to 12 µg ml^{-1} after 16 days. Contrary to *An.fertilissima*, phenol content in the other two species, *Aul.fertilissima* and *W.prolifica* exhibited a relative increase by 59 and 3% respectively in response to 60 µg ml^{-1} and 40 µg ml^{-1} concentrations of the insecticide respectively (Fig. 2k-l). It was affirmed that the primary oxidative pathway for metabolism of the insecticide parathion involved an initial hydrolysis to yield diethylthiosphosphoric acid and p-nitrophenol [26]. [27] suggested that all types of toxic stresses 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.

Open Science Index, Biotechnology and Bioengineering Vol:5, No:3, 2011 publications.waset.org/1309/pdf

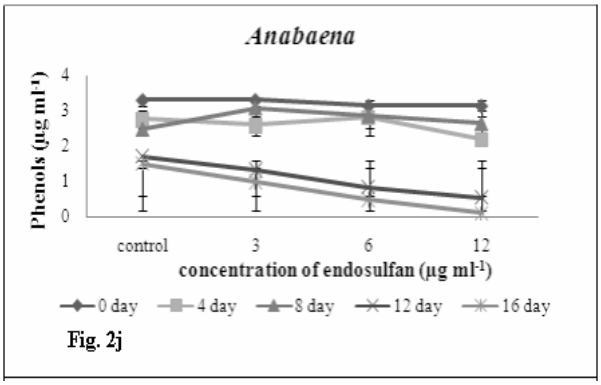


Fig. 2 (j) Concentration (µgml^{-1}) of phenol in *An.fertilissima* at different doses of Endosulfan

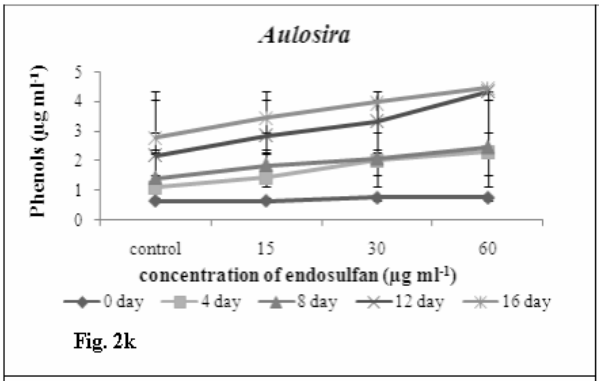


Fig. 2 (k) Concentration (µgml^{-1}) of Phenol in *Aul.fertilissima* at different doses of Endosulfan

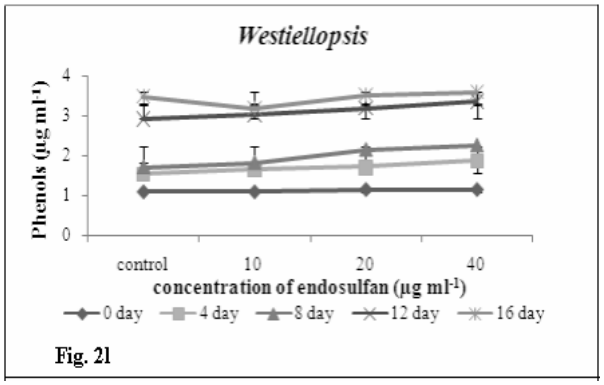


Fig. 2 (l) Concentration (µgml^{-1}) of Phenol in *W.prolifica* at different doses of Endosulfan

C. Enzymes

Nitrate reductase (NR) enzyme activity in all treated cyanobacterial species *An.fertilissima, Aul.fertilissima* and *W.prolifica* expressed a significant decline and reduced by 77, 90 and 95% respectively to the higher concentration after 16 days which corroborated with the findings who studied the effect of carbamate insecticide Sevin on the growth, survival and nitrogen fixation of *Anabaena spp*. and *W. Prolifica* [28].

Ammonia assimilating enzyme – Glutamine Synthetase (GS) enzyme activity was shown a sharp reduction by 75, 89 and 90% in *An.fertilissima*, *Aul.fertilissima* and *W.prolifica* respectively after 16th day. A marked diminution in glutamine synthetase activity of *Tolypothrix scytonemoides* in response to pesticides Bavistin, Monocrotophos and Nimbicidin was also noted [29].

Succinate dehydrogenase (SDH), an enzyme involved in the conversion of succinate to fumarate, plays a vital role in the respiration of an organism. Enzymatic activities of three selected organisms in response to Organochlorine insecticide have been represented in figures 3(a-i).The enzyme was present in the thylakoid of the cyanobacterium and observed to be highly sensitive to the insecticide, endosulfan and reduced by 95, 82 and 95% in *An.fertilissima*, *Aul.fertilissima* and *W.prolifica* respectively. Similarly, 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 [30].

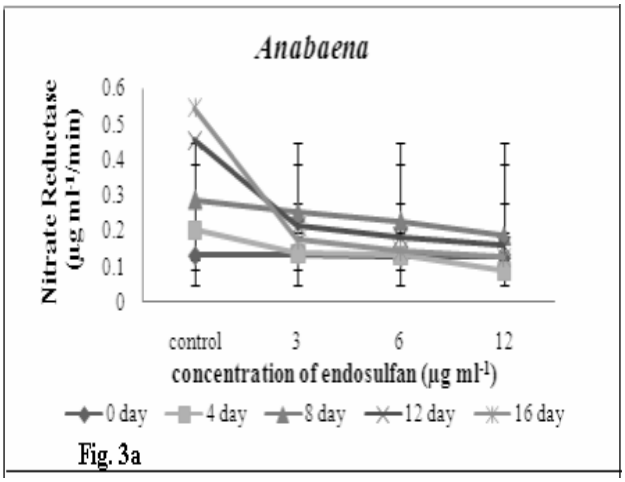


Fig. 3 (a) Nitrate reductase enzyme activity (µgml^{-1}/min) in *An.fertilissima* at different doses of Endosulfan

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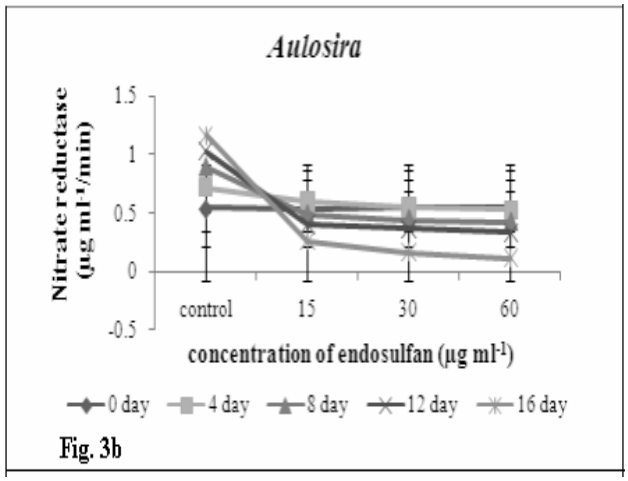


Fig. 3 (b) Nitrate reductase enzyme activity (µgml^{-1}/min) in *Aul.fertilissima* at different doses of Endosulfan

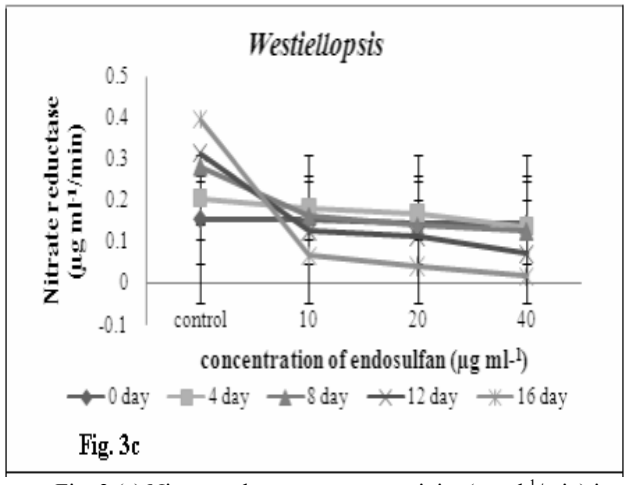


Fig. 3 (c) Nitrate reductase enzyme activity (µgml^{-1}/min) in Wes.*prolifica* at different doses of Endosulfan

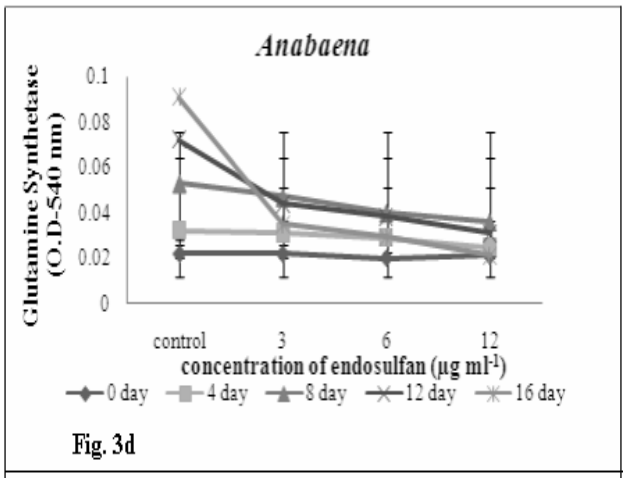


Fig. 3 (d) Glutamine synthetase enzyme activity in *An.fertilissima* at different doses of Endosulfan

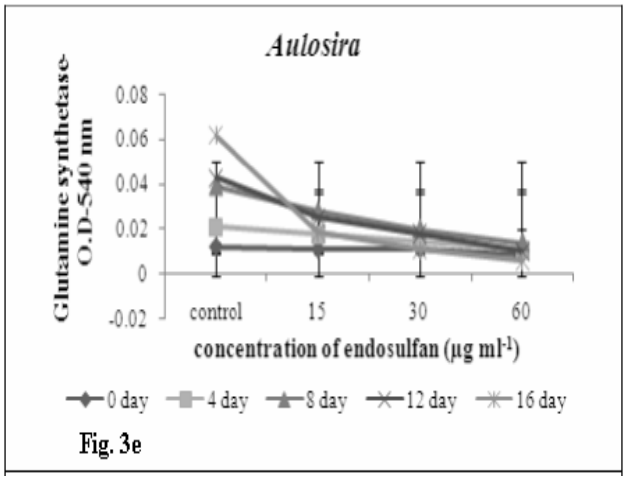


Fig. 3 (e) Glutamine synthetase enzyme activity in *Aul.fertilissima* at different doses of Endosulfan

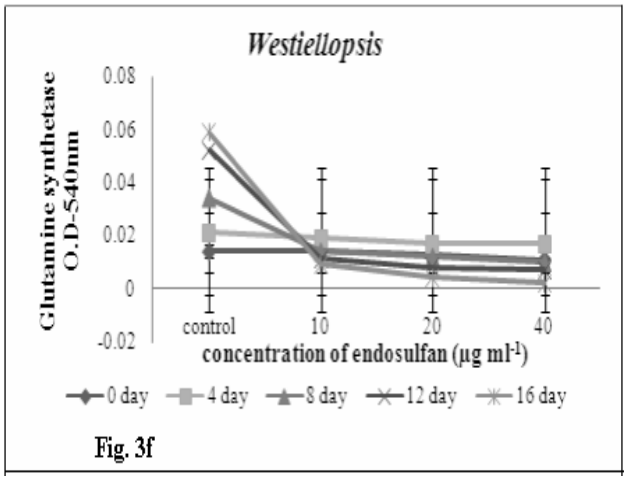


Fig. 3 (f) Glutamine synthetase enzyme activity in *W. prolifica* at different doses of Endosulfan

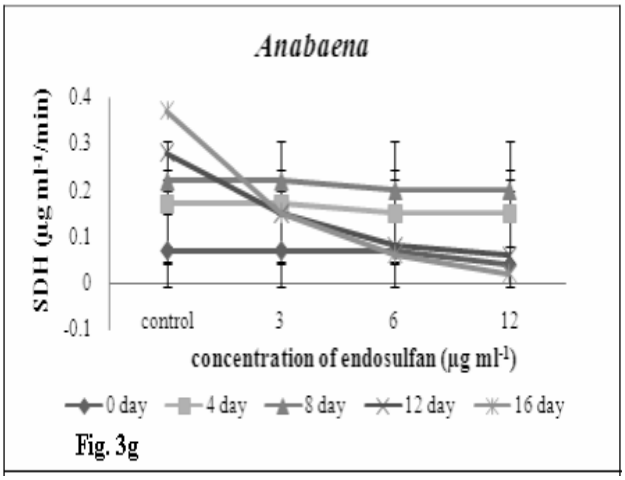


Fig. 3 (g) Sucinate dehydrogenase enzyme activity (µgml^{-1}/min) in *An.fertilissima* at different doses of Endosulfan

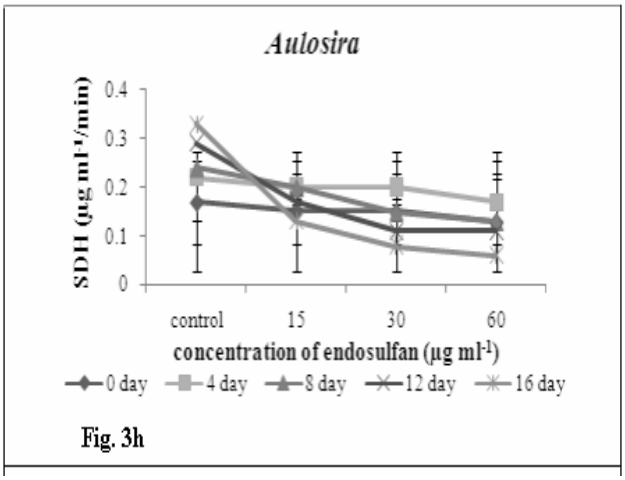


Fig. 3 (h) Succinate dehydrogenase enzyme activity (µgml^{-1}/min) in *Aul.fertilissima* at different doses of Endosulfan

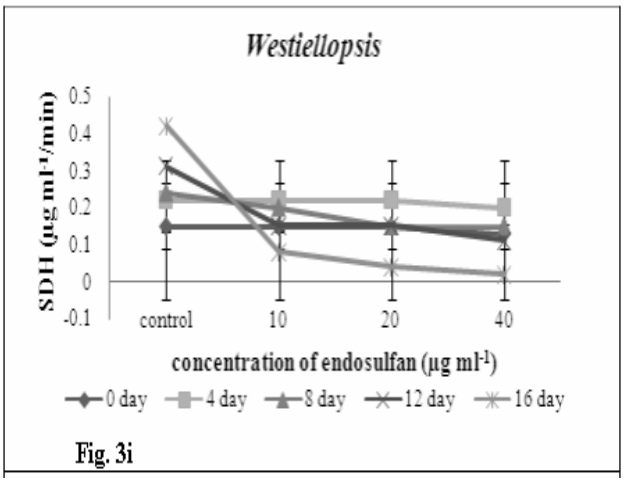


Fig. 3 (i) Succinate dehydrogenase enzyme activity (µgml^{-1}/min) in *W.prolifica*at different doses of Endosulfan

D. Statistical Analysis

Significant positive correlations (r = 0.338 to 0.654, r = 0.187 to 0.792, r = 0.021 to 0.688) were found between chlorophyll-a, carotenoids, phycocyanin, allophycocyanin, phycoerythrin, carbohydrates, proteins, amino acids, phenols, nitrate reductase, succinate dehydrogenase and glutamine synthetase of *An.fertilissima, Aul.fertilissima* and *W. prolifica* respectively treated with the Organochlorine insecticide doses.

IV. Conclusion

6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepine-3-oxide (endosulfan); a widely used insecticide in the paddy fields is indeed inhibitory to the useful Cyanobacteria. It was recorded that endosulfan not only inhibited the photosynthetic and accessory pigments but also affected metabolic activities like Carbohydrates, Proteins, Amino acids, Phenols as well as enzymes such as Nitrate reductase, Glutamine synthetase and Succinate dehydrogenase at doses of 3, 6 and 12 µg ml^{-1}, 15, 30 and 60 µg ml^{-1} and 10, 20 and 40 µg ml^{-1} for *An. fertilissima, Aul. fertilissima* and *W. prolifica* respectively.

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