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ORIGINAL ARTICLE

Photosynthetic, biochemical and enzymatic investigation of *Anabaena fertilissima* in response to an insecticide-hexachloro-hexahydro-methano-benzodioxathiepine-oxide.

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A study on the heterocystous, nitrogen fixing cyanobacterium, *Anabaena fertilissima* was carried out to investigate the effect of an organochlorine insecticide (hexachloro-hexahydro-methano-benzodioxathiepine-oxide, called as endosulfan) at different concentrations of 3, 6 and 12 μgml^{-1} on the photosynthetic pigments-Chl-*a*, Carotenoids and Phycobiliproteins-phycocyanin, allophycocyanin and phycoerythrin, stress metabolites such as carbohydrates, proteins, amino acids, phenols and enzyme activities-nitrate reductase and glutamine synthetase. The insecticide- Endosulfan showed to be deleteriously affecting the activities in the cyanobacterium. As early as the 4th day, chl-*a* and carotenoids reduced by 38% and 20% respectively. The phycobiliproteins 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, phenols and amino acids and enzymes-nitrate reductase and glutamine synthetase. Despite of deleterious effects of Endosulfan on the cyanobacterium *Anabaena fertilissima,* a unique regenerating ability in presence of the insecticide was observed by the end of 12 days in the lower doses of insecticide.

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Cyanobacteria are photosynthesizing and nitrogen fixing micro-organisms, which contribute significantly towards soil fertility and crop yield (Jha et al. 1999). Most paddy soils have a natural population of cyanobacteria which provides a potential source of nitrogen fixation at no or low cost (Mishra and Pabby, 2004). A large number of pesticides are used in rice fields to protect the rice seedlings and crops, and selectively destroy the pests, but the indiscriminate use of pesticides causes great

danger to the rice field cyanobacteria and other beneficial micro-organisms (Da Silva et al., 1975). Under water logged conditions as commonly observed in rice fields, pesticides may induce many cellular disorders in cyanobacteria (Singh et al., 1986). Depending on the type and concentration of pesticides and composition of growth media, pesticides have been found to exert stimulatory, inhibitory or no effect on the growth and nitrogen fixing ability by cyanobacteria (Tamilselvam et al. 2002).

The toxicity of BHC, Lindane and Endrin towards nitrogen fixing cyanobacteria- *Cylindrospermum* sp., *Aulosira fertilissima* and *Plectonema boryanum* have been studied (Lakshmi and Annamalai, 2007). Study on the pesticides like Dimecron-100, Ekaluk-EC and Dithane revealed that they were more toxic at higher concentrations but could be noticed lesser toxic at lower concentrations to heterocystous cyanobacteria (Anand and Veerappan, 1980). It has been also studied that insecticide; carbofuran reduced the growth of cyanobacteria *Oscillatoria* sp., and *Westiellopsis prolifica* considerably (Ravindran et al., 2000).

A hexa-chlorinated insecticide, Endosulfan has a broad spectrum of activity and is widely used on different crops such as rice (Goebel *et al,* 1982). Owing to an extensive usage of Endosulfan in various tropical and subtropical countries to control the insect population, it also declines the growth of microflora including cyanobacteria considerably (Shetty et al., 2000). Present study investigates the effect of Endosulfan insecticide on the pigment contents (Chl-*a*, Carotenoids and Phycobiliproteins-phycocyanin, allophycocyanin and phycoerythrin), metabolites (carbohydrates, proteins, phenols, amino acids) and enzymes (nitrate reductase and glutamine synthetase) of the cyanobacterium *Anabaena fertilissima*.

Materials and methods:

Organism and culture conditions: for the present study, filamentous, nitrogen-fixing, heterocystous cyanobacterium *Anabaena fertilissima* was used and raised to axenic culture. The culture was grown in BG-11 medium (pH 7.5) in the culture room at 25±2°C under 3000 lux light with a photoperiod of 14: 10 h. Exponentially grown cyanobacterial cells were used throughout the experiment. Each experiment was conducted in replicates of three and their ±SE values were calculated.

Endosulfan treatment: Endocel (35% EC, Endosulfan (6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepine-3-oxide), an organochlorine insecticide manufactured by Excel Crop Care Ltd, Gujarat, India, was used for the present study. On the basis of a series of experiments for LC_{50}, the effective doses of 3, 6 and 12 µg ml^{-1} of Endosulfan were selected. Various concentrations (0, 3, 6 and 12 µg ml^{-1}) of Endosulfan were prepared from the stock solution (200 µg ml^{-1}) by dissolving it into the sterilized nutrient medium. Exponentially growing 2 ml of the culture was inoculated to each effective dose and made up to 20.0 ml. For each experiment, the solution of Endosulfan was freshly prepared.

Photosynthetic, biochemical and enzymatic response of *Anabaena fertilissima* to Endosulfan was studied at an interval of every four days up to sixteen days. At the end of every four days, the treated as well as untreated cultures were assayed for various characters including photosynthetic pigments-chlorophyll-*a*, carotenoids and phycobiliproteins-phycocyanin, allophycocyanin and phycoerythrin, metabolites like carbohydrates, proteins, amino acids, phenols and also enzyme-nitrate reductase and glutamine synthetase. Analytical grade (Merck Ltd, and Himedia Ltd, India) chemicals were used throughout the study.

Photosynthetic pigments measurement: Chlorophyll *a* and carotenoids were extracted in 80% acetone and determined spectrophotometrically by measuring the absorbance at 663, 630nm and 480 nm, respectively (Jeffrey and Humphrey, 1975; Parsons and Strickland, 1963). Phycobilin content was

extracted in 50mM potassium phosphate buffer (pH 7.0) after repeated freezing and thawing and measured at 562, 615 and 652 nm, respectively (Bennett and Bogorad, 1973).

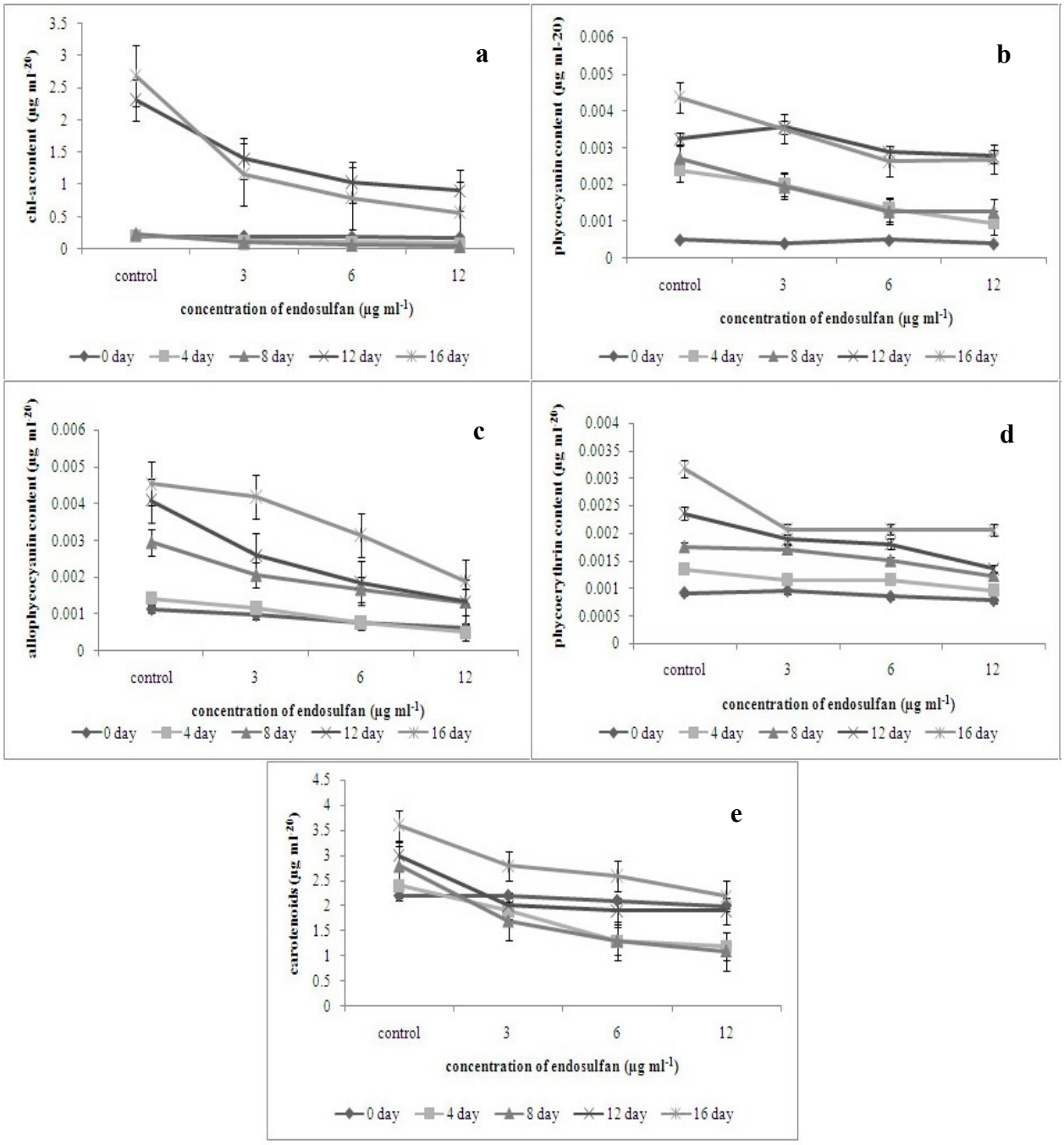

Fig 1 shows the photosynthetic response of *Anabaena fertilissima* to the insecticide Endosulfan: (a) Chl-*a*, (b) phycocyanin, (c) allophycocyanin,(d) phycoerythrin, (e) carotenoids,

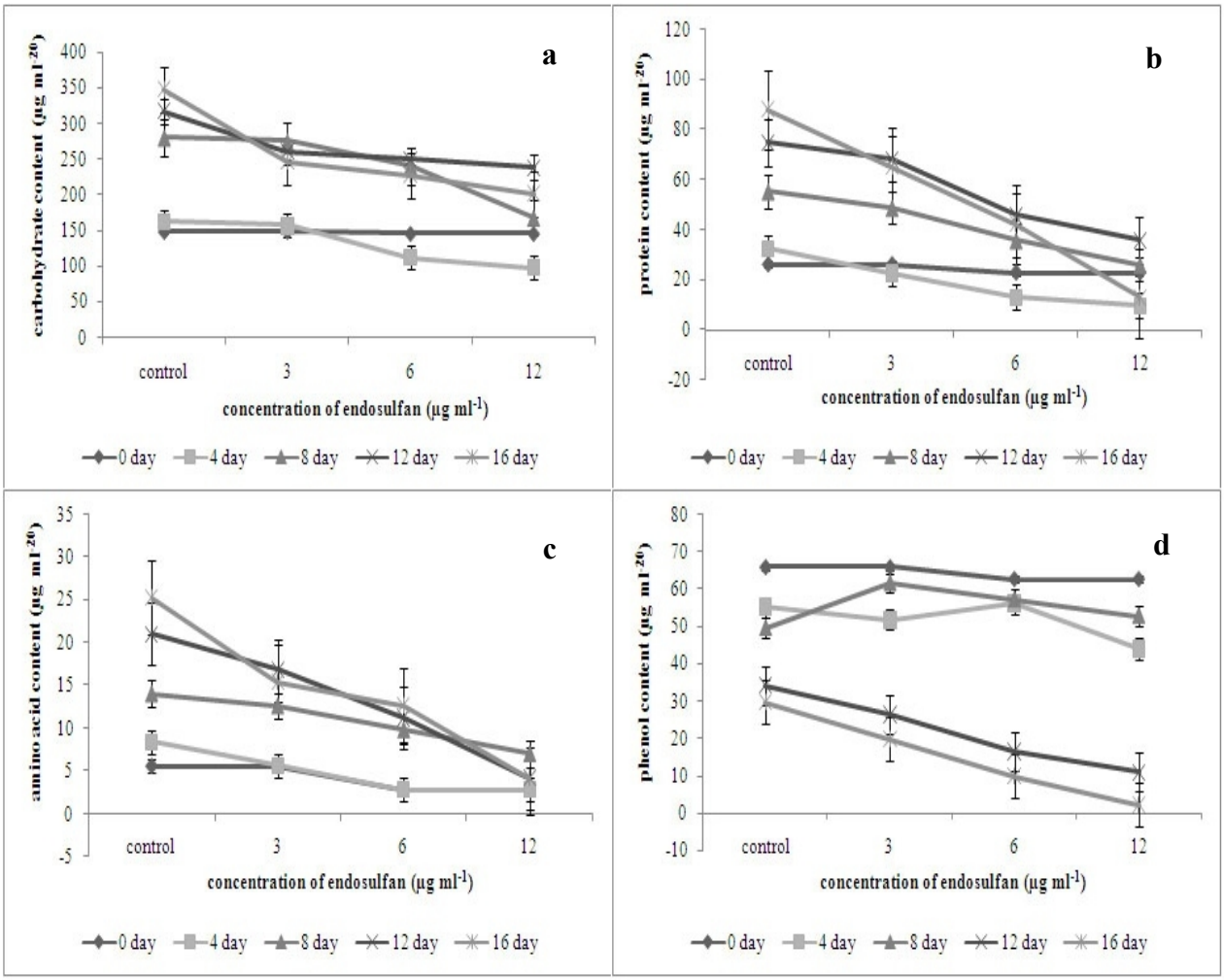

Fig: 2 shows the metabolic response of *Anabaena fertilissima* to the insecticide Endosulfan: (a) carbohydrates, (b) proteins, (c) amino acids, (d) phenols

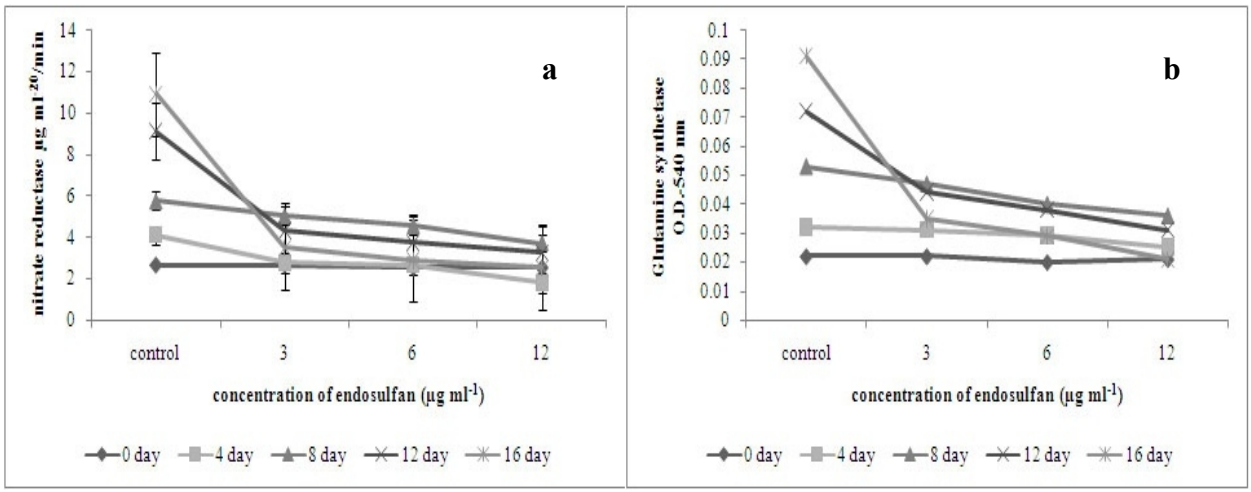

Fig: 3 shows the enzymatic response of *Anabaena fertilissima* to the insecticide Endosulfan: (a) nitrate reductase, (b) glutamine synthetase

Biochemical estimation: The total carbohydrates, proteins, amino acids and phenols were extracted in 80% ethanol. Carbohydrates were estimated by the method of Hedge and Hofreitte (1991) using sucrose as a standard. Protein estimation was done by Lowry *et al* (1951) with Bovine Serum Albumin (BSA) as the standard. The total amino acids were measured using ninhydrin reagent (Lee and Takahanshin, 1966). Phenols estimation has been carried out as per Malick and Singh (1980).

Enzyme assays: Nitrate reductase enzyme was extracted using cysteine buffer (pH 8.8). The enzyme activity was assayed spectrophotometrically following the protocol given by Sempruch et al, (2008). Glutamine synthetase enzyme was extracted in Tris HCl buffer (pH 7.5). The enzyme assay was determined by the Mn^{2+} γ-glutamyl transferase activity and estimated by a slight modification of the method described by Yuan et al, (2001). The end product γ-glutamyl hydroxamate formed was measured spectrophotometrically at 540 nm and represented in optical density readings.

Results and discussion:

Pigments:

The cyanobacterium *Anabaena fertilissima* showed inhibitory growth response against the insecticide endosulfan. The inhibitory effect of endosulfan on photosynthetic pigments of *A. fertilissima* was found to be dose dependent (Figures 1a-e). Within 4 days of treatment a low concentration (3 µg ml^{-1}) of insecticide reduced Chl *a* and carotenoid contents by 38% and 20% respectively. Moreover, the phcobiliproteins also were found to decrease significantly with respect to increasing endosulfan concentrations as well as an increase in days of exposure The lowest concentration of 3 µg ml^{-1} reduced the Phycocyanin, Allocphycocyanin and Phycoerythrin content by 16, 18 and 14 % respectively. The declining trend in the pigment contents continued with the rising concentration of insecticide as 12 µg ml^{-1} of endosulfan sharply lowered chlorophyll *a* and carotenoid contents by 58% and 50% respectively. Phycocyanin, Allocphycocyanin and Phycoerythrin also reduced significantly by 60%, 64% and 28% respectively. Such decrease in chlorophyll *a*, carotenoid and phycobilin contents may be ascribed to the inhibition of pigment synthesis directly by the insecticide or accelerated degradation of pigments. However, by the end of the 12th day the cultures began to show signs of a change in the colour turning from yellow to green. Moreover, the observation was further supported by pigment analysis where 12 µg ml^{-1} of insecticide after 12 days of exposure exhibited less reduction in Chl-*a* and carotenoid content (32% and 36 % respectively), thus showing a significant recovery in the levels of chlorophyll *a* and carotenoids. Prasad et al (2005) also quoted similar observations while studying on the growth, photosynthesis, active oxygen species and antioxidants responses of paddy field cyanobacterium *Plectonema boryanum* to Endosulfan stress.

Metabolic changes:

Total carbohydrate content showed drastic reduction with increasing concentrations of Endosulfan (fig. 2a). The reduced carbohydrate levels ranged from 3% to 39% by the end of the fourth day when exposed to 3, 6 and 12 µg ml^{-1} of the insecticide. Towards the end of 16 days of endosulfan exposure, carbohydrate levels dropped down to 41% when treated with 12 µg $ml^{-1}$of the insecticide. These results have been well corroborated with those of Kumar et al, (2008). The retardation in the carbohydrate content might be due to interference of chemicals with photosynthesis process (Padhy, 1980).

Protein content also exhibited decreasing trend with increasing exposure days and Endosulfan concentration (Fig. 2b). The protein content reduced as low as 85% by the end of 16 days when treated with 12 µg $ml^{-1}$of endosulfan. The results were

further confirmed by Shehata et al (2001). Thiel (1990) emphasized that decrease in protein content in *Anabaena variabilis* was observed in starved cells.

Similarly, Amino acid content reflected a significant increase in the percent reduction with respect to increasing concentrations and days of exposure. Amino acid percent reduction increased consistently ranging from 10% to 83% with increasing endosulfan concentration and exposure time (days) (Fig. 2c). Soliman et al. (1993) reported that the synthesis of some major amino acids depended on the provision of carbon skeleton from TCA cycle, which can be indirectly affected by the herbicide.

In contrast to other metabolites, phenol content in respective treated cells as compared to the control showed a relative decrease in the percentage reduction until the 8th day (Fig. 2d). However, from the 12th day onwards, phenol reduction level expressed an increase with respect to higher concentrations of the insecticide as well as exposure periods. The percentage reduction in case of phenols increased up to 92% by the end of the 16th day when treated to 12 µg ml^{-1}. Mallick and Rai (1994) also substantiated the findings earlier that phenol could be used as protectants to the organisms during stress or drought conditions.

Enzyme assays:

Nitrate reductase (NR) enzyme declined radically from 32% - 55% by the 4th day (Fig. 3a). A 76% reduction in the nitrate reductase activity was estimated for an increased insecticide concentration (12 µg ml^{-1}) when incubated for a period of 16 days. The decrease in the nitrate reductase enzyme also simultaneously indicated a fall in the nitrogen fixing ability of *Anabaena spp.* Adhikary et al, (1984) studied the effect of carbamate insecticide Sevin on the growth, survival and nitrogen fixation of *Anabaena spp*. and *Westiellopsis prolifica* stated that higher concentrations of the insecticide showed an inhibitory effect on the NR.

Glutamine synthetase (Fig. 3b) determined a consistent decrease with raise in insecticide concentrations and exposure (days). The percentage reduction ranged from 3% - 21% and 61% - 76% for respective concentrations by the 4th day and 16th day respectively. The depletion in glutamine synthetase activity in response to pesticides has also been reported by Rajendran et al, (2007).

Conclusion:

The present study revealed that Endosulfan-6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepine-3-oxide treatments adversely affected the photosynthetic pigments (Chl-*a*, Carotenoids and Phycobiliproteins-phycocyanin, allophycocyanin and phycoerythrin), stress metabolites (carbohydrates, proteins, amino acids and phenols) and enzyme activities (nitrate reductase and glutamine synthetase) in the isolate, *Anabaena fertilissima* even at concentrations as low as 3 µg ml^{-1}. However, the cells also displayed a unique regenerating ability against the insecticide Endosulfan towards the end of the 12th day in the lower doses.

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References:

Adhikary, S.P., Das, P. and Pattnaik, H. (1984) Effect of carbamate insecticide Sevin on *Anabaena* sp. and *Westiellopsis prolifica*. *J.Gen. Appl. Microbiol*., **35**, 335-338.

Shehata, A.I., Arif, I.A.W. and Aqeel, F.Al. (2001) Isolation and characterization of DNA and protein from endosulfan resistant mutant (endo^{r}) of the cyanobacterium anabaena flos-aquae. *Saudi.J.Biol.Sci*., **8**(1).

Anand, N and Veerappan, B. (1980) Effect of pesticide and fungicide on blue-green algae. *Phykos*, **19**, 210-212.

Bennett, A. and Bogorad, L. (1973) Complementary chromatic adaptation in a filamentous blue-green alga. *The J. of Cell Biol*., **58**, 419-435.

Da Silva, E. J., Henricksson, L. E., and Henricksson, E. (1975) Effect of pesticides on blue green algae and nitrogen fixation. *Arch. Environ. Contam. Toxicol*., **3**, 193–204.

Goebel, H., Gorbach, S., Knauf, W., Rimpau, RH. and Huttenbach, H. (1982) Properties, effects of residues and analytic of the insecticide endosulfan. *Residue Rev*., **83**, 1-165.

Hedge, J E & Hofreitte, B T. (1991) Carbohydrate chemistry. In Sadasivam, S & Manickam, A. (eds), *Biochemical Methods for Agriculture Sciences*. Wiley Estern Ltd. Pub, pp. 8.

Jeffrey, S.W., Humphrey, G.F. (1975) New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural populations. *Biochem. Physio. Pflanzen*., **167**, 191-194.

Jha, MN., Sriram, S., Venkataraman, GS. (1999) Cyanobacteria a potential bionitrogen source in integrated nutrient management systems for rice crops. In: Jha, MN., Sriram, S., Venkataraman, GS., Sharma, SG. (eds.), *Agroicrobes*. Today and Tomorrow Publishers, New Delhi, pp. 191.

Kumar, S., Habib, K., Fatma, T. (2008) Endosulfan induced biochemical changes in nitrogen-fixing cyanobacteria. Sci Total Environ., **403**(1-3), 130-8.

Lakshmi, P.T.V and Annamalai, A. (2007) Biochemical Studies on the Response of Organo-phosphorus Insecticide and Release of Extra Cellular Products by Cyanobacteria Research. *Journal of Fisheries and Hydrobiology*, **2**(1), 13-17.

Lee, Y & Takahanshin, T .(1966) An imported colorimetric determination of amino acids with the use of ninhydrin. *Anal Biochem.,* **14**, 71-77.

Lowry ,O H., Rosenbrough, N H., Farr, A L. & Randall, R J.(1951) Protein measurements with folinphenol reagent. *J. Biol. Chem.,* **193**, 265-275.

Malick, C P. & Singh, M B. (1980) Plant Enzymology and Histo Enzymology. Kalyani Publishers, New Delhi, pp. 286.

Mallick, N. and Rai, L C. (1994) Kinetic studies of mineral uptake and enzyme activities of *A.doliolum* under metal stress. *Journal of General and applied Microbiology*, **40**(2), 122-133.

Mishra, U and Pabby, S. (2004) Cyanobacteria: a potential biofertilizer for rice. Resonance, 6-10. Padhy Rabindra N. (1980). *Cyanobacteria and pesticides Residue reviews*, **95**, 1-44.

Parsons, T.R., Strickland, J.D. (1963) Discussion of spectrophotometric determination of marine plant pigments with revised equations for ascertaining chlorophylls and carotenoids. *J.Mar.Res.,* **21**, 155-163.

Prasad, S. M., Kumar, D. and Mohd. Zeeshan. (2005) Growth, photosynthesis, active oxygen species and antioxidants responses of paddy field cyanobacterium *Plectonema boryanum* to endosulfan stress. *J. Gen. Appl. Microbiol.*, **51**, 115–123.

Rajendran, U.M., Kathirvel, E., Narayanaswamy, A. (2007) Effects of a fungicide, an insecticide, and a biopesticide on *Tolypothrix scytonemoides. Pesticide Biochemistry and Physiology*, **87**(2), 164-171.

Ravindran, C. R. M., Suguna, S., and Shanmugasundaram, S.(2000) Tolerance of *Oscillatoria* isolates to agrochemicals and pyrethroid components. *Indian J. Exp. Biol*., **38**, 402–404.

Shetty, P. K., Mitra, J., Murthy, N. B. K., Namitha, K. K., Savitha, K. N., and Raghu, K. (2000) Biodegradation of cyclodiene insecticide endosulfan by *Mucor thermohyalospora* MTCC 1384. *Curr. Sci.*, **79**, 1381–1383.

Sempruch, C., Ciepiela, A. P., Sprawka,I., Chrzanowski, G. (2008) Purification ans some physicochemical properties of nitrate reductase isolated from winter triticale seedlings. *Electronic journal of polish agricultural universities*, **11**(1).

Singh, L. J., Tiwari, D. N., and Singh, H. N. (1986) Evidence for genetic control of herbicide resistance in rice field isolate of *Gloeocapsa* sp. capable of aerobic diazotrophy. *J. Gen. Appl. Microbiol.*, **32**, 81–88.

Soliman, A.I., Shaaban-Dessouki, S.A., Mansour, F.A.and Hussein, M.H. (1993) Physiological effects of rice filed herbicides: Butachlor, Oxadiazon and Thiobencarb, on *Nostoc kihlmani* and *Anabaena oscillarioides*. IV-Response of cellular phosphorous content and malate-dehydrogenase activity to herbicide treatment. *Mans. Sci. Bull. (B. Biology).,* **22**(1), 73-86.

Tamilselvam, B., Gopalswamy, G., Kannaiyan, S. (2002) Influence of butachlor on the growth, cholorophyll content and ammonia excretion by acid tolerant cyanobacteria. *Indian J Weed Sci.,* **34**, 158-159.

Thiel T. (1990) Protein turn over and heterocyst differentiation in the cyanobacterium *Anabaena variabilis*-under condition of starvation. *J.Phycol.,* **26**(1), 50-54.

Yuan, H.F., Min-wang, C., Kung, H.W. (2001) Purification and characterization of glutamine synthetase from unicellular cyanobacterium *Synechococcus* RF-1. *Bot. Bull. Aca. Sin*., **42**, 23-33.