Chapter 5: Discussion

The successful agriculture production is increasingly dependent on the use of pesticides to eliminate pathogenic organisms. However, the extensive use of pesticides tends to raise the concentration of chemical residues in the product and/or the environment to the levels that might inhibit the growth of autochthonous microorganisms like cyanobacteria in paddy fields. Anything that affects these primary producers can be expected to affect the ecological balance of the whole rice field agro-ecosystem. Studies on the possible effects of pesticides on green algae have been reported by Ma et al. (2004). However, relatively little attention has been paid on the toxicological aspects of commonly used pesticides on potentially beneficial cyanobacteria (Sabater and Carrasco, 2001). Hence the toxicity of pesticides to aquatic microorganisms, especially cyanobacteria is an area of research requires further study.

In the literature morphological, biochemical and physiological parameters are consistently used to estimate the toxicity of pesticide on cyanobacterial. Such preliminary test like photosynthetic pigment content, metabolite and enzyme variation analyses may, however, fail to detect more subtle, yet functionally important, changes in microbial communities like cyanobacterial in response to pesticide exposure. Although a number of new valuable molecular biological tools are developed but very little literature is available on pesticide induced molecular changes like functional group variation, protein expression and genetic alteration on cyanobacteria.

The results indicate that the morphological characters, pigmentation and macromolecular cell constituents of cyanobacteria show variable response to different pesticide treatments. In present study, the effect of 2,4-D ethyl ester and pencycuron has been considered to be inhibitory at higher concentration which is in agreement to earlier studies reported by Mostafa and Helling (2002). Data obtained in the present investigation revealed that most of the parameters studied in all the three selected strains of cyanobacteria were more susceptible to 2,4-D ethyl ester than pencycuron. The observations on the effect of two pesticides on the tested cyanobacterial species have been discussed as follow:
5.1. Effect of pesticides on morphological characteristics and heterocyst frequency

Morphological characters like vegetative cell size, color, heterocyst differentiation and heterocyst frequency, etc are some biological parameters commonly associated with the filamentous nitrogen fixing cyanobacteria that have been taken into consideration in the present study. The morphology of all three selected cyanobacteria under higher concentrations of both the pesticides was adversely affected by induction of changes in cell size, bleaching of the cells, disintegration and arrangement of vegetative cells in the filaments (irregular arrangement). In *Anabaena fertilissima* even the lower concentrations of 2,4-D was not suitable since the change in color and size was observed after 16-days of exposure. In contrast, moderate effect of both the pesticides was registered in *Aulosira fertilissima* and *Westiellopsis prolifica* when compared with *Anabaena fertilissima*. Panigrahy and Padhy (2000) worked on the toxicity-studies of two carbamate insecticides carbaryl and carbofuran; and three carbamate fungicides ziram, zineb and mancozeb (commercial formulations) with the N$_2$-fixing filamentous cyanobacterium *Cylindrospermum* sp. Toxicity to vegetative cells, heterocysts and nitrogen fixation, as well as akinete-formation and akinete-germination were recorded. Average number of vegetative cells between two polar heterocysts was affected by all the pesticides used in study. It was observed that three fungicides (ziram, zineb and mancozeb) had damaging roles on vegetative cells, heterocyst activity and N$_2$-fixation whereas ziram was the most toxic among three fungicides administered. Moreover, the number of vegetative cells in the filaments was significantly affected by the toxicity of a commercial formulation of parathion-methyl to *Cylindrospermum*, sp (Panigrahi et al., 2003). Huang et al. (2002) also examined the morphological and physiological response of cyanobacterium *Synechocystis* sp. strain PCC 6308 under acid stress conditions. They found that lethal acid stress, at a pH below 4.4, resulted in the formation of aggregates of denatured proteins as granules near the cell periphery, the disruption of the trans-membrane pH gradient, cell color changed to blue, and damage to photosystem II.

The nitrogen-fixation in algae is dependent upon the development of specialized cells, called heterocyst, under aerobic growth conditions. It was recorded in the present study that 2,4-D ethyl ester and pencycuron suppressed the heterocyst frequency. However, the decrease in frequency of heterocyst was more prominent at the end of the experiment at highest concentration for all the three cyanobacterial species. The earlier referred changes in the
vegetative cells like change in color, size (constriction), disintegration from long trichomes to smaller ones and irregular arrangement of vegetative cells in the filaments might have affected the heterocyst differentiation since the heterocysts and vegetative cells are mutually interdependent for their requirements as the heterocyst lack photosystem II and carbon fixation, and are dependent on vegetative cells for the source of reductant and carbon (Cumino et al., 2007). In return, vegetative cells obtain fixed nitrogen in the form of amino acids from the heterocysts (Meeks and Elhai, 2002). Popa et al. (2007) observed that in *Anabaena oscillarioides*, the newly fixed nitrogen is rapidly exported from heterocysts and distributed to nearby vegetative cells. The exchange of metabolites and intercellular signals that control the regulated spacing of the heterocysts require movement of molecules between cells along the filament, possibly through a continuous periplasm via diffusion (Flores et al., 2006). Since all these evidences propose a very undeviating relation between vegetative cells and heterocyst differentiation, so any changes in vegetative cells should affect the frequency, differentiation and distribution of heterocysts (Zhao and Wolk, 2008).

The heterocyst formation requires nitrogen-starvation, organic carbon supply for new wall synthesis and fresh protein synthesis (David, 2000). Therefore any substance which interferes with meeting any one of the above requirements would decrease/inhibit the formation of heterocyst. As observed in the present case, vegetative cells were influenced by the presence of pesticide stress and the heterocyst frequency was altered correspondingly. This strongly corroborates the earlier findings that heterocyst and vegetative cells are affected by any metabolic changes in each other (Flores et al., 2006).

5.2 Effect of pesticides on photosynthetic pigments

The results in the present investigation indicated the involvement of these pesticides with some metabolic processes responsible for pigment formation because with both the pesticides used, a reduction in photosynthetic pigments has been observed at different concentrations of pesticides. The declining trend in chlorophyll *a* content continued with the rising concentration of pesticides and sharply lowered at the end of the experiment. Similar, reduction in chlorophyll *a* content of *Protosiphon botryoides* with thiobencarb treatment was reported by Eladel et al. (1999) although the tolerance limits varied from organism to organism. Kobbia et al. (2001)
verified an inhibition of chlorophyll \(a\) synthesis in *Anabaena variabilis* and *Protosiphon botryoides* with all tested concentrations (0.2–0.8 mg L\(^{-1}\)) of simazine. Complete lysis of *Anabaena variabilis* at 8 mg L\(^{-1}\) of purified atrazine; 4 mg L\(^{-1}\) of formulated atrazine in wild type and MHR; 0.6 mg L\(^{-1}\) (wild type); 0.5 mg L\(^{-1}\) MHR of DCMU between 6 to 10 days of incubation has been reported by Singh et al. (2011). Moreover, maximum inhibition in chlorophyll \(a\) content of 74-80% in wild type, 68-77% in MHR was observed at sub-lethal dosages of atrazine pure, formulated DCMU treated cells at 8\(^{th}\) day of growth (Singh et al., 2011). Our results are also analogous to those obtained by Mohapatra et al. (2003) who worked with *Anabaena doliolum* and found a progressive decrease in chlorophyll content after short exposure to the pyrethroid insecticide cypermethrin by taking 20 \(\mu\)M and 50 \(\mu\)M of the chemical as treatment concentrations. The present results are also analogous to those obtained by Gonzalez-Tome (1996) who worked with *Nostoc* sp. and found a progressive decrease of chlorophyll \(a\) content using 100 and 200 times more bentazon than recommended levels for field application. The decrease of chlorophyll \(a\) in the cells treated with pesticide was probably resulted from degradation of lipid complexes associated mainly with pigments in thylakoid membranes of the cyanobacterium. On the contrary, stimulation in chlorophyll \(a\) synthesis upto 10 mg L\(^{-1}\)and survival upto 100 mg L\(^{-1}\) of arozin in *Anabaena variabilis* ARM 310 but not in *Tolypothrix tenuis* ARM 76 was observed by Goyal et al. (1991).

It is well established that carotenoids are involved in cyanobacterial photosynthesis in various ways: they act as light harvesting pigments, contribute to the structure of thylakoid membranes and play an important role in photooxidative protection (Schagerl and Muller, 2006). Both the tested pesticide affected total carotenoids of the *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica*. The content of these accessory pigments was decreased after 16 days, mainly with the highest pesticide concentration. These results are in agreement with that of Gonzalez-Barreiro et al. (2004) who recorded inhibition in total carotenoid content of *Synechococcus elongatus* after 96 h of atrazine treatment. Minimum cellular content of total carotenoid i.e. 0.05 pg cell\(^{-1}\) was observed in cultures treated with 0.75 \(\mu\)M of atrazine. Further Galhano et al. (2009) also verified the adverse effect of molinate on carotenoids of *Anabaena cylindrica*. They noticed that the content of carotenoids was very slightly but significantly stimulated in cyanobacterial cells after 72 h with high doses of bentazon (3.8% and 12.2% with
1.5 and 2 mM, respectively). Nevertheless, carotenoids in *Anabaena cylindrica* revealed a different picture in molinate treated cultures, being significantly depressed at all concentrations in a time-dose response manner. At the end of the experiment, the values were reduced by 24.5%, 74.2% and 90.6% relative to control with 0.75 mM, 1.5 mM and 2 mM molinate, respectively. Similarly in the present study 2,4-D ethyl ester and pencycuron exhibited inhibitory effect on carotenoid content of cyanobacteria towards the end of the experiment. For populations of *Oscillatoria agardhii*, inhibition of carotenoid accumulation was also approximately twice as great as the inhibition of chlorophyll accumulation, particularly at the greatest fluridone concentration analyzed (Millie et al., 1990). According to Bhattacharyya et al. (2011) loss of carotenoid pigments might be the reason to protect against the generation of reactive oxygen species (ROS) and inhibition in photosynthetic electron transfer.

In cyanobacteria, phycobiliproteins that are attached to the stromal surface of thylakoid membranes serve as the primary light harvesting antenna for PS2. The composition and function of phycobiliproteins in cyanobacteria changed in response to stress conditions (Sundaram and Soumya, 2011). In 2,4-D ethyl ester and pencycuron treated cultures, the phycobilin pigments were more adversely affected than other pigments in all the three cyanobacteria. These results are in consonance with the injurious effect of other pesticides on phycocyanin (Anand and Subramaniam, 1997). The inhibitory effect of pesticide on phycobiliprotein has been reported by many phycobiologists in *Anabaena variabilis* under arozin, alachlor and butachlor (Singh and Dutta, 2005), in *Anabaena dolilolum* under cypermethrin (Mohapatra et al., 2003), in *Synechocystis* PCC6803 under organophosphate (Mohapatra and Scheiwer, 2000), in *Nostoc linckia* under endosulphan stress (Satish and Tiwari, 2000). The highest reduction of phycobilin pigments suggested that under pesticide stress there was a diversion to meet the nitrogen demand possibly through the induction of proteolytic enzymes (Singh and Datta, 2006). Xia (2005) stated that a decrease in phycocyanin, phycoerythrin and allophycocyanin content implied that the phycobilisomes could have been partially damaged by high thiobencarb concentration, which resulted in a decrease of light energy absorbed by phycobilisomes.

Further, Porsbring et al. (2009) suggested that the reduction in chlorophyll *a*, carotenoids, and phycobiliproteins within marine microalgal communities were due to the deleterious effects of clotrimazole fungicides. Such decrease in chlorophyll *a*, carotenoid and phycobilin contents...
may be ascribed to the inhibition of pigment synthesis directly by pesticide or accelerated degradation of pigments due to increased active oxygen species (AOS) formation at the various sites of the photosynthetic electron transport chain during stress (Prasad et al., 2005). It was also suggested by the same author that the external localization of phycobiliprotein on intracellular thylakoid membrane gave more exposure of pesticides, causing more damage effect on phycobiliproteins and resulting in their detachment.

5.3. Effect of pesticides on metabolites

Differences in the amount of metabolites indicate that the pesticides affect the physiological and biochemical events, which favor more utilization of carbohydrates for nitrate reduction and synthesis of amino acids and proteins (Foyer and Noctor, 2002). Biosynthesis of a carbohydrate shell or a protective carbohydrate coat is a common cyanobacterial tactic for self-defense against the environmental stress (Hoiczyk and Hansel, 2000).

Reduction in total carbohydrates of *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica* was observed under 2,4-D ethyl ester and pencycuron exposure. Upon raising the concentration of both the pesticides the carbohydrate content diminished depicting a concentration-dependent inhibition. Similar observations were quoted by Kumar et al. (2008) while studying endosulfan induced biochemical changes in *Aulosira fertilissima*, *Anabaena variabilis* and *Nostoc muscorum*. Highest concentration of the herbicide simazine was reported to reduce the carbohydrate content and gross photosynthesis in treated algae i.e. *Protosiphon botryoides* and *Anabaena variabilis* (Kobbia et al., 2001). Further, Galhano et al. (2009) also demonstrated decrease in total carbohydrates from 0 to 24 h in bentazon treated *Anabaena cylindrica* cultures in a time-dose response manner. While in a comparative study conducted by Kapoor and Arora (2000) very low concentrations of benzene hexa chloride (BHC) were found to affect the total nitrogen and total carbohydrate content of treated *Cylindrospermum majus*, Kutz. The study on the acute toxicity of commercial herbicide on three freshwater chlorophytes revealed that carbohydrate contents of paraquat treated *Scenedesmus dimorphus*, *Scenedesmus quadricauda* and *Ankistrodesmus falcatus* were hindered with the same sequence of inhibitory effect as observed in growth (Ibrahim, 1990).
On the other hand, contradictory observations have been reported by Mansour et al. (1994) for *Nostoc kihlmansi* and *Anabaena oscillariodes* where the lower concentration of thiobencarb showed increase in the contents of reducing sugar, sucrose, polysaccharides and total sugars but higher concentration of the pesticide showed significant decrease. Similarly moncrotophos have been reported to cause an increase in the carbohydrate content of treated *Platymonas* sp. which became higher with promoted intensity and prolonged time of stress (Tang et al., 1998).

A consistent decrease in the amino acids content of the selected organisms was registered in response to the treated concentrations of the both the pesticides. Reduction in amino acid content of *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica* with respect to highest concentrations of pesticides were more prominent after 16 days. Lakshmi and Annamalai (2007) have reported comparable trend of reduction in total amino acids under Divap 100 stress and highest amino acids was recorded in control cultures of *Anabaena ambigu*a (A100) and *Oscillatoria foreaui* (A1340), especially on the 24th (40μg mL⁻¹) and 20th day. However, the culture of *Oscillatoria foreaui* (A1340) resisted the lower concentration (1ppm) of Divap 100, which showed the results similar to that of control culture. Similar observations were recorded by Nirmal Kumar et al. (2008), on treatment of *Tolypothrix tenuis* with fertilizer industrial effluents. When reflecting upon a decline in protein and amino acids content as a result of pyraclostrobin, propiconazole, and tebuconazole use, Ochoa-Acuna et al. (2009) suggested that these fungicides directly impact unicellular algae (*Pseudokirchneriella subcapitata*).

The 2,4-D ethyl ester and pencycuron suppressed the total protein content of all three organisms in comparison to control values. Although, negligible increase in protein levels was recorded during 4 days by 2,4-D ethyl ester exposure which was followed by consistent period of suppression. At lower concentrations of pesticide, raise in the protein content suggests that lower concentrations of pesticide stimulated the synthesis of stress retarding proteins. Increase in protein content of *Anabaena sphaerica* due to the effect of 50 μg/ml bavistin and 1 μg/ml nimbicidin have also been demonstrated by Rajendran et al. (2007). Whereas in case of lindane treated *Anabaena sp.* (0.5–2 μg/ml) decrease in protein content was recorded by Babu et al. (2001). In the present study also it was noticed that lower concentrations enhanced the synthesis of protein on the other hand, the higher concentrations failed to reach near the control. Battah et
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al. (2001) also supported present findings, that the high herbicide concentration (10 mgL⁻¹ thiobencarb) depletes the protein content of *Anabaena variabilis*. Moreover, Sinha et al. (1997) suggested that decrease in protein content during pesticide stress might be due to of proteases and loss in phycobiliprotein contents. Whereas Leitao et al. (2003) affirmed that the decrease in protein content may also be due to increased level of reactive oxygen species (ROS) or increased protease activity.

Phenols are important aromatic metabolites formed during stress conditions which trigger various biochemical processes of the organisms. The release of phenols was significantly stimulated by both the pesticides throughout the experimental exposures and was higher in all treated cultures as compared to untreated cultures (control) in all the test species. The findings also corroborated with those of Nirmal Kumar and Rita Kumar (2002) who confirmed that phenols could act as protectants in the organisms under stress or drought conditions. Whereas Mallick and Rai (1994) suggested that increase in phenol content could be due to the possible conversion of primary metabolites into phenols as well as to the accumulation of fungicide during stress conditions, which substantiate with our findings. Nirmal Kumar et al. (2010) studied the chronic response of cyanobacterium, *Westiellopsis prolifica* Janet to a triazole fungicide tebuconazole, at different concentrations. The influence of tebuconazole on release of phenols was analyzed and rise in phenol content with increase in fungicide concentrations was recorded. Effect of systemic fungicides (topsin-M) and insecticides (dimecron) on phenolic content of *Vigna radiata* was examined by Siddiqui and Khan (2001) and revealed that phenolic contents were stimulated by the application of systemic fungicide and insecticide at lower rates while showed harmful effects at higher concentration.

5.4. Effect of pesticides on enzymes

Nitrate assimilation is the major process of nitrogen acquisition in cyanobacteria. It is transported into the cells by an active transport system and reduced to ammonium by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR) prior to fixation into amino acids through the glutamine synthetase (GS) pathway (Herrero et al., 2001). In cyanobacteria, as in most prokaryotes, glutamine synthetase plays a key role in nitrogen assimilation. The vegetative cells of cyanobacterial supply glutamate to heterocysts which was then converted to glutamine and other amino acids (Martin-Figueroa et al., 2000). The enzyme
catalyzes the ATP-dependent biosynthesis of glutamine from NH₄ and glutamate and thus represents a key point for the interaction of carbon and nitrogen metabolism (Nigel et al., 1995). Experiments conducted on three cyanobacterial species revealed that different concentrations of 2,4-D ethyl ester (herbicide) inhibited the synthesis of NR, GS and SDH. Moreover, the highest doses of pencycuron (fungicide) were more suppressive to the activities of the three enzymes. The displacement of an essential metal ion forming the central and functional part of the enzyme protein may be one of the reasons for the inhibition of nitrate reductase and secondly, interference with sulphhydryl (SH) groups which often determine the secondary and tertiary structure of proteins (Awashti, 2005). Megharaj et al. (1998) studied the effect of pentachlorophenol pollution towards microalgae and microbial activities in soil. They noticed that among the enzymes tested dehydrogenase was the most sensitive with total inhibition followed by urease and nitrate reductase accounting for 96 and 95% inhibition, respectively, in the highly polluted soil compared to the unpolluted soil. However Chen et al. (2010) suggested that nitric oxide (NO) stimulated the activity of plasma membrane H+-ATPase, 5'-nucleotidase, peroxidase, ascorbate peroxidase and glutathione reductase in ultraviolet B (UV-B) irradiated Chlorella pyrenoidosa. It also boosted the activity of nitrogen-metabolism enzymes such as nitrate reductase, nitrite reductase, glutamine synthetase, which were inhibited by UV-B irradiation.

Besides other toxic effects, the elevated levels of Cu²⁺ and Zn²⁺ are also toxic to NO₃⁻ uptake and nitrate reductase (NR) activity of Scenedesmus sp (Tripathi et al., 2004) where these two metals inhibited NR and NO₃⁻ uptake in concentration-dependent manner, with the latter process being inhibited more strongly than the former. After withdrawal of metal stress, NR and NO₃⁻ uptake activity recovered in a metal ion concentration-dependent manner. Marco and Orus (1993) investigated the toxic effect of trichlorfon (insecticide) on nitrate and ammonium uptake in cyanobacteria. A drastic reduction in the rate of uptake was detected 3 h after the addition of the insecticide to batch cultures of Anabaena PCC 7119. Nitrate uptake rate and chlorophyll and phycobiliprotein contents were also reduced by insecticide treatment in the cyanobacteria Synechococcus UAM 211, GloeocthecePCC 6501, Plectonema calothricoides, Nostoc UAM 205 and Chlorogloeopsis PCC 6912. Even nitrate reductase (ferredoxin: nitrate reductase, EC 1.7.99.4) activity of cyanobacteria was lowered as a result of insecticide action.

An investigation of differential effects of 2,4-D and Pencycuron on three species of Cyanobacteria in-vitro – A Biochemical and Molecular approach
In case of *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica*, 2,4-D ethyl ester and pencycuron treatment suppressed the *in vitro* GS activity in a time-dose response manner. The inhibition of glutamine synthetase can be attributed either to exhaustion of energy-yielding substrates or direct inactivation of the enzyme complexes (Rai et al., 1990). Lee et al. (2007) reported that butachlor treated cultures (*Rhodobacter capsulatus* and *Rhodospirillum rubrum*) showed 1.1~1.2 times higher GS activity, while the other 5 strains (*Rhodobacter sphaeroides*, *Rhodopseudomonas acidophila*, *Rhodopseudomonas blastica*, *Rhodopseudomonas viridis* and *Rhodomicrobium vannielii*) showed lower activities. In the butachlor treated cultures, the presence of pyruvate was associated with 1.1~1.4 times higher GS activities than those seen in the presence of malate. The GS activities in *Rhodopseudomonas acidophila* cultures treated with butachlor were only 21~24% of the untreated control values which has also been further supported by findings of Rajendran et al. (2007). According to them the activities of nitrogenase and glutamine synthetase were affected by bavistin (fungicide), monocrotophos (insecticide), and nimbicidin (biopesticide) treatments but nitrogenase activity was enhanced in the presence of bavistin. Glutamine synthetase activity was found to decline in response to all the pesticide treatments in the present investigation. Nimbicidin proved to be the most inhibitory causing a total loss of activity in cells grown at concentrations >1.0ppm.

Succinate dehydrogenase is a major respiratory enzyme responsible for the conversion of succinate to fumarate in the tricarboxylic acid (TCA) cycle. But the treatment of 2,4-D ethyl ester and pencycuron lowered the SDH activity of three studied cyanobacterial species. The current findings also corroborated with those of Phillips and Rejda-Heath (1993) who substantiated earlier that succinate dehydrogenase of *Rhizoctonia solani* (Kuhn) was inhibited due to fungicide toxicity. Moreover, Nirmal Kumar et al. (2009) examined the effect of graded concentrations of common rice field herbicide (2,4-D) on pigments, metabolic contents, enzyme activities and tolerance potential of cyanobacterial strains *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica* when compared at periodic intervals of 4th and 16th days after treatment. Cyanobacterial strains showed progressive declination of enzymatic activity such as nitrate reductase (62-78%), glutamine synthetase (69-97%) and succinate dehydrogenase (73-89%).
Kalam and Banerjee (1995) elucidated the action of the fungicide tridemorph on glucose, lactate and succinate dehydrogenase activities of some tridemorph-sensitive and -resistant bacteria. It was found to inhibit strongly glucose and lactate dehydrogenase activities in 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*. Growth of these bacteria was inhibited by tridemorph at concentrations between 7 and 60 mg L\(^{-1}\). In contrast, similar dehydrogenase activities in other gram negative organisms, *Escherichia coli* and *Azotobacter vinelandii*, showed no growth inhibition even at 200 mg L\(^{-1}\) tridemorph. Similarly, succinate dehydrogenase activity in *Rhodococcus* sp. AK 1 was strongly inhibited than that in *E. coli* by tridemorph. In cell-free extracts of *Rhodococcus* sp. AK1 and *E. coli*, lactate dehydrogenase activity was also inhibited by tridemorph to a much greater extent in the sensitive strain (63%) than in the resistant ones (8%). The results of present work agree with the above mentioned report, as 2,4-D ethyl ester rendered decrease in succinate dehydrogenase activity in dose and time dependent manner.

5.5. **Effect of pesticides on functional groups:**

A challenge in the life sciences today is the understanding of processes in living organisms at the molecular level. The cell wall of cyanobacteria includes carboxyl, hydroxyl, carbonyl, sulphahydryl, thioether, sulphonate, amine, imine, imidazole and phosphodiester groups (Davis et al., 2003). Cell wall of microbes contain proteins, carbohydrates and phenolic compounds which possess different functional groups such as, hydroxyl, amine, and carboxyl that are responsible for the binding of metal ions (Majumdar et al., 2008). The cyanobacterial cells have developed natural methods of responding to toxin like heavy metals and other xenobiotic compounds through passive accumulation in cells and through surface binding to various functional groups (Gardea-Torresdey et al., 1998). Moreover, these functional groups, present on the cell surface, can be identified by FTIR as each group has a unique absorption band but very scanty literature is available on functional group variation on cyanobacteria.

In the present study *Anabaena fertilissima* when treated with three different concentrations of 2,4-D ethyl ester generated new peaks of O-H of phenols and alcohols, N-O symmetric stretch of nitro compounds, N-H wag of 1°, 2° amines and C-H of aromatics, respectively. However when treated with different concentrations of pencycuron *Anabaena*
*Anabaena fertilissima* produced three new groups viz. aromatic amines (C-N stretch), nitro compounds (N-O symmetric stretch) and (alkynes (-C≡C- stretch)). Khambhaty et al. (2009) studied the response of *Aspergillus niger* with reference to variation in pH, temperature, metal ion concentration and biomass concentration with a view to understand the effect of these parameters on biosorption of Cr (VI) and observed the presence of –OH groups, –CH stretching and N–H stretching which are also accordance with present study. The observation that a depression of 1703 cm⁻¹ which attributed to C=O of α, β unsaturated aldehydes, ketones was consistently absent in *Anabaena fertilissima* after the treatment with both the pesticides used, which is corroborated by the earlier findings of Park et al. (2005) where the peak around 1740 cm⁻¹ (C=O stretching) disappeared from the chromium-loaded biomass of the brown seaweed, *Ecklonia sp.*

In 2,4-D ethyl ester treated *Aulosira fertilissima*, new peaks of nitro compounds; N-O symmetric stretch, 1°, 2° amines; N-H wag, aromatic amines; C-N stretch, aromatics; C-C stretch (in-ring) and 1° amines; N-H bend were observed, whereas pencycuron treatment induced alcohols, carboxylic acids, esters, ethers; C-O stretch, 1° amines (N-H bend) and aliphatic amines; C-N stretch. Bayramoglu and Arica (2008) explained the mechanism of metal ions biosorption (i.e., Hg²⁺, Cd²⁺ and Zn²⁺ ions) by live and heat inactivated fungus pellets (*Lentinus edodes*) and revealed the presence of N–H stretching vibrations masked with the broad band of C–O stretching which coincided with the above observations. *Spirulina platensis* both in free and immobilized forms were studied by Gaur and Dehankhar (2009) and functional groups involved in zinc biosorption were identified using Fourier Transform Infra Red spectroscopy on algae revealed the presence of carboxyl, hydroxyl, amino, amide and imine groups, which were responsible for biosorption of zinc ions. Similarly Nirmal Kumar et al. (2010) reported the sorption of Hg and Pb from mono-metal and bi-metal solution under different concentrations (20ppm–80ppm) using dried *Aspergillus niger* biomass. They found decrease in band from control 1875 to 1743 in the mono-metal treated biomass and absence of band in the bi-metal treated biomass. Further the FTIR analyses verified that carbonyl and amine groups play an important role in biosorption of Pb and Hg.

After 2,4-D ethyl ester treatment in *Westiellopsis prolifica*, the occurrence of functional groups like aromatic amines (C-N stretch), aliphatic amines (C-N stretch), nitro compounds (N-O symmetric stretch) and aromatics (C-H “oop”) were recorded. While, in pencycuron treated
Westiellopsis prolifica new functional groups such as aromatics (C-C stretch (in-ring)), nitro compounds (N-O symmetric stretch), 1° amines (N-H bend), carboxylic acid (O-H bend) and 1°, 2° amines (N-H wag) were produced. The study conducted by Majumdar et al. (2008) revealed that the chemical interaction of copper with Mucor rouxii biomass (MRB) revealed the amide I band as primarily a C=O stretching mode while the amide II band was a combination of N–H bending and C–N stretch.

5.6 Biotransformation of pesticides by cyanobacteria:

Cyanobacteria are free living, photoautotrophic microorganisms and have shown their capabilities to degrade both naturally occurring compounds and synthetic chemicals, especially pesticides (Yan et al., 1998). Therefore, cyanobacteria have been considered as the potent alternative organisms for the chemical and physical treatments to transform the environmentally persistent, toxic xenobiotic materials. Most of the published literature on pesticide degradation has been focused on bacteria, while the role of algae and cyanobacteria in transforming these xenobiotic compounds received little attention.

Biotransformation and enzymatic responses of 2,4-DCP have been reported in the diatom Skeletonema costatum by Yang et al. (2002). Although cytochrome P-450 is considered to be a pivotal enzyme in phase I (the initial oxidation) of transformation and metabolism (Roy et al., 1995), in this experiment, the cytochrome P-450 system appeared to be unimportant in the detoxification of 2,4-DCP (Yang et al., 2002). In the present report, GC-MS study revealed different by-products of 2,4-D ethyl ester when highest concentrations of herbicide were liberated to three cyanobacterial species. Isobutyric acid allyl ester, 3-bromobutyric acid and 2,4-D butyl ester were the products of biotransformation of 2,4-D ethyl ester by Anabaena fertilissima; whereas hydroxyurea, trifluoroacetic acid, 2-methyl propyl ester and acetic acid 2-propenyl ester were resulted by Aulosira fertilissima, while acetic acid (2,3-dichlorophenoxy) and 2,4-D methyl ester were produced by Westiellopsis prolifica. Chlorinated agrochemicals are widely used for pest control. Chlorophenols and their degradation by-products (from industrial wastes and degradation of chlorinated pesticides) have become a serious environmental concern (Petroutsos et al., 2007). Ghasemi et al. (2011) reported the potential of microalgae in transformation, conversion, remediation, accumulation, degradation and synthesis of various
organic compounds. These biotransformations converted organic contaminants to obtain carbon or energy for growth or as co-substrates. Whereas Caceres et al. (2008) found two oxidation products of fenamiphos i.e. fenamiphos sulfoxide (FSO) and fenamiphos sulfone (FSO (2)) which were non toxic to *Pseudokirchneriella subcapitata* and *Chlorococcum sp.* up to 100 mg L$^{-1}$. However bioaccumulation of both fenamiphos and its metabolites (fenamiphos sulfoxide (FSO), fenamiphos sulfone (FSO(2)), fenamiphos phenol (FP), fenamiphos sulfoxide phenol (FSOP) and fenamiphos sulfone phenol (FSO(2)P)) were observed in case of *Chlorococcum sp.* while only metabolites of fenamiphos were observed to be accumulating in case of *P. subcapitata*.

El-Bestawy et al. (2007) reported that the cyanobacteria *Synechococcus, Oscillatoria, Nostoc, Nodularia,* and *Cyanotoce* were able to degrade the pesticide lindane at a very fast rate. Also, Megharaj et al. (1994) had demonstrated the potential of two green unicellular algae like *Chlorella vulgaris and Scenedesmus bijugatus* and four cyanobacteria like *Nostoc linckia, Nostoc muscorum, Oscillatoria animalis,* and *Phormidium foveolarum* to metabolize methyl parathion, a structurally closely related organophosphorus pesticide, to fenamiphos. In the present investigation in pencycuron treated *Anabaena fertilissima* and *Aulosira fertilissima* there was no change as not a single unique by-product was detected while in *Westiellopsis prolifica* one new by-product i.e. benzoxazole was recorded. On the other hand, residues of fenamiphos and its metabolites were detected in algae and cyanobacteria at the end of incubation period of 96-h by Caceres et al. (2008). *Scenedesmus sp.* MM1, *Chlamydomonas sp.* and *Nostoc sp.* MM2 were able to accumulate appreciable quantities of the parent compound fenamiphos and, also, its primary oxidation product FSO. No residues were detected in the extract from *Nostoc sp.* MM3. Thus, two green algae *Selenastrum sp.* and *Chlorococcum sp.*, were able to bioaccumulate fenamiphos and its metabolites (Caceres et al., 2008).

5.7 **Effect of pesticides on protein profile:**

Blue-green algae either produce or modify proteins (or both) to improve their adaptation to stress conditions (Sinha and Hader, 1996). To overcome pesticide stress, the functioning of some proteins is altered or lost and that of others is enhanced or induced (Castielli et al., 2009). Geeta (2000) demonstrated the polypeptide pattern of blue-green algae grown in BG11 medium amended with different concentrations of NaNO$_3$. Exposure to heavy metals resulted in a
qualitative and quantitative regulation of individual proteins. Changes in protein profiling and newly formed proteins might be helping cyanobacterium to tolerate adverse conditions (Zutshi, 2009). There are large numbers of specific proteins reported in various genera of bacteria that exhibited increase in their level of expression, upon adverse conditions, such as heat, toxic elements and nutrient limitations (Shilev et al., 2007).

The present study found that *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica* had 4 basic differences in terms of protein content under the stress conditions used: 1. Production of some new proteins not present in the untreated cultures; 2. Inhibition of production of some proteins that are produced by the untreated cultures; 3. Increase in the level of expression of some proteins; 4. Decrease in the level of expression of some proteins that are present in the untreated cultures. All four of the above differences are directly associated with the response of different blue-green algal species to 2,4-D ethyl ester and pencycuron stress conditions. Our results tent to agree with Rajendran et al. (2007), who reported that new polypeptides of ≈280, 152, and 25 kDa (in 250 ppm bavistin), ≈58 and 28 kDa (in 0.3 and 0.2–0.4 ppm monocrotophos, respectively) and ≈31, 28, and 26 kDa (in 0.5 and 1.0 ppm nimbicidin) were detected in the treated cells of *Tolypothrix scytonemoides* and also stated that the production of novel proteins or the increased production of already existing proteins under stress conditions was due to stress response. However (Kim et al., 1999) suggested that the decrease in production or the inhibition in production of certain proteins could be the result of high levels of protein modification or gene regulation, caused by a decrease in metabolic activity when treated with different chemicals.

Kumar Saha et al. (2003) had documented that marine cyanobacterium *Oscillatoria willei* BDU 130511 respond to stress by synthesizing new proteins; our results indicate that the pesticide treated cyanobacteria also responded in the similar manner. The new proteins observed in the cell extracts of blue-green algae, grown at different pesticide concentrations suggest that these proteins were the modified or induced proteins, which might have some role in resistance, and transport and/or storage of pesticide and needs further investigation. The results indicated that protein synthesis in the test organism eventually succumbed under stress condition which has also been reported by Nicholson et al. (1991). Our results are also in comparable with the findings of Thacker and Madamwar (2005), who had reported that a protein with molecular
weight 30 kDa was induced under stress of chromium and this may be associated with reduction of chromium by bacterial culture (DM1) which was identified in *Ochrobactrum* sp.

The electrophoretic studies were carried out by El-Gamal (2008) on three selected algal species, namely *Chrococcus dispersus*, *Microcystis flosaquae* and *Microcoleus steenstruqi* exposed to two different concentrations (low = 0.5 & high = 2 ppm) of cadmium. Protein profile analyzed by SDS PAGE gel electrophoresis showed differential expression of several proteins. Considerable changes in the number and the percentage of protein fractions were found in each alga. In addition, each organism reacted differently and individually in response to heavy metal exposure. Novo et al. (2000) suggested that cadmium treatments resulted in complete elimination of some protein bands in *Thiobacillus ferrooxidans*. On the other side, Surosz and Palinska (2005) also observed the similar results and reported that the treated test organisms (*Anabaena flos-aquae*) responded to stress by repressing the synthesis of some protein bands under heavy metals tested (copper and cadmium). The response of *Spirulina platensis* to high salt stress was investigated by Sudhir et al. (2005). NaCl treatment of cells resulted in the alterations of other thylakoid membrane proteins: most prominently, a dramatic diminishment of the 47 kDa chlorophyll protein (CP) and 94 kDa protein, but accumulation of a 17 kDa protein band were observed in SDS-PAGE. From the current study, it is evident that each organism reacted differently against 2,4-D ethyl ester and pencycuron treatments. These responses of organism could be attributed to the reaction against the pesticide as well as its concentration, by dissociation of some protein fractions, which moved to the lower molecular weight area. These findings are prettily corroborated with the results conducted by Yoshida et al. (2006) who reported that proteins were involved with protection against cadmium and the impact of some environmental conditions affected the protein profiles expressed under stress.

5.8 Effect of pesticides on DNA profile:

Genetic variability within a species offers a valuable tool for studying mechanisms of stress tolerance (Nguyen et al., 2004). Several PCR-based technologies have been developed to assay genetic polymorphism at the DNA level. Among these, RAPD have been used increasingly as it is accessible and quickly provide a large number of polymorphic markers with universal reagents and assay protocols (Williams et al., 1990). The presence of large number of decamer sequences in a large genome forms the basis of this technique, which utilizes random primers so
that just by chance at several unpredictable locations, two primers will anneal sufficiently close to one another on opposite strands of the template to amplify the intervening regions. This technique is sensitive and specific because the entire genome of an organism is used as the basis for generating the DNA profile. The RAPD technique does not require previous information and knowledge of an organism's gene sequences (Prabina et al., 2005).

It is evident that 2,4-D ethyl ester and pencycuron exposure indeed affected the template activity of DNA, due to the structural damage to DNA (Jenkins et al., 2000). These data provide evidence that the RAPD-PCR method is useful for screening and characterization of genomic regions that have undergone alterations as the result of pesticide treatment. Changes in the genome that were observed in this study were mainly variations in RAPD band intensity in the profiles generated by 2,4-D ethyl ester and pencycuron-exposed culture cells. Additional bands in Westiellopsis prolifica or loss of bands in Anabaena fertilissima and Aulosira fertilissima were also observed after both pesticide treatments. These results suggest that short-term (4 days) 2,4-D ethyl ester treatment induces mainly DNA damage in Anabaena fertilissima, which resulted in disappearance of the specific RAPD band. However, in Aulosira fertilissima and Westiellopsis prolifica short-term treatment with 2,4-D ethyl ester and pencycuron did not found to induce permanent genomic variations or changes in banding pattern. Inhibition of growth or complete killing of cells might be due to inactivation of a number of physiological and biochemical processes (Ashok et al., 2003). Sikorsky et al. (2004) recently reported that a single 8-oxo-7,80-dihydro-20-deoxyadenosine lesion, a basic site, or a T–T dimer markedly reduced amplification efficiency of a DNA template. In addition to DNA damage type, the relative position of the DNA lesions within the template also influences polymerase progression. For example, a single 8-oxodG did not significantly perturb amplification, but two tandem 8-oxodGs did so substantially. These findings suggest that pesticide-induced oxidative DNA damage and DNA–protein cross-links might block Taq polymerase processing and decrease intensity of some specific RAPD bands produced by the primer. It is interesting to note the change of RAPD band patterns did not exhibit a concentration-dependent tendency to 2,4-D ethyl ester and pencycuron in Westiellopsis prolifica. Recently, similar study was conducted by Cenkci et al. (2009) who used RAPD-PCR to analyze the genotoxicant (Hg, B, Cr and Zn)-induced DNA damage in Phaseolus vulgaris. This phenomenon is probably due to RAPD-PCR a method for qualification
rather than for quantification for DNA damage. In fact, the nature and amount of DNA impact in RAPD band can only be obtained by sequencing or probing (Atienzar and Jha, 2006).

Besides the limit of RAPD-PCR, 16 days of pesticide exposure may also not be a reliable time point to induce a relatively stable DNA alteration which is supported by Becerril et al. (1999) who reported the consistency of the changes of RAPD-band patterns dependent upon exposure period of mitomycin C. In Aulosira fertilissima appearance of 1000 bp fragment after 16 days of penecuron treatment seems intriguing and unusual but it is possible that certain region(s) of DNA strand is resistant to penecuron which in turn allows binding of primer resulting in amplification of this particular DNA fragment as it is not a degraded product of template DNA. Nevertheless, the results of our study suggest that pesticide-induced genomic DNA damage and alterations are reflected by the RAPD-PCR method. Moreover, three informative 10-mer primers; OPAH-02, OPG-04, and OPB-09 were also found that might have potential for detecting pesticide-induced specific genomic alterations.

Prabina et al. (2005) analysed the electrophoretic patterns for 17 different cyanobacterial cultures derived from 6 different decamer primers in order to provide diagnostic fingerprints for each culture and their genetic distances based on RAPD markers who had reported that the primers OPB 09, OPG 04 and OPAH 02 generated markers specific for Nostoc cultures while the primer OPF 03 generated specific markers for Tolypothrix temuis. Furthermore Westiellopsis was found to be distinct from other cyanobacterial cultures in the RAPD profile obtained with the primer OPAH 02 and Fischerella cultures could be identified with the primers OPB 09, OPAG 03 and OPF 05.

5.9. Effect of pesticides on 16S rDNA sequence:

In view of the limitations of taxonomic, physiological and biochemical approaches in biodiversity assessment, the molecular approach is currently being preferred (Schafer and Muyzer, 2001). Genetic fingerprinting has been applied to detect pollutant-induced changes in the microbial communities (Polymenakou et al., 2005). One of most important molecular methods for determining bacterial species identities was an approach advocated by Wayne et al. (1987), who employed DNA re-association values of >70% to indicate a single species. This method was followed by the work of Stackebrandt and Goebel (1994), in which 16S rDNA
sequence similarity and DNA re-association values were correlated. The authors found that 16S rDNA sequence similarities above 97% may or may not belong to the same species, but values under 97% were not of the same species. While Doolittle (1982) stated that when considering phylogenetic inferences, the rate of mutation and evolution seen in rRNA sequences is markedly slower than that seen in other biological molecules.

The results obtained by 16S rDNA sequencing confirmed that 16S rDNA region of *Anabaena fertilissima* was more affected by 2,4-D ethyl ester as there was no homology in the region of 39 basepairs also several mismatches and gaps were observed, whereas less difference in 16S rDNA was observed in case of *Aulosira fertilissima* and *Westiellopsis prolifica*. However, after 16-days of exposure to pencycuron treatment there was no significant change in the sequence pattern of all the three test organisms. Similarly Widenfalk et al. (2008) studied the effects of pesticides i.e. captan, glyphosate, isoproturon, and pirimicarb at environmentally relevant and high concentrations on sediment microorganisms. According to Saker et al. (2009) internal base pairing of approximately 70% gives a high degree of structural complexity to the rRNA. Advances in technology related to the sequencing of DNA have made intensive analysis of 16S rRNA gene a standard procedure.

In the case of 16SrRNA gene, distinct environmental conditions such as salinity and rocky shores rich in organic matter have contributed to the genetic relatedness leading to discernible ecological trends among the isolates as observed by Miller et al. (2007). The experiments of Han and Hu (2007) suggested that 16S rRNA structures of the desiccation-tolerant *Nostoc* strains were more stable than that of planktonic *Nostocaceae* species. The adaptive strategies included replacement of GC with other types of base pairs in the DNA sequence. However, the stability and biophysical properties of macromolecules inside desiccated cells are still poorly understood. Even though modifications of genome DNA such as hypermethylation, have been observed in old desiccated sample of *Nostoc* species (Shirkey et al., 2003). Gremion et al. (2004) studied the effects of heavy metals and phytoextraction practices on a soil microbial community during 12 months, using a hyperaccumulating plant (*Thlaspi caerulescens*) grown in an artificially contaminated soil. The 16S rDNA genes of the bacteria and the β-Proteobacteria and the *amoA* gene (encoding the a-subunit of ammonia monooxygenase) were analysed and the data revealed that: (i) the heavy metals had the most...
drastic effects on the bacterial groups targeted, (ii) the plant induced changes which could be observed in the amoA and in the bacteria 16S rRNA gene patterns.

Dudu et al. (2011) amplified and studied rDNA sequences from four related salmonid species. DNA sequences were aligned using CLUSTAL W. These researchers observed gaps from the sequence alignment which represented mutations occurred in 16S rDNA genes and these gaps were inferred as phylogenetic signals of evolution and not as missing data. In current study, 16S rDNA sequence of 2,4-D ethyl ester treated cyanobacterial showed 6% gap in Anabaena fertilissima, 5% gap in Aulosira fertilissima and 0% gap in Westiellopsis prolifica. The gaps in the two sequences results from mismatching of purines and pyrimidines, depurination or mismatching of nitrogen bases, formation of dimeric products, single strand breaks and double strand breaks. According to DeSantis et al. (2006) alignments are useful when gaps have been appropriately added to mark an inference of an insertion or deletion event where one sequence has a base while another sequence lacks a base at the corresponding position. However, after pencycuron treatment no gaps were found in the sequence alignment of three selected cyanobacterial species. Similarly, Mylvaganam and Dennis (1992) analyzed two nonadjacent ribosomal RNA operons, designated rrnA and rrnB, in Haloarcula marismortui. The 16S rRNA genes within these operons were 1472 nucleotides in length and there were no nucleotide gaps in the alignment of the two sequences; however, the two 16S sequences differ by nucleotide substitution at 74 positions which were randomly distributed.