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Toxicological effect of pretilachlor on some physiological processes of cyanobacterium *Synechocystis* sp. strain PUPCCC 64

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ABSTRACT

The toxicological effects of herbicide Pretilachlor on photosynthesis, respiration and nitrogen assimilation of the unicellular cyanobacterium *Synechocystis* sp. PUPCCC 64 has been studied. Treatment of the test organism with Pretilachlor (10, 15 and 20 mg L^{-1}) negatively affected its growth, soluble proteins, photosynthetic pigments, photosynthesis and respiration in a dose dependent manner. Soluble cellular proteins decreased in range of 14-52%. Although herbicide affected all the photosynthetic pigments, maximum effect was observed on carotenoids (76% decrease) followed by allophycocyanin (61% decrease). Pretilachlor caused 49% decrease in photosynthetic rates. Studies on photochemical activity revealed that the herbicide affected both photosystems (PS-I and PS-II) as well as whole chain photosynthetic electron transport activity. The rate of respiration decreased in the range of 24-59% in the presence of herbicide. Decrease in photosynthetic as well as respiration rates ultimately resulted in decreased nitrogen assimilation as revealed by 50% reduction each in nitrate and nitrite uptake and 33% reduction in ammonium uptake along with 21-32% decrease in nitrate reductase, nitrite reductase and glutamine synthetase activity. The main toxic effect of Pretilachlor on the test organism appears to be on photosynthesis, and the effect on other physiological processes is a consequence of toxic effects of Pretilachlor on photosynthetic machinery.

1. INTRODUCTION

It is estimated that by 2050 the world population will be approximating 9 million and to provide food to these population will be a major challenge for human being [1]. The production of sufficient food for the world's population in 2050 will be possible only if agriculture shall be based on high-tech seeds and low-tech farming practices [2]. Rice is the most important cereal crop in the developing world and a staple food for more than half of the world's population. Research and development programmes in rice are crucial for the development of strategies to increase global food security [3]. The application of herbicides in rice fields to eradicate weeds allowing rice plant to grow and gain a competitive advantage is one of the strategies [4]. However, the use of herbicides in rice fields results in serious environmental contamination threatening the integrity and stability of ecosystems [5]. The cyanobacteria, being an important component of microbial community in rice field ecosystem, significantly contribute to fertility of the soil as natural biofertilizer [6,7,8]. These microorganisms increase organic matter, water holding capacity, nitrogen status and release vitamins or plant growth stimulating hormones, extracellular polysaccharides etc. [9, 10] and even some metabolize pesticides [11, 12]. Thus, supplementation of cyanobacterial biofertilizer is considered to be a good management of paddy field since this not only adds to fertility of the soil but is also eco-friendly. Detailed investigations on deleterious effects of herbicides on cyanobacteria are required, since the utilization of cyanobacterial biofertilizer in rice fields requires the strains tolerant to a variety of routinely used agrochemicals including herbicides. Effects of some herbicides like Thiobencarb [13], Atrazine, Molinate and Bentazon [14, 15], Butachlor [16,17], Glyphosate, 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [18,19] and Anilofos [12, 20] on few cyanobacteria have been reported. Pretilachlor (2-chloro-N-(2,6-diethylphenyl)-N-(2-propoxyethyl) acetanilide) is the active chemical of the commercial grade herbicide Rifit, which is used on a large scale in the rice fields of Punjab state. This herbicide belongs to the chloroacetanilide group and is used as pre-emergence and early post-emergence herbicide for the control of annual grasses and some broad-leaved weeds such as *Echinocloa crusgalli* and *Ischaemum rugosum* in both seeded and transplanted fields at the rate of 300 gm active ingredient per acre [21, 22]. The chemical structure of Pretilachlor is shown in figure 1.

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The aim of the present investigation was to study the toxicological effect of Pretilachlor on photosynthesis, respiration and nitrogen assimilation of a unicellular cyanobacterium *Synechocystis* sp. strain PUPCCC 64.

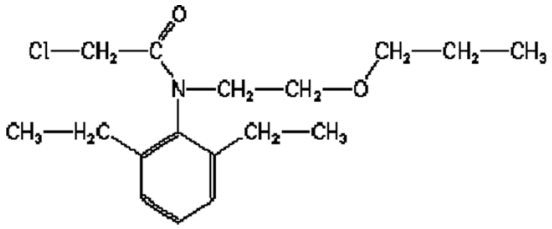

Fig. 1: Chemical structure of Pretilachlor.

2. MATERIALS AND METHODS

2.1 Organism and Culture Conditions

The cyanobacterium *Synechocystis* sp. strain PUPCCC 64 was isolated from paddy fields of village Derabassi (30°58′72″ N; 76°8′28″ E) of district Mohali, Punjab, India [11]. The organism was grown in slightly modified Chu-10 medium [23] supplemented with micronutrients. One litre nutrient medium contained 0.232 g $CaCl_2.2H_2O$, 0.025 g $MgSO_4.7H_2O$, 0.02 g Na_2CO_3, 0.044 g $Na_2SiO_3.5H_2O$, 0.01 g K_2HPO_4, 0.0035 g of ferric citrate and citric acid and 1 g KNO_3. Stock and experimental cultures were maintained in a culture room at 28±2 ˚C and illuminated with fluorescent tubes giving photon flux of 44.5 µmol m^{-2} s^{-1} (µE) with light/dark cycle of 14/10 h. Exponentially growing (10 days old) cultures were used throughout the study and each experiment was repeated thrice.

2.2 Tolerance limit of test organism to Pretilachlor

The tolerance limit of *Synechocystis* towards herbicide was determined by growing it in graded concentrations of Pretilachlor (10 to 40 mg L^{-1}). These concentrations were prepared in 250 mL Erlenmeyer flasks containing 100 mL Chu-10 medium from the stock solution of commercial grade Pretilachlor (50% Emulsifiable Concentrate). Exponentially growing stock cultures, after two washings with sterilized double distilled water, were inoculated in herbicide containing medium to get an initial absorbance of 0.1 at 680 nm (110 x10^6 cells mL^{-1} culture). At regular intervals of 2 d, extending up to 12 d, 10 mL samples were withdrawn and growth was measured as an increase in absorbance of the cultures at 680 nm with the help of spectrophotometer (Spectronic 20D+, USA). The per cent inhibition in growth of the organism was calculated by taking growth data of control culture on day 6 as 100%. Specific growth rate constant and generation time were determined from the linear portion of growth curve following Myers and Kratz [24]. Protein content of the cultures was determined as per the method of Lowry et al. [25].

2.3 Effect of Pretilachlor on photosynthetic pigments

Ten milliliter of experimental cultures were withdrawn and number of cells per mL of cultures were counted. The cells were centrifuged at 5000 g, washed thrice with double distilled water and to the cell pellet same volume of 80% acetone was added. The mixture was shaken vigorously and incubated in a refrigerator for 12 h. The contents were centrifuged at 5000 g, attained the same volume of supernatant by adding sufficient volume of 80% acetone and the absorbance was taken at 660 nm, 645 nm and 450 nm.

Chlorophyll *a* (Chl *a*) was calculated following Holm [26] as per the equation given below:

$$\text{Chl } a \text{ (µg per } 10^6 \text{ cells)} = (9.76 \times A_{660}) - (0.99 \times A_{645})$$

where, A_{660} = absorbance at 660 nm, A_{645} = absorbance at 645 nm

Total Carotenoids were quantified following Myres and Kratz [24] as per the equation given below:

$$\text{Carotenoids (mg per } 10^6 \text{ cells)} = A_{450}/200$$

Where, A_{450} = absorbance at 450 nm

The phycobiliproteins were extracted in phosphate buffer (50 mM, pH 7.0) by freeze-thaw method. Pellet of cyanobacterial cells, obtained after centrifugation at 5000 g, was washed and re-suspended in phosphate buffer containing lysozyme (1 mg mL^{-1}). The mixture was incubated at 37 ˚C in a water bath for 1 h with occasional stirring. The spheroplasts obtained were gently washed by centrifugation at 5000 g and resuspended in phosphate buffer. The contents were then subjected to 10-12 freeze-thaw cycles till all the water soluble pigments were released from the cells. Absorbance of supernatant, after centrifugation at 5000 g, was noted at 565 nm, 615 nm and 652 nm and phycobiliproteins (µg per 10^6 cells) were quantified following Bennet and Bogorad [27] as per the equations given below.

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

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

$$\text{Phycoerythrin} = \frac{(A_{565}) - (2.41 \times PC) - (0.849 \times APC)}{5.62}$$

2.4 Measurements of photosynthetic, respiratory and photochemical activities

Rates of photosynthesis and dark respiration were measured in terms of oxygen evolution and oxygen consumption in light and dark, respectively. Pretilachlor treated and untreated control cultures were concentrated by centrifugation at 5000 g and thick suspension of biomass (10 µg chl *a* mL^{-1}) was taken in a reaction vessel fitted with oxygen electrode of oxygen analyzer (Model 5300 A, YSI Bioanalytical Products, USA). Increase in amount of dissolved oxygen was followed for 5 min after switching on light source (225 µE on the surface of vessel). The rate of dark respiration was measured for 10 min by following the decrease in dissolved oxygen when the reaction vessel was incubated in dark.

Photochemical activities of cultures were measured according to the method of Chen et al. [28]. Exponentially growing culture treated with 10, 15 and 20 mg Pretilachlor L^{-1} for

6 h and untreated cultures were harvested by centrifugation at 5000 g and re-suspended in fresh Chu-10 medium. Photochemical activities of whole cells were assayed under light intensity of 225 µE and at 28±2 °C using oxygen electrode. PS-II activity was measured as oxygen evolution with H_2O as the electron donor and *p*-benzoquinone (*p*-BQ) as the electron acceptor in 3 mL culture medium containing 25 mM bis–tris propane (BTP, pH 7.8) and 1 mM *p*-BQ. PS-I activity was measured as light dependent oxygen uptake in 25 mM BTP (pH 7.8), containing 0.1 mM 2,6-dichlorophenol indophenol (DCPIP) as the electron donor, 5 mM ascorbate as reductant to reduce DCPIP to $DCPIPH_2$), 0.1 mM methyl viologen (MV) as the electron acceptor, 1 mM NaN_3 (inhibiting respiration), and 10 µM DCMU (inhibiting PS-II activity). The whole chain photosynthetic electron transport activity (WCA) was determined by monitoring the light-dependent oxygen uptake with H_2O as the electron donor and MV as the electron acceptor in 3 mL Chu-10 medium containing 25 mM BTP (pH 7.8), 1 mM NaN_3, and 0.1 mM MV.

2.5 Nitrogen source uptake by the organism

The uptake of nitrate, nitrite or ammonium by the organism was studied by their depletion of N source from the liquid medium with time. Cyanobacterial suspension after two washings with double distilled water was added separately in media containing nitrate as potassium nitrate, nitrite as potassium nitrite (100 µmol L^{-1} each) or ammonium as ammonium chloride (200 µmol L^{-1}), and incubated under light for 6 h. After separating the cells by centrifugation at 5000 g, the residual amount of nitrate, nitrite or ammonium in the medium was determined according to the methods of Robinson *et al*. [29], Nicholson and Nason [30] and Solarzano [31], respectively. The nitrate, nitrite and ammonium uptake was expressed as µmol nitrate/ nitrite/ ammonium mg^{-1} protein.

2.6 Enzyme assays

The whole cell nitrate reductase (NR) activity was assayed as rate of nitrate reduction with sodium dithionite and reduced methyl viologen as the electron donor [32]. One millilitre cell suspension, after three washings with double distilled water, was treated with 20 µL toluene by agitating for 3 min and toluene treated permeablized cells were used as enzyme extract. The reaction mixture contained in a final volume of 1 mL, 0.2 mL enzymes extract; $NaHCO_3$-Na_2CO_3 buffer (pH 10.5, 100 µmol); KNO_3, 20 µmol; methyl viologen, 4 µmol; and 10 µmol of sodium dithionite (freshly prepared in 0.3 M $NaHCO_3$ buffer). The reaction mixture was incubated at 30 °C for 10 min and amount of nitrite formed was estimated. NR activity is expressed as nmol nitrite formed mg^{-1} protein min^{-1}. The same procedure was followed for measuring nitrite reductase (NiR) activity except that KNO_3 was replaced by KNO_2 in the reaction mixture and decrease in the amount of nitrite was estimated. NiR activity is expressed as nmol nitrite decreased mg^{-1} protein min^{-1}.

Whole cell glutamine synthetase (GS) activity was measured following Shapiro and Stadtman [33]. Washed cyanobacterial cells were suspended in 1 mL of imidazole-HCl buffer (50 mM, pH 7.0). To this suspension, 20 µL of toluene was added and agitated for 3 min. The toluene treated cells were separated by centrifugation at 5000 g and resuspended in imidazole-HCl buffer and used directly as enzyme extract. The assay mixture in a total volume of 2 mL contains 0.8 mL 50 mM imidazole-HCl buffer (pH 7.0), 1 mL assay mixture (50 mM Imidazole buffer, 0.1 M glutamine, 0.1 M manganese chloride, 0.01M ADP, 1 M sodium arsenate and 2 N hydroxylamine hydrocholoride neutralized with 2 N sodium hydroxide), and 0.2 mL enzyme extract. The assay mixture was incubated at 37 °C for 30 min. The reaction was terminated by adding 4 mL stop mixture (prepared by mixing 4 mL ferric chloride (10%), 1 mL of trichloro-acetic acid (24%), 0.5 mL of 6 N HCL and 6.5 mL of double distilled water). The absorbance of brown colour developed was measured at 540 nm. L-glutamic acid γ-monohydroxamate was used to construct a standard curve. The GS activity is expressed as µmol of γ-glutamyl hydroxamate formed mg^{-1} protein min^{-1}.

2.7 Chemicals

All chemicals used in media preparation and analytical assays were obtained from Merck, India. Commercial grade herbicide Pretilachlor (Rifit 50% EC) manufactured by Syngenta Chemicals Limited, Maharashtra, India was used in the present study.

2.8 Statistical Analysis

Data were statistically analyzed by applying one way ANOVA and Tukey's post-hoc test at 95% confidence level ($p < 0.05$) using GraphPad Prism 5.0 version 5.4. Data are average±SD of three independent experiments.

3. RESULTS AND DISCUSSION

3.1 Tolerance limit of organism towards Pretilachlor

The cyanobacterium *Synechocystis* sp. PUPCCC 64 employed in the present study is a natural inhabitant of paddy fields of Punjab, India and is an isolate of our laboratory [11]. The strain was grown in graded concentrations (5-40 mg L^{-1}) of Pretilachlor to study its tolerance limit. The results revealed that the herbicide caused concentration dependant decrease in the growth of the test microorganism. The microorganism exhibited 15, 35, 55, 80, 90 and 92% inhibition in growth in 5, 10, 15, 20, 25, and 30 mg L^{-1} of Pretilachlor, respectively (Fig. 2). The organism did not grow in 40 mg Pretilachlor L^{-1}. Microscopic observations of cultures grown in this concentration of herbicide revealed that nearly 99% of cells were lysed and pigments were released in medium. Pretilachlor concentration dependent increase in generation time from 110 h in 5 mg L^{-1} to 235 h in 25 mg L^{-1} with simultaneously decrease in growth rate constant confirm dose dependent inhibition of growth of the test organism (Fig. 3). Similar inhibitory effects by other herbicides in cyanobacteria have been reported. The herbicides shown to affect growth of

cyanobacteria include Butachlor, Bensulfuron-methyl and Dimethoate in *Nostoc* [16,28], Glyphosate in *Anabaena* sp., *Leptolyngbya boryana*, *Nostoc punctiforme*, *Microcystis* sp. and *Microcystis aeruginosa* [34,35], Molinate and Bentazon in *Anabaena cylindrica* and *Nostoc muscorum* [14,15], Atrazine and DCMU in *Anabaena variabilis* [18], Anilofos in *Oscillatoria simplicissima* [36] and *Anabaena torulosa* [20]. The inhibition of growth varied with the type of cyanobacteria and herbicide employed.

The inhibition in growth of the present strain may be due to the damaging effect of herbicide on photosynthetic machinery, protein and or DNA molecules [37, 38]. Since the organism exhibited nearly 55% growth in 15 mg Pretilachlor L^{-1} which is 11 times the recommended field application concentration of Pretilachlor as compared to control cultures, it indicated that organism exhibit significant tolerance to herbicide.

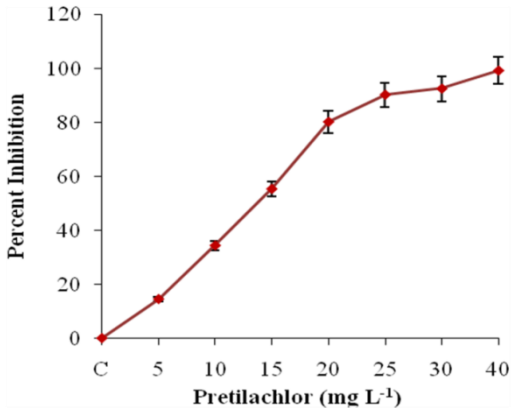

Fig. 2: Inhibition in growth (A_{680}) of *Synechocystis* in presence of Pretilachlor on day 6.

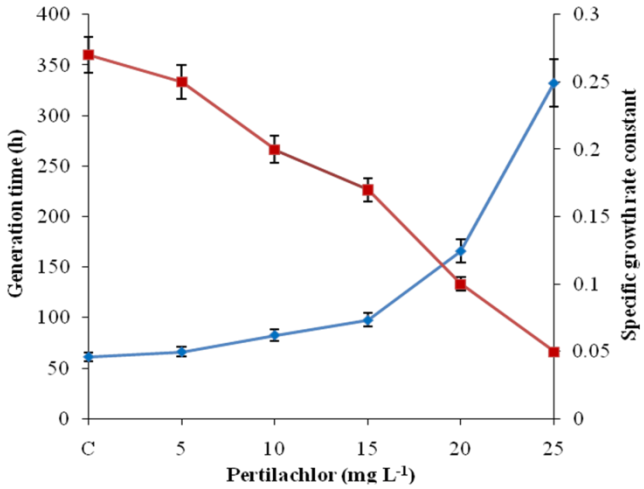

Fig. 3: Specific growth rate constant and generation time of *Synechocystis* in presence of Pretilachlor.

In order to understand the mechanism of growth inhibition in *Synechocystis* sp., the impact of Pretilachlor on total soluble proteins, photosynthetic pigments, photosynthesis, respiration and N assimilation were analysed. For this, three concentrations of Pretilachlor i.e. 10, 15 and 20 mg L^{-1} were chosen.

3.2 Protein content

The total protein content of control cultures of *Synechocystis* increased from 4.46 µg per10^6 cells on zero day to 10.5 µg 10^6 $cells^{-1}$ on day 6. Pretilachlor concentration dependent decrease in protein content of the organism was observed. The test organism exhibited 14.5, 33.8 and 52.4% decrease in total protein content compared to control cultures in 10, 15 and 20 mg herbicide L^{-1} (Fig. 4). Supplementation of Butachlor (8-20 mg L^{-1}) in culture medium of *Nostoc muscorum* resulted in decrease of protein content by 27-89% whereas 14 and 63% decrease in protein content in 5 and 8 mg Thiobencarb L^{-1} was reported [39]. Of the three herbicides studied, Glyphosate, Pretilachlor and Propanil, only Propanil (0.187-1.5 mg L^{-1}) significantly inhibited protein content of *Anabaena fertilissima* [40]. Carbaryl (40 mg L^{-1}) decreased content of protein by 40% in a paddy field cyanobacterium *Calothrix brevissima* [41]. The decrease in protein content of the test organism in varied concentration of Pretilachlor may be due to its interference with structure and function of membrane proteins or photosynthetic machinery of cells such as photosynthetic pigments, PS-I and PS-II or nitrogen assimilation [12, 42, 43]. Thus, the affect of Pretilachlor on photosynthetic pigments, photosynthesis, respiration and nitrogen assimilation was studied.

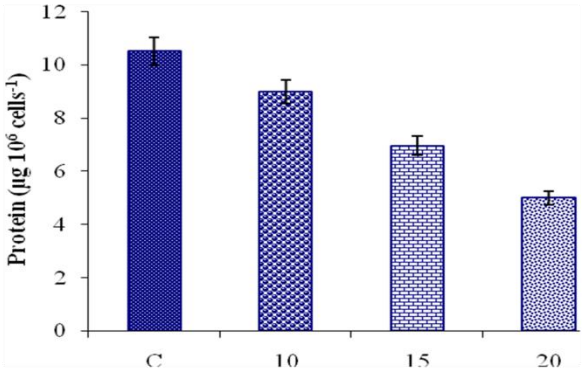

Fig. 4: Effect of Pretilachlor (mg L^{-1}) on total protein content of *Synechocystis* on day 6. C: Control. Data in figure are significantly different from each other at 95% confidence level ($p < 0.05$).

3.3 Photosynthetic pigments

Pretilachlor caused a significant decrease in chlorophyll (Chl) *a*, carotenoids, phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE) content in a dose dependent manner compared to control with more pronounced effects at higher concentrations of Pretilachlor (Figs 5 and 6). Chl *a* content of *Synechocystis* sp., on day six, in 20 mg Pretilachlor $L^{-1}$was decreased by 49%. In the same concentration of the herbicide, carotenoids decreased by 76% while PC, APC and PE were decreased by 52%, 61% and 50%, respectively, compared to control cultures. The order of decrease in photosynthetic pigments was Carotenoids > APC > PC > PE > Chl *a*. It is interesting to

note that in 10 mg Pretilachlor L^{-1} which is 7 times the recommended field application dose, levels of photosynthetic pigments decreased in the range of 13-25% only (Fig. 5 and 6). Growth inhibition of *Synechocystis* with increase in Pretilachlor concentration could be explained on the basis of damaging effect of herbicide on photosynthetic pigments. Bensulfuron methyl, Butachlor and Dimethoate inhibited photosynthetic pigments of *Nostoc* [28]. Treatment of *Anabaena cylindrica* with Bentazon (2 mM) for 72 h decreased total phycobiliproteins by 58% where as Molinate at highest tested concentration (2 mM) completely suppressed phycobiliproteins after 24 h treatment [15]. Anilofos inhibited all photosynthetic pigments of *Anabaena torulosa* [20], *Oscillatoria simplicissima* [36] and even of the test organism [12].

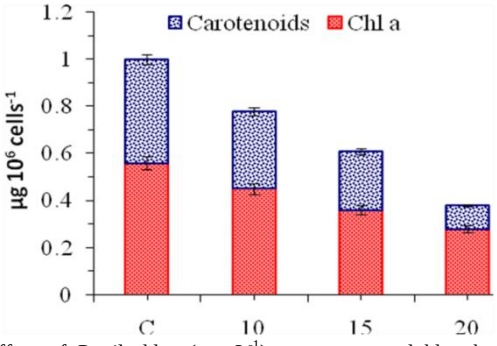

Fig. 5: Effect of Pretilachlor (mg L^{-1}) on acetone soluble photosynthetic pigments of Synechocystis on day 6. C: Control. Data in figure are significantly different from each other at 95% confidence level ($p< 0.05$).

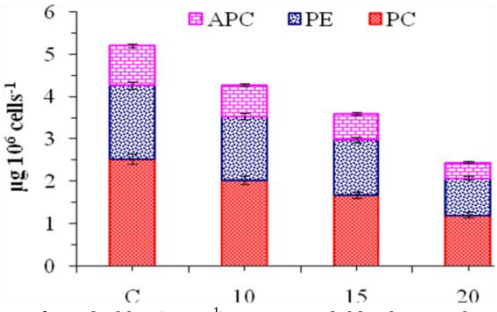

Fig. 6: Effect of Pretilachlor (mg L^{-1}) on water soluble photosynthetic pigments of *Synechocystis* on day 6. C: Control. Data in figure are significantly different from each other at 95% confidence level ($p< 0.05$).

3.4 Photosynthetic and respiratory activities

The growth and development of photoautotrophs depend upon photosynthesis [44]. Decreased growth of the *Synechocystis* in the presence of Pretilachlor may also be as a result of low photosynthetic activities due to the loss of photosynthetic pigments. To ascertain this, the effect of Pretilachlor on photosynthetic rate was studied. Whole cell photosynthetic O_2 evolution and photosynthetic electron transport activity of the test organism were significantly affected by Pretilachlor in a dose dependent manner. Treatment of the organism with 20 mg Pretilachlor L^{-1} for 12 h caused 48.8% reduction in rate of photosynthesis over control cultures (Table 1). Decrease in photosynthetic O_2 evolution in *Anabaena torulosa* has also been reported in the presence of Anilofos [20]. Kim and Lee [45] observed that Bensulfuron-methyl (5 mg L^{-1}) suppressed photosynthesis of *Nostoc commune* by 32%. The treatment with pure (8 mg L^{-1}) and formulation (4 mg L^{-1}) forms of Atrazine and DCMU (0.6 mg L^{-1}) caused 80-87% inhibition in photosynthetic O_2 evolution in wild and multiple herbicide resistant strain of *Anabaena variabilis* [18]. Herbicides inhibit photosynthesis mainly by preventing electron flow from PS-II both in algae as well as in higher plants [46-48]. In higher plants, herbicides have been shown to be uncouplers of photophosphorylation [49]. The effect of Pretilachlor on photosynthetic electron transport activity (PS-I, PS-II and WCA) of the strain PUPCCC 64 was dose dependent. It was observed that 20 mg Pretilachlor L^{-1} reduced PS-I, PS-II activities and WCA by 39%, 43% and 38%, respectively (Table 1). It appears that Pretilachlor reduced the energy transfer from phycobilisomes to PS II, as reported earlier in *Anabaena doliolum* in the presence of Glyphosate [50]. The photochemical activities of the *Anabaena torulosa* were significantly inhibited by herbicide Anilofos [20]. Significant decrease in photochemical activities of the test microorganism under Pretilachlor stress may lead to the reduction of photophosphorylation as reported in *Plectonema boryanum* under Endosulfan stress [51].

The inhibitory effect of Pretilachlor on respiration in *Synechocystis* sp. was also dose dependent. The test microorganism exhibited 59% decrease in the rate of respiration in 20 mg Pretilachlor L^{-1} compared to control cultures (Table 1).

Table 1: Effect of Pretilachlor (mg L^{-1}) on photosynthesis, respiration and photochemical activities of *Synechocystis* after 12 h treatment.

Treatments	Photosynthesis (µmol O_2 evolved mg^{-1} Chl *a* min^{-1})	Photosystem I (µmol O_2 consumed mg^{-1} Chl *a* min^{-1})	Photosystem II (µmol O_2 evolved mg^{-1} Chl *a* min^{-1})	Whole Chain Activity (µmol O_2 evolved mg^{-1} Chl *a* min^{-1})	Respiration (µmol O_2 consumed mg^{-1} Chl *a* min^{-1})
C	70.00±2.78	35.2±2.65	54.7±3.15	77.4±3.20	54.40±3.28
10	61.00±2.17**(15.80)**	25.8±1.67**(26.70)**	46.1±2.16**(15.72)**	72.6±2.65**(6.20)**	46.30±2.15**(24.00)**
15	55.80±2.99**(28.85)**	24.9±2.27**(29.26)**	35.2±2.19**(35.64)**	54.4±2.67**(29.71)**	42.13±2.16**(46.69)**
20	42.80±1.85**(48.85)**	21.4±1.81**(39.20)**	31.1±2.17**(43.14)**	47.9±2.75**(38.11)**	35.40±2.87**(58.82)**

Data given in parenthesis indicate percent inhibition over untreated control culture. C: Control. Data in each row and column are significantly different from each other at the 95% confidence level ($P< 0.05$).

Table 2: Effect of Pretilachlor (mg L^{-1}) on nitrogen assimilating enzymes of *Synechocystis* after 12 h treatment.

Treatments	NR Activity (nmol nitrite formed mg^{-1} protein min^{-1})	NiR Activity (nmol nitrite decreased mg^{-1} protein min^{-1})		GS Activity (nmol γ-glutamyl hydroxamate formed mg^{-1} protein min^{-1})		
	Nitrate	Nitrate	Nitrite	Nitrate	Nitrite	Ammonium
C	37.86±3.61	33.33±3.27	48.62±3.29	2.67±0.86	3.25±1.21	3.6±1.16
10	34.98±3.76**(7.60)**	30.57±2.65**(8.28)**	45.46±3.26**(6.49)**	2.31± 0.78**(13.48)**	2.86±0.23**(12.0)**	3.14±1.21**(13.88**)
15	32.38±3.27**(14.47)**	27.76±2.14**(16.71)**	42.10±3.17**(13.41)**	2.07±0.94**(22.47)**	2.56±0.38**(21.23)**	2.73±0.35**(25.0)**
20	28.81±2.17**(23.90)**	24.87±2.15**(25.38)**	38.57±3.22**(20.67)**	1.75±0.049**(30.71)**	2.20±0.43**(32.30)**	2.34±0.47**(36.0)**

Data given in parenthesis indicate percent inhibition over untreated control culture. C: Control. Data in each column are significantly different from each

These observations indicate that Pretilachlor affected not only photosynthesis but respiration of the organism as well though to less extent. Similar observations have been reported in other organism with other pesticides. Anilofos (1.25-5.0 mg L^{-1}) caused a decrease in respiration rate in *Anabaena torulosa* [20]. Chen et al. [28] also reported decrease in the rate of respiration in *Nostoc* when treated with Bensulfuron methyl, Butachlor, and Dimethoate. Thus, it would be interesting to study at what step(s) the Pretilachlor interferes with the respiration process of the test organism.

3.5 Nitrogen uptake studies

Cyanobacteria may use nitrate, nitrite or ammonium as a nitrogen source for growth and development. Many of them, especially heterocystous forms, are able to fix atmospheric nitrogen [52]. Nitrogen assimilation in cyanobacteria is photosynthesis dependent. Thus, the effect of Pretilachlor on nitrogen assimilation was studied. Pretilachlor caused dose dependent effect on nitrogen up take by *Synechocystis* sp. The treatment of the test organism with 20 mg Pretilachlor L^{-1} caused nearly 50% inhibition in nitrate and nitrite uptake (Fig. 7).

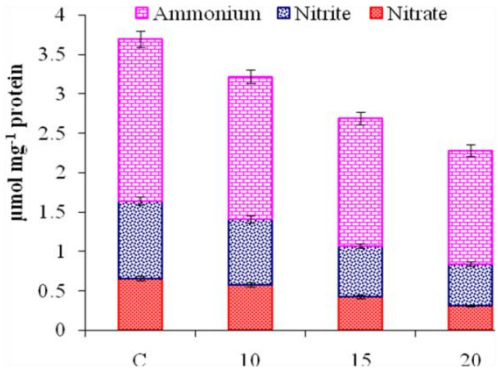

Fig. 7: Effect of Pretilachlor (mg L^{-1}) on nitrogen uptake by *Synechocystis* after 6 h treatment. C: Control. Data in figure are significantly different from each other at 95% confidence level ($p < 0.05$).

Photosynthetically reduced ferredoxin serves as an electron donor for nitrate reduction in cyanobacteria [53]. Thus, decrease in photosynthesis caused by Pretilachlor may have resulted in low uptake and reduction of nitrate in this organism. Ammonium uptake by the test organism was also significantly reduced (32.88%) by 20 mg Pretilachlor L^{-1} (Fig. 7). It has been demonstrated that ammonium uptake in cyanobacteria is a membrane potential driven transport process [54]. It may be that Pretilachlor interfered with the membrane potential of this organism and resulted in reduced ammonium uptake.

3.6 Activity of nitrogen assimilation enzymes

Three enzymes, nitrate reductase (NR), nitrite reductase (NiR) and glutamine synthetase (GS) of *Synechocystis* sp. strain PUPCCC 64 were chosen to study the effect of Pretilachlor on N assimilation. Activities of both NR and NiR were significantly reduced by Pretilachlor in a dose dependent manner (Table 2).

The reduction in NR activity was 24% in 20 mg Pretilachlor L^{-1} treated cultures compared to control cultures. Effect of Pretilachlor on NiR activity of the strain PUPCCC 64 was studied in nitrate and nitrite grown cells. The test organism exhibited 21% inhibition in NiR activity of nitrite grown cells. NR and NiR are membrane bound enzymes and their activity depends on reduced ferredoxin produced during photosynthesis [55]. Decreased activities of these enzymes in presence Pretilachlor may be due to its interference with ferredoxin reduction or at uptake level of nitrate or nitrite. The progressive decrease in NR and NiR activities of *Nostoc muscorum* by carbaryl (5-50 mg L^{-1}) and *Anabaena fertilissima* by Endosulfan (3-12 mg L^{-1}) has been reported [56,57]. Ammonium produced by the activities of NR and NiR or taken up directly is assimilated by the activity of glutamine synthetase (GS) through GS-GOGAT cycle [52]. Thus, effect of herbicide on GS activity of cells grown in nitrate, nitrite or ammonium was studied. Similar to the effect on NR and NiR, GS activity also decreased by 31% in 20 mg Pretilachlor L^{-1}as compared to control cultures. The pattern of effect of Pretilachlor on GS activity of nitrite and ammonium grown cells of the test organism was similar to nitrate grown cells. GS activity decreased by 32% when cells were grown in nitrate medium with 20 mg Pretilachlor L^{-1}. In ammonium grown cultures, GS activity of *Synechocystis* decreased by 36% in presence of herbicide (Table 2). Among all the nitrogen sources, the effect of Pretilachlor was more pronounced on GS activity of cells grown in ammonium supplemented medium. GS activity depends upon the amount of ammonium ions taken up by the cells or produced by reduction of nitrate by NR followed by NiR reduction. Thus, decrease in GS activity appears to be correlated with reduction in nitrate/

ammonium uptake rather than direct toxic effects of Pretilachlor on GS activity. The decrease in GS activity in cyanobacteria caused by other pesticides has been reported [56, 58]. Treatment of *Anabaena torulosa* with 1.25- 5 mg Anilofos L^{-1} caused 9-22% decrease in GS activity [20].

4. CONCLUSIONS

Pretilachlor at 20 mg L^{-1} concentration which is 15 times higher than recommended field application dose, adversely affected the growth of *Synechocystis* by reducing photosynthetic pigments, photosynthesis and respiration. The suppression of photosynthesis under the influence of Pretilachlor appears to be responsible for low uptake of nitrogen compounds and lowered activity of nitrogen assimilation enzymes. Since at low dose (10 mg L^{-1}) of Pretilachlor, these physiological processess were not adversely affected, the test organism can be made a part of biofertilizer programme for Pretilachlor applied fields.

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