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Research Article

# Relative Tolerance and Nitrogenase activity of Several Heterocystous Cyanobacteria to Herbicide, Hiltachlor, 50 EC.

Sahu J K[1], Bhattacharyya S[2], Deep P R[2], Nayak B[2*]

[1]*Department of Botany, Trust Fund Degree College, Bargarh-768028, Odisha, India*
[2]*Cyanolab, School of Life Sciences, Sambalpur University, Burla-768019 , Odisha, India*

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**ABSTRACT**
Effect of various doses of herbicide, Hiltachlor on growth, chlorophyll *a* content and nitrogenase activity (Acetylene reduction activity, ARA) was studied on two species each of genus *Nostoc*, *Anabaena*, *Aulosira* and *Calothrix*. *Nostoc sp.* UU29130 and *Aulosira sp.* UU25118 tolerated higher concentration of Hiltachlor and both the species of *Calothrix* having sheath are found to be more sensitive. Chlorophyll *a* content of the experimental organisms followed a similar trend like that of influence of Hiltachlor on growth. Sublethal dose of the herbicide completely ceased ARA of all organisms except *Nostoc sp.* UU29130 and *Calothrix sp.*UU24112. Further, the growth and nitrogenase activity under influence of herbicide on the experimental organisms does not go parallel. Response of Hiltachlor varied between the two different species belonging to the same genus.

**Keywords:** Acetylene reduction assay, Carotenoid, Chlorophyll-*a*, Hiltachlor

## INTRODUCTION

Cyanobacteria are diverse group of Gram negative prokaryotes[1] belong to ancient group of organisms that are recorded even from Precambrian microfossils[2,3] and dominate over a wide range of diverse habitat[4,5]. Rice fields of several countries including India have been investigated in which a dominant presence of these organisms[6-9] have been reported. Majority of them are nitrogen fixers and their presence in the rice fields add nitrogen to the soil[10]. These organisms have been used as biofertilizer in rice based cropping system[11-13].

In modern agricultural practices, large number of pesticides and herbicides are used to combat pests, weeds and diseases of rice crop. But, application of these agrochemicals and their persistence in rice fields brings harms to many nontarget beneficial microorganisms including cyanobacteria which is considered to be a natural nitrogen fixer. Reduction of cyanobacterial flora in rice field ecosystem has been observed earlier[14]. In the last few decades a number of studies have been made to see the effect of these agrochemicals on various cyanobacterial species [15-22]. In most cases single species toxicity test have been carried out but little emphasis has been paid to the diversity response among cyanobacterial taxa to various pesticides and herbicides. Though, several reports on eco-toxicological impact assessment of pesticide have been made[23-27] rare studies have been made on toxicity of herbicide in rice field ecosystem in particular.

Besides several pesticides, application of herbicide like Hiltachlor, 50 EC is widely used in paddy cultivation of Orissa state. In order to find out the tolerance of several cyanobacterial species, eight species belonging to four different genera were selected and their growth response in terms of Chlorophyll *a* content and Acetylene reduction activity to different concentration of Hiltachlor, 50 EC was studied.

## METHODS

Eight species of cyanobacteria belonging to the genera *Anabaena*, *Nostoc*, *Calothrix* and *Aulosira* were used as the experimental material. One commercial grade herbicide Hiltachlor, 50EC (Hindustan Insecticides Ltd., New Delhi was used in the investigation. Fresh stock solutions of these pesticides were prepared in double distilled water and added to the culture medium to obtain the desired concentration. pH of all the media was adjusted to 7.8 prior to sterilization. Experiments were conducted in 15x100 mm Borosil test tubes containing 10ml of medium containing equal amount of homogenized culture suspension (absorbance of the inoculum of each organism from their experimental growth phase at 760nm was 0.4 always). The medium containing 10, 50, 100, 200, 300, 400 and 500 ppm concentrations of herbicide Hiltachlor (50EC). Growth was recorded as absorbance of the homogenized culture suspension at 760nm in a Systronics 105 Spectrophotometer[28]. The EC50 concentration of the herbicide for each organism was inversely estimated from the regression lines of percentage inhibition of growth on log dose of agrochemicals.

Chlorophyll *a* pigment content of the cyanobacterial cells was extracted with 80% acetone. At 660nm, absorbance of the acetone extract was recorded. The quantity of chl *a*

**Author for Correspondence*

Table 1: Growth response (absorbance of the culture at 760 nm) of several species of nitrogen fixing cyanobacteria of rice fields to the herbicide, Hiltachlor (50 EC). Cultures were incubated at 25± $1^0$ C under continuous light 7.5 W/$m^2$ up to 10 days. Absorbance of culture of each species soon after inoculation into the experimental vessels at 760nm = 0.04; Values represent mean of three replicates ± S.D., L= lethal concentration (filamentous structure lost and the organism was bleached

| Organism | Concentration of Hiltachlor(50 EC), ppm | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | 10 | 50 | 100 | 200 | 300 | 400 | 500 |
| *Anabaena* sp. UU2493 | 0.44 ± 0.06 | 0.43± 0.03 (-2.0) | 0.23± 0.01 (-48.0) | 0.11± 0 (-75.0) | L | | | |
| *Anabaena* sp. UU147 | 0.58 ± 0.04 | 0.45± 0.03 (-22.0) | 0.4± 0.01 (-31.0) | 0.37 ± 0.02 (-36.0) | 0.09± 0 (-84.0) | L | | |
| *Nostoc* sp. UU24100 | 0.71± 0.08 | 0.55± 0.02 (-23.0) | 0.5 ± 0.01 (-30.0) | 0.44± 0.04 (-38.0) | 0.33±0.03 (-54.0) | 0.12± 0.01 (-83.0) | L | |
| *Nostoc* sp. UU 29130 | 0.52 ± 0.03 | 0.5± 0.005 (-4.0) | 0.48 ± 0.04 (-8.0) | 0.45± 0 (-13.0) | 0.32±0.03 (-38.0) | 0.24±0.005 (-54.0) | 0.07±0.01 (-87.0) | L |
| *Calothrix* sp.UU24112 | 0.34± 0.05 | 0.25± 0 (-26.0) | 0.14± 0.01 (-59.0) | 0.11 ± 0.005 (-68.0) | L | | | |
| *Calothrix* sp.UU29139 | 0.49± 0.03 | 0.2± 0.005 (-59.0) | 0.15± 0.01 (-69.0) | 0.08 ± 0 (-84.0) | L | | | |
| *Aulosira* sp.UU25118 | 0.3 ± 0.03 | 0.39±0.01 (-23.0) | 0.35± 0.005 (-14.0) | 0.31 ± 0.03 (-3.0) | 0.28± 0 (-7.0) | 0.27 ± 0.02 (-10.0) | 0.12 ±0 (-60.0) | L |
| *Aulosira* sp.UU29147 | 0.61± 0.05 | 0.22 ± 0.02 (-64.0) | 0.18± 0.01 (-70.0) | 0.08± 0.005 (-87.0) | L | | | |

was determined using extinction coefficient of Mackinney [29].

Acetylene reduction activity of the herbicide treated cultures was measured after 24 hours and 10 days of

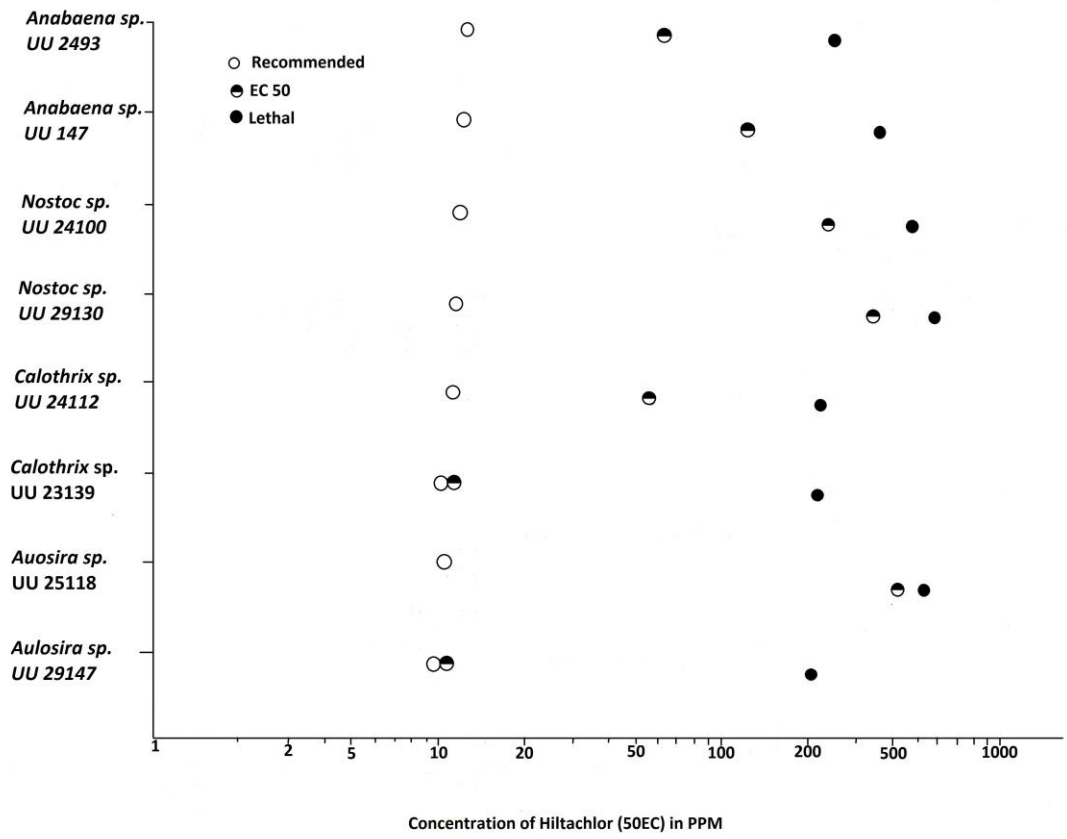


Fig1: Recommended, EC 50 and lethal dose (ppm) of Hiltachlor (50 EC) on growth of eight different species of Cyanobacteria.

incubation. Three selected concentrations of herbicide were selected for such experiments. Nitrogenase activity was measured by acetylene reduction assay (ARA) [30]. Tubes containing cyanobacterial suspension were sealed with selected concentrations of herbicide, Hiltachlor. Two ml of air was removed from each tube and then same quantity of acetylene was injected followed by incubation under light at intensity of 7.5 $Wm^{-2}$. 100µl of the gas phase was removed from the tubes using a Hamilton gas tight syringe and fed to an Amil Nucon 5765 microprocessor based gas chromatograph with FID detector fitted with porapak T column (50-100 mesh), carrier gas Nitrogen =30ml/min and Temperature 100ºC, Injector temperature 110ºC, detector temperature 120ºC). Quantity of acetylene reduced to ethylene was calculated using external standard 105 VPM ethylene in Argon (EDT research, 14 Trading Estate Road London). The Nitrogenase activity was expressed as nmol of $C_2H_4$ / µg Chl *a* / hr.

**RESULTS**

Growth response of eight different species to different concentration of herbicide Hiltachlor, 50 EC is given in table.1. The commercial grade herbicide Hiltachlor 50 EC inhibited the growth of *Anabaena* sp. UU2493, *Aulosira* sp. UU29147 and both the *Calothrix* species when the medium contained 200 ppm of the chemical. *Anabaena* sp. UU147 tolerated up to 200 ppm Hiltachlor, *Nostoc* sp. UU24100 up to 300 ppm, *Nostoc* sp. UU29130 and *Aulosira* sp. UU25118 up to 400 ppm of the herbicide. Further increase in the dose of agrochemical was lethal. Lower concentration of the Hiltachlor up to 100 ppm did not decrease growth of *Aulosira* UU25118 over the control rather at lower concentration of 10 ppm increased the growth of the organism by 30 per cent than the control. Presence up to 200 ppm of the herbicide did not show any adverse effect and 500 ppm of the chemical was lethal for the entire organism.

EC 50 and lethal concentrations of herbicide Hiltachlor, 50 EC to eight different species of cyanobacteria was calculated and given in Fig.1. Wide range of variation in the tolerance of different cyanobacteria to the herbicide Hiltachlor EC 50 was also observed. Fifty percent inhibition in growth of *Calothrix* sp. UU29139 and *Aulosira* sp. UU29147 was observed in presence of the recommended dose of 10 ppm Hiltachlor in the medium. EC 50 dose of *Anabaena* sp. UU2493 and *Calothrix* sp. UU24112 was about 50ppm of the herbicide. EC 50 dose for *Anabaena* sp. UU147 was 88 ppm. Both the species of *Nostoc* and *Aulosira sp.* UU25118 tolerated the higher concentration of the herbicide. EC 50 dose of the chemical for *Nostoc sp.*UU24100 was about 200 ppm and for *Nostoc sp*. UU29130 was nearly 300 ppm, at which concentration most of the organisms except *Aulosira sp.*

Table 2 Chlorophyll-a content (µg/ml) of several species of nitrogen fixing cyanobacteria of rice fields to the herbicide, Hiltachlor (50 EC). Cultures were incubated at 25± $1^0$ C under continuous light 7.5 $W/m^2$up to 10 days. Values represent mean of triplicate determination± S.D.; Values in parenthesis indicate the chlorophyll-a content of the initial inoculum of the respective species.

| Organisms | | Concentration of Hiltachlor(50 EC),ppm | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | 10 | 50 | 100 | 200 | 300 | 400 | 500 |
| *Anabaena* sp. UU2493 | (0.20) | 1.60± 0.18 | 1.51 ± 0.07 (-6.0) | 0.93 ± 0 (-42.0) | 0.20 ± 0.02 (-88.0) | 0 | | | |
| *Anabaena* sp. UU147 | (0.46) | 1.36 ±0.09 | 0.68 ± 0.05 (-50.0) | 0.40 ± 0 (-71.0) | 0.27 ± 0.02 (-80.0) | 0.10 ± 0.005 (-93.0) | 0 | | |
| *Nostoc* sp. UU24100 | (0.26) | 1.67± 0.13 | 1.18 ± 0.09 (-29.0) | 0.64 ± 0.05 (-62.0) | 0.53± 0 (-68.0) | 0.20± 0.01 (-88.0) | 0.16 ± 0.01 (-90.0) | 0 | |
| *Nostoc* sp. UU 29130 | (0.15) | 1.20 ± 0.08 | 1.02± 0.10 (-15.0) | 0.94± 0 (-22.0) | 0.66± 0.04 (-45.0) | 0.40±0.02 (-67.0) | 0.29 ± 0.03 (-76.0) | 0.20 ± 0.005 (-83.0) | 0 |
| *Calothrix* sp. UU24112 | (0.13) | 0.80± 0.06 | 0.40±0.02 (-50.0) | 0.30 ± 0.003 (-63.0) | 0.13± 0 (-84.0) | 0 | | | |
| *Calothrix* sp. UU29139 | (0.46) | 0.94 ± 0.13 | 0.45±0.03 (-52.0) | 0.28 ± 0.01 (-70.0) | 0.07±0.004 (-93.0) | 0 | | | |
| *Aulosira* sp. UU25118 | (0.13) | 0.60± 0.05 | 0.80 ±0.05 (-25.0) | 0.71±0.07 (-15.0) | 0.67± 0.04 (-10.0) | 0.53± 0.005 (-12.0) | 0.42± 0.04 (-30.0) | 0.37±0 (-38.0) | 0 |
| *Aulosira* sp. UU29147 | (0.25) | 1.74 ± 0.11 | 0.78± 0.03 (-55.0) | 0.57± 0.04 (-67.0) | 0.13 ±0.003 (-93.0) | 0 | | | |

Table 3: Nitrogenase activity (n mol.$C_2H_4$/µgChl-a/h) of several species of nitrogen fixing cyanobacteria of rice fields in presence of the herbicide, Hiltachlor (50EC).Cultures were incubated at 25± $1^0$ C under 7.5 W/$m^2$light intensity up to 24 hours or 10 days. Figures in parenthesis represent percent increase or decrease of ARA by the experimental organisms after exposure to various concentration of the herbicide over their respective control values. Figures in brackets under EC 50 and sublethal dose column represent the EC 50 and sublethal concentrations of the herbicide for respective organism

| Organism | Concentration of Hiltachlor (50EC) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 24 hours | | | | 10 days | | | |
| | Control | Recommended dose (10ppm) | EC 50 dose | sublethal dose | Control | Recommended dose (10ppm) | EC 50 dose | sub lethal dose |
| *Anabaena* sp. UU2493 | 3.47 ± 0.1 | 0 | 0[50.6] | 0[100] | 0.41± 0.03 | 0.38± 0.02 (-7.3) | 0.33± 0.02 [50.6] (-19.5) | 0 [100] |
| *Anabaena* sp. UU147 | 0.18 ± 0.01 | 0.17 ± 0.01 (-5.5) | 0 [10] | 0 [200] | 2.16 ± 0.02 | 3.53± 0.03 (+63.4) | 1.37 ±0.01 [10] (-36.6) | 0 [200] |
| *Nostoc* sp. UU 24100 | 0.54 ± 0.03 | 0.88 ± 0.05 (-62.7) | 0[30] | 0 [300] | 1.72 ± 0.01 | 1.22± 0.01 (-29.1) | 0.24± 0.02 [30] (-86.04) | 0[300] |
| *Nostoc* sp. UU 29130 | 0.98 ± 0.005 | 1.08± 0.08 (+10.2) | 0.72± 0.05 [120] (-26.5) | 0.36± 0.04 [400] (-63.2) | 2.96 ±0.1 | 2.36 ± 0.02 (-20.3) | 1.18 ± 0.08 [120] (-60.1) | 0.88± 0.05 [400] (-70.2) |
| *Calothrix* sp. UU24112 | 2.96 ± 0.01 | 1.48± 0.06 (-50) | 1.18± 0.1 [10] (-60.1) | 0.59 ±0.05 [100] (-80.1) | 5.14± 0.3 | 4.11± 0.03 (-20) | 1.61± 0.1 [10] (-68.6) | 0.75±0.04 [100] (-85.4) |
| *Calothrix* sp. UU29139 | 0.12 ± 0.01 | 0.05± 0.005 (-58.3) | 0.04±0.001 [10] (-66.6) | 0 [100] | 12.45± 0.7 | 14.52 ± 0.08 (+16.6) | 12.45± 0.09 [10] (±0) | 0[100] |
| *Aulosira* sp. UU25118 | 0.53 ± 0.05 | 0.26 ±0.03 (-51) | 0[400] | 0 [400] | 2.66± 0.3 | 2.66± 0.01 (±0) | 1.1 ± 0.1 [400] (-58.6) | 0 [400] |
| *Aulosira* sp. UU29147 | 2.12 ± 0.02 | 0 | 0 [10] | 0 [100] | 0.78± 0.05 | 1.04 ± 0.01 (+33.3) | 0.26 ± 0.01 [100] (-66.6) | 0[100] |

UU25118 were killed in the culture. EC 50 dose of the latter species was about 400 ppm and lethal dose of the chemical for the same organism was 500 ppm.

Chlorophyll *a* content of all the experimental organisms in presence of various concentrations of the herbicide followed a similar trend like that of their influence on growth with minor deviations (Table.2). In Hiltachlor supplemented media 22 and 34 percent decrease in Chlorophyll *a* content have been observed in most resistant *Nostoc* sp. UU29130 and *Aulosira* sp. *UU* 25118 whereas most significant decrease in Chl *a* content at EC 50 dose was observed in *Anabaena* sp.UU147 and *Nostoc sp*.

UU24100. For *Calothrix* sp. UU29139 and *Aulosira* sp. UU29147 recommended and EC 50 dose are almost same and Chl *a* content also followed the same trend like the growth curve.

Nitrogenase activity (Acetylene Reduction activity) of the experimental organisms after 24 hours or 10 days of exposure to recommended EC 50 and sub-lethal dose of the herbicide, Hiltachlor has been given in Table-3. In almost all the organisms except both the species of *Calothrix* and *Nostoc* sp. UU29130 nitrogenase activity was completely stopped within 24 hours of exposure to EC 50 dose of the herbicide Hiltachlor. Under similar experimental conditions even recommended dose of the chemical inhibited the ARA of *Anabaena sp*. UU2493 and *Aulosira sp.* UU29147 (Table.3). In both the *Calothrix* sp. ARA was inhibited by 50% within 24 hours of their exposure to recommended dose of Hiltachlor, but the same concentration of the herbicide stimulated the ARA of *Nostoc* sp.UU29130 at recommended dose followed by a decrease in the activity in the presence of higher concentration of the chemical within 24 hours of incubation in light. However, with prolonged incubation in the presence of different concentrations of the herbicide up to 10 days, an adverse effect of the herbicide on the ARA of almost all organisms was considerably removed. On $10^{th}$ day, recommended dose of Hiltachlor did not show any adverse effect on the ARA of *Aulosira* sp. UU25118 and increased the ARA of *Calothrix* sp. UU29139, *Aulosira* sp. UU29147 and *Anabaena* sp. UU147 by 17, 33 and 63 per cent respectively over the control (Table.3). ARA of all the organisms except that of

*Nostoc sp*. UU29130 and *Calothrix* sp. UU24112 was completely ceased in the presence of sublethal dose of the herbicide. However, ARA of *Aulosira sp.* UU29147, *Aulosira sp.* UU25118, *Calothrix sp.*UU24112 and *Nostoc sp*. UU24100 was decreased by 59, 67, 69 and 86 percent in presence of EC 50 dose of Hiltachlor in 10 days respectively. *Nostoc sp.*UU29130 and *Aulosira sp*.UU25118 two herbicide tolerant species among the test organisms behave differentially on ARA activity at 24 hours and 10 days of incubation. Increase in ARA activity of *Nostoc* sp.UU29130 was observed up to 10% at recommended dose on 24 hours exposure whereas; at same condition in *Aulosira* sp.UU25118 ARA activity was decreased drastically. But, during 10 days ARA activity at the recommended dose was reverse. ARA activity of *Nostoc* sp.UU29130 ceases by 20% whereas in *Aulosira* sp.UU25118 ARA activity remain unchanged.

## DISCUSSION

Hiltachlor, a systemic herbicide showed a great deal of variation on toxicity to eight test algae. Out of eight nitrogen fixing cyanobacteria used in the experiment, *Nostoc* sp. UU29130 and *Aulosira* sp. UU25118 were comparatively more tolerant species to the herbicide. *Aulosira sp.* UU29147 along with both the species belong to genus *Anabaena* and *Calothrix* were found less tolerant. Inhibitory effect on algal growth has been reported for many herbicides [26,31-35]. The herbicides are known to interfere with electron transport[36] which is closely associated with PS II. Reactions coupled with PS II and intermediates of noncyclic electron transport system (ETS), get inhibited [37]. Herbicide concentrations inhibiting growth also inhibit Chl a accumulation and nitrogense activity.

Reports on the growth response, Chl *a* content and nitrogenase activity of cyanobacteria to herbicide showed a great deal of variation among the species of the same genus and between the genera [38]. Further, herbicide tolerant species were never accompanied by protection to their nitrogen fixing capacity with increase in the herbicide dose. These, results indicate that growth and nitrogenase activity of rice field cyanobacteria under the influence of various doses of herbicide did not go parallel (Fig1).Thus, a specific herbicide applied to control a target organism showed adverse effect on the growth of a non-target species.

Species which possesses a well-defined sheath around their trichomes could tolerate higher concentration of herbicide[16,18,39,40]. But, present investigation on the effect of Hiltachlor on the growth (Table.1) and Chl *a* content (Table.2) on eight tested cyanobacteria showed that well-defined external envelope of genus *Calothrix* seldom protect their cells from the adverse effect of the herbicide than that of unsheathed form like *Nostoc.* Further, the toxicity of particular cyanobacteria also varied between two different species under the same genus suggesting that pesticide tolerance by a specific organism is genetically determined and is not affected due to the presence of external envelope layer always.

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