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Volume 10(6)

Potential Use of Rice Field Cyanobacterium *Nostoc muscorum* in the Evaluation of Butachlor Induced Toxicity and their Degradation

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Received May 26, 2014; Accepted May 28, 2014; Published June 30, 2014

Abstract:
In the present study, butachlor (5, 10, 20, 40 and 80 ppm) induced toxicity in *Nostoc muscorum* and their degradation was evaluated. The dose of butachlor dependent decreased in the cell survival and growth of *N. muscorum* was noticed. Scanning electron microscopy revealed the adverse impact on the cell size and shapes. Low concentrations of butachlor (10 and 20 ppm) induced the over expression of a polypeptides of 31.0 K Da and 42.7 K Da, respectively which could be responsible for developing resistance in the organism up to certain level. Further, the degradation product of butachlor as a result of metabolic activities of *N. muscorum*, identified by GC-MS analysis includes phenols and benzene dicarboxylic acid indicating the utilization of herbicide during active growth.

Keywords: Bioremediation, Butachlor, Cyanobacteria, GC-MS, SDS-PAGE, SEM

Background:
Butachlor, belongs to the chloroacetanilide class of herbicides, are being used as a pre-emergence pesticide in modern agriculture to control weeds, crop losses and production losses in rice field **[1].** Extensive use of herbicides in the agricultural land has been reported to adversely affect the diversity, biology or even sustainability of cyanobacteria **[2].** They persist in agricultural soils, ground and surface waters, as organic pollutants **[3, 4].** Its bioaccumulation in the ecosystem's primary producers and subsequent propagation through the trophic chain is a major concern **[5].**

Cyanobacteria are an important component of microbial population in wetland soils, especially in rice paddy fields, where they significantly contribute to building-up soil fertility as a natural biofertilizer **[5].** They have been shown to degrade organic pollutants including herbicides and transform their original forms into other more or less toxic forms. Ateeq *et al*., **[6]** demonstrated that the toxicity of butachlor is not only due to parent compound, but also its degradation product such as dialkylquinoneimine. Herbicides persist in fertile layer of soil and harms non-target organisms of soil including cyanobacteria by inducing oxidative stress leading to considerable losses in crop yield **[7, 8].** The effect of butachlor on growth and various metabolic activities have been well documented. However, the use of rice field cyanobacterial strain for the remediation of butachlor and toxicity assessment is less explored. Therefore, the objectives of the present study are to investigate the tolerant mechanism of cyanobacterium *Nostoc muscorum* to butachlor treatment and degradation of the butachlor.

Methodology:

Organism and culture conditions

Cyanobacterium *Nostoc muscorum* was originally obtained from Ranjan Plant Physiology and Biochemistry Laboratory, Department of Botany, University of Allahabad, Allahabad. The culture was maintained in the culture room at 27 ± 2°C under 75 µmol m^{-2} s^{-1} photon flux density (PFD) with a photoperiod of 14:10 h. in the Department of Biosciences, Integral University, Lucknow. CHU-10 medium was used without extra supplementation of combined nitrogen for the growth. The exponentially grown cyanobacterial cells were used throughout the experiment.

Butachlor treatment

The stock solution of butachlor was prepared by dissolving it into the sterilized nutrient medium (Chu -10).Various required concentrations of butachlor (5, 10, 20, 40 and 80 ppm) were prepared from stock solution. For each experiment, the solution of butachlor was freshly prepared and sterilization was done by passing through a Millipore membrane filter (0.22 mm).

Survival and growth determination

The survival of *Nostoc muscorum* against butachlor was determined by viable cells count. Cyanobacterial cells (0.05 mL) obtained from exponential phase, were inoculated onto agar plates containing 5, 10, 20, 40 and 80 ppm of butachlor. After 15 days of incubation under growth conditions, the survival was recorded by counting the colonies of cyanobacterium with respect to untreated control. Growth of cyanobacterium was measured by estimating dry mass at regular intervals up to 10 days of butachlor treatment as per the method of Sheeba *et al*., **[9].**

Scanning Electron Microscopy

Morphological alteration in the cells of cyanobacteria was determined by Scanning electron microscopy (SEM) by following the modified method of Goldstein *et al*. **[10]** and Hayat **[11].** The cells of *Nostoc muscorum* (treated and control) after centrifugation were chemically fixed with an equal amount 2 % glutaraldehyde (1, 5-glutaraldehyde) in a 0.1 M cacodylate buffer (pH 7.2) for 20 min on ice bath. The samples were then washed with same buffer and then by distilled water. Cyanobacterial samples were then dehydrated in ethanol followed by critical point drying. Afterwards, samples were placed on stubs and coated with a layer of Gold –Palladium alloy at 18 mA for 160 sec by Polaron Sputter Coater SC7640. Finally the images were magnified to 25.00 KX and viewed with LEO 430 Cambridge Scanning electron microscope.

SDS-PAGE analysis of proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done on 12 % polyacrylamide resolving gel according to the method of Laemmli **[12].** Approximately 25 µL containing 25 µg protein from treated and untreated extracts were loaded in each well.

GC-MS analysis

2 µL of cyanobacterial extract in dichloromethane was subjected to GC-MS/MS analysis. GC-MS/MS analysis was carried out on a Trace GC Ultra coupled to TSQ Quantum XLS mass spectrometer (Thermo Scientific, USA). The separation was achieved on a TG-5MS capillary column (30 m length × 0.25 mm I.D. × 0.25 µm film thickness). Oven temperature was programmed as follows: initial 70 °C (1.00 min); increased to 210 °C at the rate of 15 °C/min; further increased to 230 °C at a rate of 2 °C/min; and finally increased up to 280 °C at the rate of 15 °C/min (5.00 min). Injection was performed in split less mode at an injector temperature of 280 °C. Helium was used as carrier gas at a flow rate of 1 mL/min. Quantification was performed in selected reaction monitoring (SRM) mode. Two transitions were used; the quantifier (237 → 160) and qualifier (176 → 146) for butachlor.

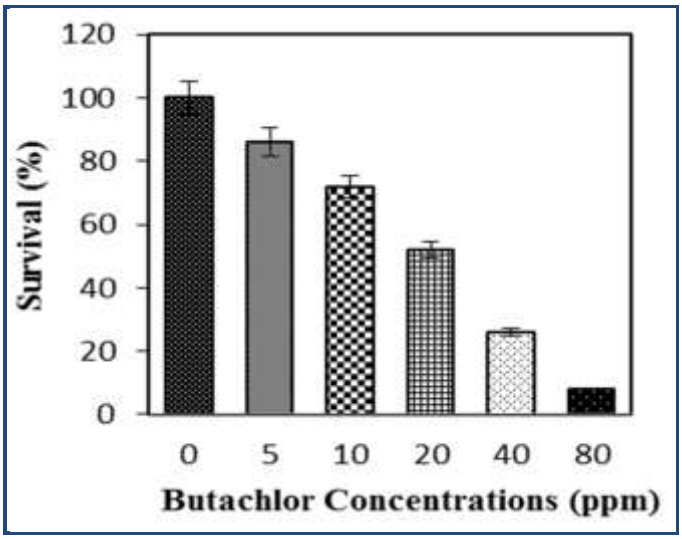

Figure 1: effects of butachalar on cell survial of *N. muscorum* measured after 15 days.Mean ± SE. All the treatments are significantly different at P<0.01.

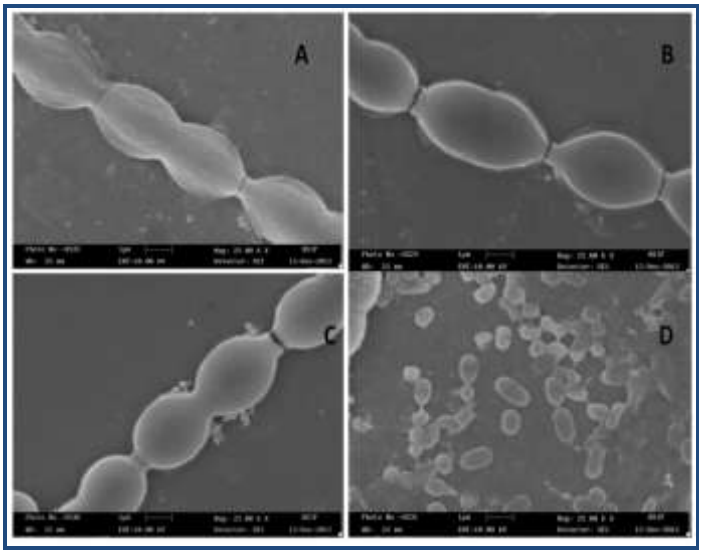

Figure 2: Scanning electron micrographs of N.muscorum treated with butachlor; **(A)** Control filament **(B)** 5ppm **(C)** 20ppm and **(D)** 80ppm.

Result:

In response to butachlor treatment, a significant decrease in the survival and growth of *Nostoc muscorum* was observed. Cell

survival of *N. muscorum* treated with different concentration of butachlor was 86 %, 72 %, 52 %, 26 % and 8 % at 5, 10, 20, 40 and 80 ppm, respectively **(Figure 1).** The viability of the cell could also be reflected by changes in cell morphology, membrane integrity, metabolic activities and the levels of biomolecules such as protein and DNA. Scanning electron microscopy observations showed that butachlor caused swelling in the cells of the filament followed by separation. At high concentration (80 ppm) of butachlor, cells collapsed and completely separated from each other indicating the potential adverse effect of butachlor on the cellular integrity and metabolic activities of *N. muscorum* **(Figure 2 A, B, C & D).**

Figure 3 shows the effect of various concentrations (0, 5, 10, 20, 40 and 80 ppm) of butachlor on the growth pattern of *N. muscorum*. It can be seen from the figure that butachlor consistently inhibited the growth of *N. muscorum* in concentration dependent manner. Upon increasing the concentration of butachlor from 5 to 20 ppm, the growth of *N. muscorum* declined from 20 to 76 % to that of control after 10 days of treatment. Further, increase in the butachlor concentration (from 40 to 80 ppm) did not show any growth even after 10 days of treatment, indicating the failure of the cell system to come back in the active forms.

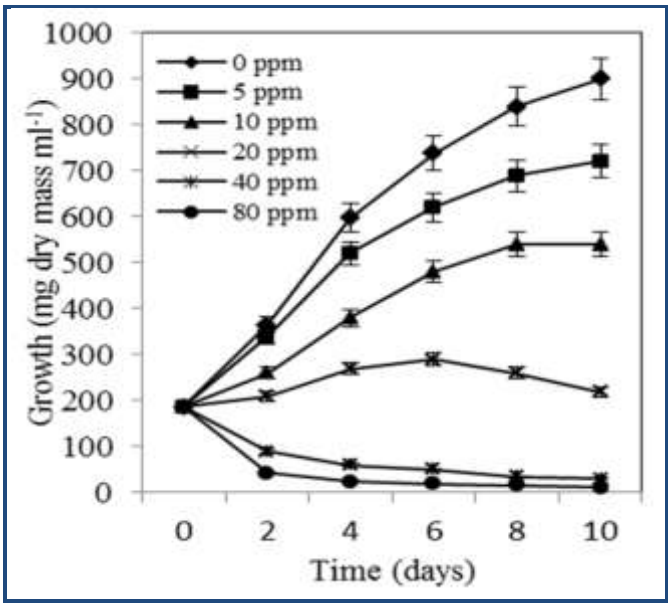

Figure 3: Growth characteristic of *N.muscorum* under different concentration of butachalor in liquid medium. Mean ± SE with n=3.

SDS – PAGE analysis of crude extract of protein isolated from the control and butachlor treated *Nostoc muscorum* after 48 h of treatment is shown in **(Figure 4)**. The bands resolved were classified according to their molecular weight markers (lane-1). Scanning of the gel indicated butachlor concentration at 10 ppm resulted in the induction of one new polypeptide of 31 KDa (lane-4) which can be seen more prominent at 20 ppm (lane-5) concentration. In addition to this on increasing the concentration, most of the polypeptides bands disappeared or inhibited as compare to control. Further, the band of 42.7 KDa was found over expressed at 10 and 20 ppm concentration of butachlor.

The GC-MS study of butachlor suspension exposed to *N. muscorum* are shown in **Table 1 (see supplementary material)** and **(Figure 5 D).** The major residues obtained after 72 h of exposure were Phenol (RT: 8.72), 1, 2-Benzenedicarboxylic acid (RT: 12.98) and Butachlor (RT: 15.68). Butachlor amount was found left 0.408 µg/mL which is 8 % of the initial amount (5ppm/5µg mL^{-1}) indicating the uptake or breakdown of butachlor by *N. muscorum* **(Figures 5 A). Figure 5 B and C** shows mass spectrum and structure of butachlor, respectively.

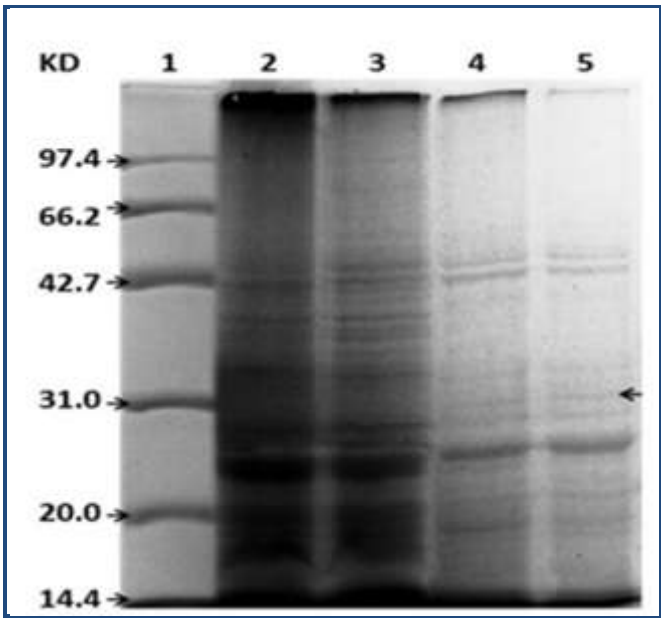

Figure 4: The SDS-page of total protein of Nostoc muscorum treated with different concentrations of butachlor, lane 2 (Control), lane 3 (5ppp), lane 4 (10ppm) and lane 5 (20ppm). The polypeptide bands which became enhanced or appared in response to butachlor is indicated by arow.

Discussion:

The awareness related to the herbicide toxicity in microorganisms, and their degradation is growing as a result of increased investigation and improved modern techniques. In the present study attempt has been made to evaluate butachlor induced toxicity in *Nostoc muscorum* exposed to butachlor treatment and its degradation.

Our findings show that *Nostoc muscorum* is sensitive to herbicide butachlor at all the concentration used. A decrease in cell survival was noticed with an increasing concentration of butachlor indicating the dose dependent effect on cell survival **(Figure 1).** It might be due to damage caused at the level of bio molecules or to the metabolic and physiological activities **[13, 9].** Decline in the cell survival could be correlated with changes in cell morphology **(Figure 2)** which reflects the status of membrane integrity, metabolic and physiological activities and on the levels of biomolecules such as ATP, proteins and nucleic acids **[14, 15].**

Like cell survival, growth of *N. muscorum* was also inhibited by increasing concentration of butachlor **(Figure 3)** which is in agreement with the results of earlier workers **[16, 9].** It has been suggested that reduced growth of cyanobacterium *N. muscorum* was due to inhibitory effect of herbicide on pigment synthesis, photosynthetic activity and nitrogen metabolism **[17, 18].**

During various biotic and abiotic stress factors the equilibrium between the production and the scavenging of ROS may disturbed and lead to sudden increase in intracellular levels of ROS which can cause significant damage to proteins, lipids, carbohydrates, DNA and thereafter cell structures **[19, 20].**

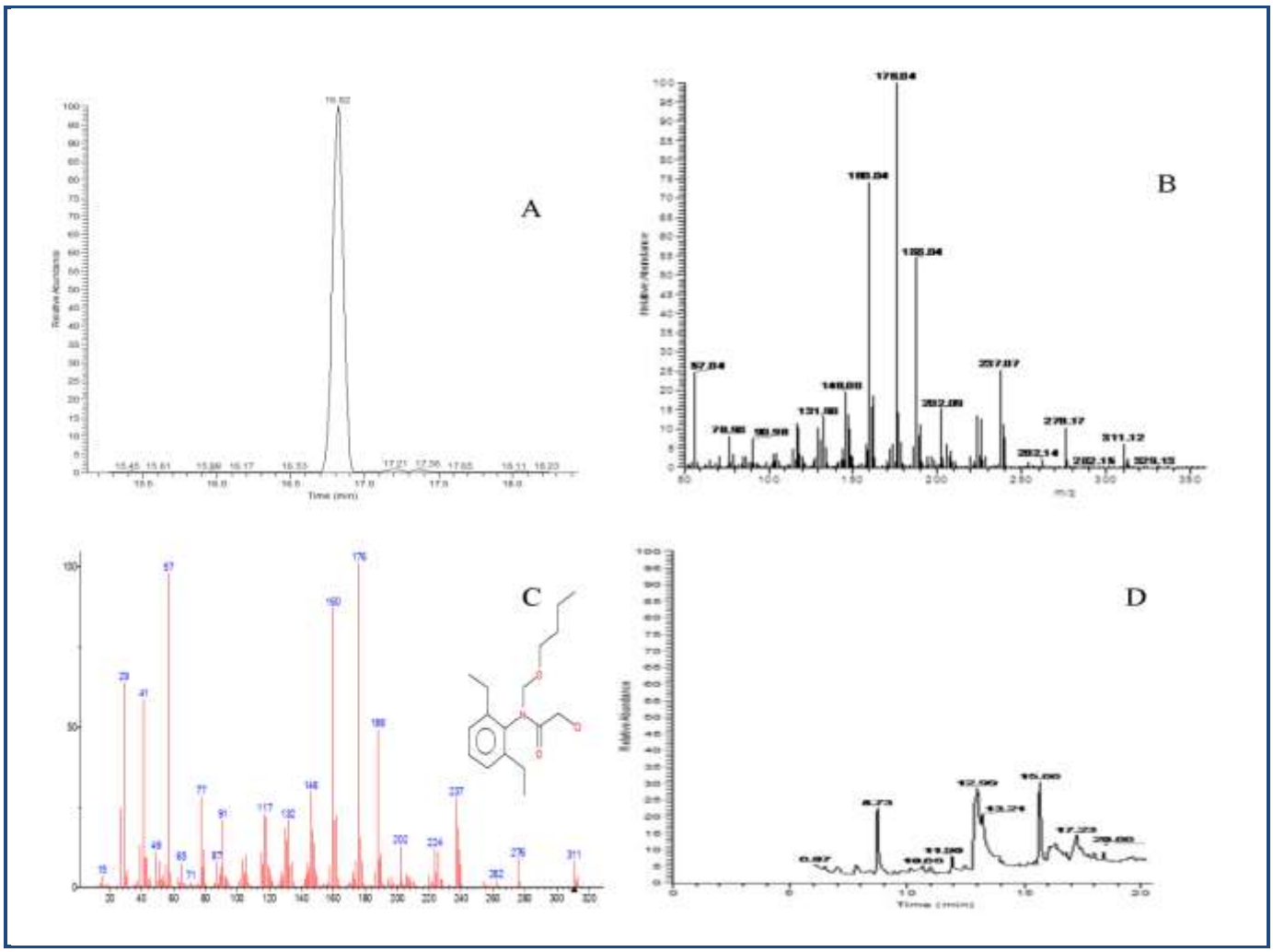

Figure 5: GC-MS determination of butachlor degradation in media including the 5ppm herbicide performed after 72hrs of incubation **(A)** Total butachlor was found to be 0.408 mg/ml. **(B)** Mass spectra of butachlor **(C)** library match of butachlor **(D)** GC-MS chromatogram in full scan.

Gel-electrophoretic investigation of protein profile demonstrated that butachlor at the concentration of 10 and 20 ppm induced a polypeptide of ~31.0 K Da and over expression of ~42.7 K Da **(Figure 4).** This may be ascribed to the utilization of the chemical itself or its degradation products providing carbon skeleton for amino acid forming protein **[21].** In another study, new polypeptides of ~280, 152, and 25 K Da (in 250 ppm Bavistin), ~58 and 28 K Da (in 0.3 and 0.2–0.4 ppm monocrotophos, respectively) and ~31, 28, and 26 K Da (in 0.5 and 1.0 ppm Nimbicidin) were detected in the treated cells of cyanobacterium **[22].** Loss of polypeptides above 42.7 K Da 10 and 20 ppm of butachlor may be correlated with the enhanced production of ROS. The reduction in the amount of butachlor from the cell suspension as observed by GC-MS analysis after 72 h **(Figure 5B)**, indicated the presence of 1, 2-Benzenedicarboxylic acid and phenol. The reason behind the degradation of butachlor at 5ppm could be due to active metabolic activities at low dose of butachlor. Gushit *et. al.,* **[23]** also found residues of 1, 2-Benzenedicarboxylic and phenol from pesticide treated plants. In conclusion, it appears that herbicide butachlor can cause damage to the biomolecules and cell components of *N. muscorum*. However, the breakdown/utilization of butachlor was observed at the low concentration (5 ppm). Further, increased concentration has adverse impact on the cell survival, morphology and protein profile indicating the ROS mediated toxicity in the cells of *N muscorum.* The toxic effects of butachlor may be due to its original form or intermediate compounds which might have induced the formation of ROS and.

Acknowledgement:
Financial assistance to Miss Sumaiya Anees as a Maulana Azad National Fellowship by UGC, New Delhi is gratefully acknowledged. Authors are thankful to the Prof. (Dr.) A.K. Srivastava, Head, Department of Biosciences for providing necessary laboratory facilities and making arrangement for the SEM and GC-MS analysis. Authors are also grateful to the Vice Chancellor and Pro-Vice Chancellor for encouragement and providing necessary facilities.

Conflict of interest:
The authors declare that there is no conflict of interest.

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Edited by P Kangueane
Citation: **Anees *et al.*** Bioinformation 10(6): 365-370 (2014)

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Supplementary material:

Table 1: Residues in the cell suspension identified after 72 hrs of butachlor exposure to *N. muscorum*.

Organism	Herbicide	Residues	Retention Time
N. muscorum	Butachlor	Phenol	8.72
		1,2-Benzenedicarboxylic acid	12.98
		Butachlor	15.68