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Impact of Pretilachlor on Wild and Mutant strains of *Nostoc spongiaeforme*

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Abstract
In present research, impact of pretilachlor herbicide on two cyanobacterial strains: wild *Nostoc spongiaeforme* and mutant herbicide resistant *Ns*(MHR)^5 was investigated. We elucidated physiological, biochemical responses of these cyanobacteria to pretilachlor and assessed potential for herbicide resistance. Results revealed that pretilachlor adversely effected growth, photosynthetic pigments and metabolic processes in both strains. *Ns*(MHR)^5 showed greater resistance and resilience to pretilachlor. Pretilachlor significantly decreased photosynthetic pigments in both strains but *Ns*(MHR)^5 exhibited lower reduction. Herbicide particularly affected photosynthetic processes with PS-II, photosynthetic chain activity, respiration and nitrogen assimilation processes more severely in *N. spongiaeforme*. Nitrate uptake was negatively impacted in *N. spongiaeforme* but not in *Ns*(MHR)^5. LD_{50} of pretilachlor to *Ns*(MHR)^5 was observed to show higher tolerance. This research highlighted the potential for herbicide resistance in *Ns*(MHR)^5. These findings shed valuable insights on broader implications of herbicide usage on aquatic ecosystems and for the evolution of resistance mechanisms in cyanobacterial populations.

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INTRODUCTION

Cyanobacteria, often referred to as blue-green algae, constitute a diverse and widespread group of prokaryotic organisms with a rich evolutionary history dating back to the Precambrian era. These remarkable microorganisms thrive in an extensive range of environments, from aquatic ecosystems to terrestrial habitats. These organisms have different morphologies including unicellular and filamentous forms as well (Castenholz 2001). While unicellular types exist as single cells, suspended or benthic or as aggregates. Filamentous types may be thin or thick, single trichome or bundles either with or without a sheath (Stal 1995). Cyanobacteria show maximum three cell types i.e. vegetative cells, heterocysts and akinetes. Structurally and functionally, the heterocysts are the ideal sites for nitrogen fixation (Haselkorn 1978). Cyanobacteria play a pivotal role in environmental management by serving as biofertilizers, biomonitors of soil fertility and water quality, and agents for the reclamation of saline and degraded lands. Cyanobacteria possess unique ability to perform oxygenic photosynthesis and nitrogen fixation within the same cells or filaments sets them apart in the biological world. Their capacity for nitrogen fixation further underscores their significance in enhancing soil fertility.

Cyanobacteria are known for their ability to adapt to various environmental conditions, including exposure to different stresses such as pollutants and herbicides. Previous studies have shown that cyanobacteria can exhibit diverse responses to herbicides, including changes in photosynthetic pigments, photosynthesis rates, and nitrogen assimilation (Elsa et al. 2015). However, the widespread use of herbicides comes with unintended consequences, affecting non-target organisms and altering ecosystems. Cyanobacteria are not immune to the impact of herbicides. Their versatility and adaptability have drawn the attention of ecologists, physiologists, biochemists, and molecular biologists, making them a subject of extensive scientific investigation. Singh and Datta (2006) assessed the effect on growth of different cyanobacteria e.g. *Nostoc punctiforme*, *N. calcicola*, *Anabaena variabilis* and *Gloeocapsa* sp. and *Aphanocapsa* sp. along with standard laboratory strain *N. muscorum* growth at the lethal concentrations of arozin. Herbicide Bensulfuron-methyl exhibited distinct effect on two freshwater cyanobacteria, *Anabaena variabilis* and *Nostoc commune*. At herbicide concentrations ranging from 8 ppm to 10 ppm, both cyanobacteria species experienced 50% inhibition in both growth and photosynthesis. A significant reduction in their nitrogenase activity was observed when these cyanobacteria were exposed to 10 ppm and 20 ppm of the herbicide. Specifically, *A. variabilis* experienced a dramatic decrease of 94-98% in nitrogenase activity and *N. commune* exhibited slightly lower reduction of 85-86% (Kim and Lee 2006). The toxic impact of the herbicide paraquat at various concentrations within the range of 15-25 ppm for a period of 10 days was investigated under controlled laboratory conditions, focusing on *Anabaena oryzae* and *Nostoc ellipsosporum*. Results of the study unveiled dose-response relationship clearly, indicating that as the concentration of the herbicide increased, it had a progressively detrimental effect on the growth kinetics and formation of heterocysts in these cyanobacteria (Pandey et al. 2011).

In modern agriculture, the use of herbicides, including pretilachlor, has become essential for weed control, increasing crop yields, and minimizing labor and machinery requirements. Pretilachlor (a-chloro-2, 6-diethyl-N-[2-propoxy ethyl] acetanilide) is a member of the acetanilide group of herbicides and is used for the control of most sedges and broadleaf weeds in direct seeded as well as transplanted paddy fields (Baki and Azmi 1992). It is a relatively new acetanilide herbicide developed for pre-emergence use on a wide spectrum of weeds, causing rapid inhibition of root and shoot growth of young plants. This widely used herbicide plays a crucial role in weed control in agricultural practices. The review of literature reveals that considerable amount of work has been reported on inhibitory effects of different herbicides but not on the pretilachlor and its effect on growth, photosynthetic pigments, photosynthesis and nitrogen fixation of

cyanobacteria. Therefore, understanding how pretilachlor affects these process in both wild-type strains and mutant variants will provide valuable information regarding the molecular and biochemical adaptations associated with herbicide resistance. The organisms employed in this study are *Nostoc spongiaeforme*, *N. muscorum*, *Anabaena naviculoides* and *A. torulosa.* These cyanobacteria are unbranched, filamentous, blue-green, mucilaginous, and heterocystous. Vegetative cells of *N. spongiaeforme* are rounded to oval, measuring 3.5-4.0 µm wide and 4 µm long. Heterocysts are 5.0 µm wide and 5.0-6.0 µm long, while spores are 6.0-8.0 µm wide and 8.0-10.0 µm long. The study aimed to elucidate the physiological and biochemical responses of cyanobacteria to pretilachlor exposure through a comparative study of herbicide-resistant mutants. By comparing the responses of herbicide-resistant mutants to their non-resistant counterparts, the study seeks to shed light on the mechanisms underlying cyanobacteria's adaptability to herbicides and their potential role in ecosystem resilience in the face of herbicide applications. Understanding these responses is critical for assessing the environmental impact of herbicides and developing sustainable agricultural practices that minimize their adverse effects.

MATERIALS AND METHODS

Organisms

The organisms employed in this study were *Nostoc spongiaeforme*, *N. muscorum*, *Anabaena naviculoides* and *A. torulosa*, which were isolated from the rice fields of Kum Kalan village (Ludhiana, Punjab) India. A mutant strain named *Ns*(MHR)^5, resistant to Pretilachlor herbicide, was raised through spontaneous mutations and used in this study. Borosil glasswares were used, pre-treated by soaking in chromic acid solution for 24 hours, washed with Teepol liquid soap, rinsed with tap water and oven-dried. All chemicals, including pretilachlor, were of analytical reagent (AR) grade and obtained from reputable suppliers.

Screening of cyanobacterial strain and isolation of herbicide resistant spontaneous mutants

Susceptibility to pretilachlor of four nitrogen-fixing cyanobacterial organisms, namely *Nostoc spongiaeforme*, *N. muscorum*, *Anabaena naviculoides* and *A. torulosa* were screened and spontaneous mutants resistant to the herbicide were isolated. These organisms were inoculated on agar plates containing varying concentrations (5-30 ppm) of pretilachlor and incubated at 28 ± 2 ˚C for 15 days in the culture room. After incubation period, colonies emerged on the agar plates were randomly selected and for growth transferred to Chu-10 medium (Safferman and Morris, 1964). Chu-10 medium was slightly modified by replacing $CaNO_3$ with equivalent amount of $CaCl_2$ and KNO_3 was used as nitrogen source. These cultures were then inoculated into medium supplemented with increasing concentrations of pretilachlor. Based on the growth response of the clones to pretilachlor, a spontaneous mutant of *N. spongiaeforme*, designated as *Ns*(MHR)^5 was identified. The nomenclature reflects its status as a *N. spongiaeforme* mutant herbicide-resistant clone, with the number 5 denoting its specific clone identification.

Growth of the organisms

The assessment for growth of organism was measured in terms of increase in absorbance, protein content and biomass dry weight of cultures. Exponentially growing cultures of organisms underwent homogenization, followed by centrifugation (5,000 g). A suitable quantity of the inoculum was introduced into test tubes containing 25 mL medium to achieve an initial absorbance of 0.1 at 720 nm. All cultures were then incubated 28 ± 2 ˚C in the incubator. The growth progression of the organisms was observed for 12 days at the interval of 3 days. The absorbance of the cultures was recorded at 720 nm using spectrophotometer (Spectronic - 20D+, Thermospectronic, USA). The same culture was utilized for determining protein content and biomass dry weight. The quantification of protein content for the cultures was assayed by following the methodology outlined by Lowry et al. (1951). Bovine Serum Albumin served as the reference for constructing the standard curve. Cell harvesting was achieved through centrifugation at 5,000 g followed by triple washing with double-distilled water. Subsequently, for protein extraction the cells were hydrolysed with 1N NaOH in boiling

water bath for 10 minutes. After cooling, centrifugation was conducted and the supernatant obtained was employed for protein determination. The absorbance for estimating protein was measured at 660 nm using a spectrophotometer. A predetermined volume of the cultures was extracted, subjected to centrifugation, and washed twice with double-distilled water. The resulting pellet was then transferred to a watch glass and kept for drying in oven at 60 ^{o}C for a duration of 24 hours. The weight of the watch glass, both with and without the dried biomass was meticulously recorded. The calculation of the dried biomass weight involved subtracting the weight of the watch glass lacking biomass from the weight of the watch glass containing the biomass.

Determination of LD_{50} and lethal doses of herbicide

LD_{50} and lethal doses of pretilachlor for the test organisms were determined by exposing them to graded concentrations of pretilachlor. The biomass dry weight and the percent decrease in growth were noted at various concentrations of herbicide. LD_{50} and lethal doses were calculated using ORIGIN software.

Herbicide effect on photosynthetic pigments

Concentration of pigments chlorophyll a and carotenoids was determined by extracting cells with acetone, and phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin) by extracting cells with water. Concentrations were estimated by measuring absorbance at specific wavelengths.

Acetone soluble pigments

For the extraction of acetone-soluble pigments like chlorophyll a and carotenoids, a specified volume of cultures underwent centrifugation. Acetone was added to the resulting pellet and the mixture was vigorously shaken before being incubated in a refrigerator for 10-12 hours. Subsequently, the contents were centrifuged, and the absorbance of the supernatant chlorophyll a and carotenoids was measured at 665 nm and 410 nm respectively. The quantification of chlorophyll a and carotenoids was done by following Kratz and Myers (1955).

Water soluble pigments

The quantification of phycobiliproteins (PBP) was conducted according to the method outlined by Bennet and Bogorad (1973). A predetermined volume of a homogenous cell suspension of the organism underwent centrifugation at 5,000 g for 10 minutes. The resulting pellet was resuspended in double-distilled water and vigorously shaken. This suspension underwent multiple cycles of freezing and thawing to ensure the release of all pigments from the cells. Subsequently, the contents were centrifuged at 5,000 g for 5 minutes. The absorbance of the supernatant was then measured at 562, 615 and 652 nm and quantification of pigment amounts (µg mL^{-1}) was determined.

Photosynthesis, respiratory and photochemical activities assay

The oxygen evolution rate and dark respiration rate were measured by monitoring the dissolved oxygen with an oxygen electrode under high light intensity and dark conditions. Photochemical activities of PS-I and PS-II were assessed by determining light-dependent oxygen uptake and oxygen evolution, respectively in the presence of specific components. Heterocyst frequency was determined by counting the number of vegetative cells and heterocysts in the filaments and expressing it as a percentage.

Photochemical activities of both test organisms were investigated in accordance with the methodology outlined by Chen et al. (2007). Exponentially growing cultures were incubated in a basal medium supplemented with pretilachlor (15.5 ppm). Subsequently, samples were harvested through centrifugation and concentrated by suspending them again in a reduced volume of the same medium. The electron transport activity of intact cells was assayed at 25 °C by subjecting them to illumination from a lamp with a light intensity of 8000 lux.

The activity of PS-I was quantified through light-dependent oxygen uptake in a solution comprising pretilachlor (15.5 ppm), 25 mM bistris propane (pH 7.8), 0.1 mM 2,6-dichlorophenol indophenol, 5.0 mM ascorbate,

0.1 mM methyl viologen as the electron acceptor, 1.0 mM NaN_3 and 10 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

The activity of PS-II was estimated by measuring the oxygen evolution with water as the electron donor and p-benzoquinone as the electron acceptor in 2.0 mL medium containing 25 mM bistris propane (pH 7.8) and 1.0 mM p-benzoquinone. The photosynthetic electron transport activity was quantified through light-dependent oxygen uptake, with water as the electron donor and methyl viologen as the electron acceptor in a 2.0 mL medium comprising of 25 mM bistris propane (pH 7.8), 1.0 mM NaN_3, and 0.1 mM methyl viologen.

Statistical analysis

Data were reported as the mean, standard deviation of three independent experiments, each with three replicates. The coefficient of correlation and LD_{50} values of selected herbicides were calculated using ORIGIN software.

RESULTS

Pretilachlor showed the effect on various parameters including growth, protein content, photosynthetic pigments, photosynthesis and respiration on *N. spongiaeforme* and *Ns*(MHR)^5 (mutant herbicide resistant).

Screening of cyanobacterial strain and isolation of herbicide resistant spontaneous mutant(s)

Nitrogen-fixing cyanobacterial organisms including *Nostoc spongiaeforme*, *N. muscorum*, *Anabaena naviculoides* and *A. torulosa*, isolated from the laboratory, were subjected to testing for their tolerance to the herbicide pretilachlor. After 15 days on colony formation, a total of 200 healthy colonies were randomly selected for further analysis, with 69 clones from *N. spongiaeforme*, 54 from *N. muscorum*, 40 from *A. naviculoides*, and 37 from *A. torulosa*. The selected clones were inoculated in liquid medium with the 5-30 ppm concentration of pretilachlor and allowed to grow for 20-25 days. Results showed that in basal medium supplemented with pretilachlor above 10 ppm concentration, none of the clones exhibited growth. However, at lower herbicide doses (5-10 ppm), 14 clones from *N. spongiaeforme* displayed growth, while clones from other organisms did not grow at any pretilachlor concentration. Further investigation involved growing the 14 clones of *N. spongiaeforme* for 3 days in graded concentrations of pretilachlor (5, 7.5, 10, and 12.5 ppm). Amongst 14 clones Ns 5, Ns 13 and Ns 22 showed superior growth. These three clones were selected for comparative study and cultivated in the basal medium supplemented with pretilachlor of 10 and 15 ppm for 9 days (Figure 1) and their growth was compared. Clone Ns 5 showed the maximum growth amongst the three clones and was consequently designated as *Ns*(MHR)^5, signifying as a *N. spongiaeforme* mutant with herbicide resistance with the number 5 denoting the clone identifier.

Growth of the organisms

The growth of experimental organisms in the presence of pretilachlor was assessed through the monitoring of absorbance, protein content, and dry weight over time. Growth experiments were conducted by inoculating cultures in basal and nitrate (10 mM) medium, supplemented with varying concentrations of pretilachlor, and adjusting the initial absorbance of the cultures to 0.1 at 720 nm. In the control cultures of *N. spongiaeforme* and *Ns*(MHR)^5 in basal medium, absorbance increased from 0.1 to 0.518 and 0.84, respectively, on day 12 (Figure 2).

In nitrate-supplemented medium, the absorbance of *N. spongiaeforme* and *Ns*(MHR)^5 cultures increased from 0.1 to 0.7 and 1.01, respectively (Figure 3a). Concentration-dependent reductions in the growth of both organisms were observed in pretilachlor-supplemented media. *N. spongiaeforme* and *Ns*(MHR)^5 exhibited growth reductions of 60%, 40.7%, 34.9%, and 15.8%, and 80.5%, 81.9%, 63.3%, and 47.7% on day 12 compared to control cultures in the presence of 5, 10, 15, and 20 ppm pretilachlor, respectively. In nitrate-supplemented medium, *N. spongiaeforme* and *Ns*(MHR)^5 showed growth reductions of 70%, 52.7%, 38%, and 13.5%, and 90.3%, 81.1%, 66.3%, and 52.3% on day 12 compared to control cultures in the presence of 5, 10, 15, and 20 ppm pretilachlor (Figure 3b). Thus, the impact of

pretilachlor on the growth of *N. spongiaeforme* and *Ns*(MHR)^5 was comparatively less in nitrate-supplemented media. Furthermore, the effect of pretilachlor on the growth of *Ns*(MHR)^5 was less pronounced compared to *N. spongiaeforme*.

In basal medium, the protein content of *N. spongiaeforme* cultures increased from 19.36 to 71.8 µg mL^{-1} on day 12 (Figure 4a), whereas *Ns*(MHR)^5 cultures exhibited an increase from 23.27 µg mL^{-1} on day 0 to 152.14 µg mL^{-1} on day 12 (Figure 4b). In nitrate-supplemented medium, the protein content of *N. spongiaeforme* and *Ns*(MHR)^5 increased to 89.4 µg mL^{-1} and 180 µg mL^{-1}, respectively, on day 12 (Figure 5a). Both organisms displayed a concentration-dependent decrease in protein content of cultures in the presence of graded concentrations of pretilachlor, consistent with the observed absorbance trends.

The protein content of *N. spongiaeforme* and *Ns*(MHR)^5 cultures showed reductions of 53.9%, 42.3%, 24.2%, and 9.0%, and 89.4%, 78.9%, 68.4%, and 34.1%, respectively, on day 12 in basal medium supplemented with 5, 10, 15, and 20 ppm of pretilachlor (Figures 4a, 4b). In nitrate medium supplemented with 5, 10, 15, and 20 ppm pretilachlor, the protein content of *N. spongiaeforme* cultures decreased by 64.8%, 48.4%, 34.8%, and 16.2%, respectively, while the protein content of *Ns*(MHR)^5 cultures decreased by 97%, 87.7%, 80.5%, and 41.6%, respectively, compared to nitrate medium (Figures 5a, 5b). Pretilachlor exhibited a more severe impact on the protein content of *N. spongiaeforme* cultures compared to *Ns*(MHR)^5.

The growth of the test organisms, as indicated by the increase in the dry weight of the cultures, mirrored the patterns observed in both absorbance and protein content. In basal medium, the dry weight of *N. spongiaeforme* and *Ns*(MHR)^5 biomass increased from 76.6 to 393.4 µg mL^{-1} and from 83.3 µg mL^{-1} to 610 µg mL^{-1}, respectively, on day 12 (Figures 6a, 6b). In nitrate-supplemented medium, the dry weight of biomass for *N. spongiaeforme* and *Ns*(MHR)^5 increased to 485 µg mL^{-1} and 754 µg mL^{-1}, respectively, on day 12 (Figures 7a, 7b). However, a decrease in dry biomass for both test organisms was observed in the presence of pretilachlor. On day 12 in basal medium with 5, 10, 15, and 20 ppm pretilachlor, *N. spongiaeforme* exhibited reductions of 96.6%, 48.3%, 38.1%, and 25.4%, while *Ns*(MHR)^5 exhibited reductions of 90.7%, 87.4%, 37.7%, and 30.0%, in dry weight compared to control cultures (Figures 6a, 6b). In nitrate medium with 5, 10, 15, and 20 ppm pretilachlor, the dry weight of *N. spongiaeforme* cultures was reduced by 87.8%, 66.5%, 43.5%, and 30%, while *Ns*(MHR)^5 cultures exhibited reductions of 91.5%, 85.8%, 41.4%, and 34%, respectively, compared to control cultures (Figures 7a, 7b). These findings indicate that pretilachlor adversely affected the dry weight of both *N. spongiaeforme* and *Ns*(MHR)^5, with varying degrees of impact depending on the concentration of the herbicide in the culture medium.

The growth of both test organisms, in the presence and absence of pretilachlor in both basal and nitrate-supplemented medium, demonstrated a positive correlation between absorbance and dry weight of biomass, as well as absorbance and protein content of the cultures at a 95% confidence level (Figures 8a and 8b).

Determination of LD_{50} and lethal doses of herbicide

To ascertain the LD_{50} and lethal dose of pretilachlor for *N. spongiaeforme* and *Ns*(MHR)^5, dry weight of the cultures was considered as a growth parameter. A plot illustrating graded concentrations of pretilachlor versus the percent inhibition in growth (in terms of dry weight) of the test organisms was generated to determine LD_{50}. In basal medium, the LD_{50} of pretilachlor for *N. spongiaeforme* and *Ns*(MHR)^5 was found to be 15.5 and 21.1 ppm, respectively. The lethal dose of pretilachlor for *N. spongiaeforme* and *Ns*(MHR)^5 was observed to be 30.07 and 42.1, respectively (Figures 9a and 9b). These results provide valuable insights into the concentration levels at which pretilachlor adversely affects the growth of *N. spongiaeforme* and *Ns*(MHR)^5, offering crucial information for assessing the potential impact of this herbicide on these organisms.

Herbicide effect on photosynthetic pigments

The impact of pretilachlor on the photosynthetic pigments of both test organisms was investigated, considering both acetone-soluble pigments (chlorophyll a and carotenoids) and water-soluble pigments (phycobiliproteins).

The chlorophyll a (Chl a) content of *Ns*(MHR)^5, a mutant strain of *N. spongiaeforme*, was significantly higher in both basal and nitrate mediums on the 9th day, measuring 11.7 and 12.3 Chl a mg^{-1} dry weight, respectively, compared to *N. spongiaeforme* cells (8.7 Chl a mg^{-1} dry weight) in corresponding conditions (Figures 10a and 10b). In both organisms, the Chl a content exhibited a decrease with an increase in pretilachlor concentration. Specifically, on the 9th day, *N. spongiaeforme* and *Ns*(MHR)^5 displayed a reduction of 19.5%, 21.8%, 35.6%, and 50.6%, and 20.3%, 20.3%, 22.1%, and 43.3% in Chl a content on a dry weight basis over control cultures in basal medium supplemented with 5, 10, 15, and 20 ppm of pretilachlor, respectively.

In nitrate medium supplemented with 5, 10, 15, and 20 ppm pretilachlor, both *N. spongiaeforme* and *Ns*(MHR)^5 showed decreases of 11.5%, 35.6%, 37.9%, and 54%, and 10.5%, 25.2%, 26.8%, and 41.4% in Chl a content, respectively. The findings indicated that the impact of pretilachlor on the Chl a of *N. spongiaeforme* was more pronounced compared to *Ns*(MHR)^5. Nitrate medium supported higher Chl a levels in both organisms. The effect of 5, 10, and 15 ppm pretilachlor on Chl a in *Ns*(MHR)^5 was comparable, while a significant decrease was observed at 20 ppm pretilachlor. The toxic effect of pretilachlor on the Chl a of *Ns*(MHR)^5 in nitrate medium was relatively less pronounced compared to basal medium cultures.

A concentration-dependent reduction in carotenoids (Car) content in the presence of pretilachlor was observed in control cultures of both *N. spongiaeforme* and *Ns*(MHR)^5. *N. spongiaeforme* and *Ns*(MHR)^5 demonstrated a decrease of 3.8%, 13.4%, 23%, and 30.8%, and 15.4%, 18.3%, 18.3%, and 29.5% in Car content, respectively, on a dry weight basis over control cultures at pretilachlor concentrations of 5, 10, 15, and 20 ppm. In nitrate medium supplemented with pretilachlor, *N. spongiaeforme* and *Ns*(MHR)^5 exhibited a decrease of 5.6%, 16.9%, 26.4%, and 35.8%, and 16.4%, 19.1%, 20.5%, and 31.5% in Car content respectively.

On the 9th day, the Car content of *Ns*(MHR)^5 (6.1 mg^{-1} dry weight) exceeded that of *N. spongiaeforme* (5.2 mg^{-1} dry weight) (Figures 11a and 11b). These results indicated that the Car content of *Ns*(MHR)^5 was comparable to that of *N. spongiaeforme*, and the impact of pretilachlor on Car was less pronounced compared to its effect on chlorophyll a (Chl a).

In basal medium cultures of *N. spongiaeforme* and its mutant strain *Ns*(MHR)^5, there was an evident concentration-dependent reduction in total phycobiliproteins. *N. spongiaeforme* and *Ns*(MHR)^5 displayed a decrease of 6.8%, 12.1%, 19.4%, and 41.4%, and 8.6%, 15.5%, 13.4%, and 18.8% in phycobiliproteins, respectively, in basal medium supplemented with 5, 10, 15, and 20 ppm pretilachlor. In nitrate medium supplemented with 5, 10, 15, and 20 ppm pretilachlor, cultures of both organisms exhibited a decrease of 4.6%, 10.1%, 24.7%, and 41.7%, and 7.8%, 13.5%, 16.8%, and 18.0% in phycobiliproteins over control cultures (Figures 12a and 12b). The total phycobiliproteins of both organisms in basal medium were nearly identical. The addition of nitrate to the basal medium did not result in a significant increase in phycobiliproteins, and the impact of 5 and 10 ppm pretilachlor was not significant. However, 15 and 20 ppm of pretilachlor significantly affected phycobiliproteins.

Photosynthesis, respiratory and photochemical activities

The impact of pretilachlor on the photosynthesis of *N. spongiaeforme* and its mutant strain *Ns*(MHR)^5 was investigated through short-term experiments. Exponentially growing cultures of both organisms were transitioned to pretilachlor (15.5 ppm) supplemented basal and nitrate (10 mM) medium, and the oxygen evolution by cultures in light was examined after 4 and 8 hours. Simultaneously, the quantities of chlorophyll a (Chl a) and phycobiliproteins in

the cultures were determined to assess the effect of pretilachlor on photosynthetic pigments during short-term exposure to the herbicide.

In control cultures, the amount of oxygen evolved by *N. spongiaeforme* and *Ns*(MHR)^5 at zero hours was 46.7 and 57.9 nmol of O_2 evolved mg-1 Chl min^{-1}, respectively. In the basal medium supplemented with pretilachlor, *N. spongiaeforme* exhibited a reduced oxygen evolution of 37.6 nmol of O_2 evolved mg^{-1} Chl min^{-1} at 8 hours, while *Ns*(MHR)^5 showed an evolution of 50.6 nmol of O_2 evolved mg^{-1} Chl min^{-1} (Figures 13a). Notably, the inhibition of pretilachlor on the photosynthetic rate of both organisms commenced at 4 hours and remained relatively constant up to 8 hours. In nitrate medium supplemented with pretilachlor, *N. spongiaeforme* and its mutant strain *Ns*(MHR)^5 exhibited oxygen evolution rates of 38.5 and 52.2 nmol of O_2 mg^{-1} Chl min^{-1}, respectively, at 8 hours. Similar to basal medium cultures, the inhibitory effect of pretilachlor in nitrate medium commenced at 4 hours and remained consistent up to 8 hours (Figures 13b). The percentage inhibition in the oxygen evolution by *N. spongiaeforme* and *Ns*(MHR)^5 in basal medium cultures supplemented with pretilachlor was 19.12% and 12.6%, respectively. In nitrate-supplemented medium cultures, this inhibition was 17.5% and 9.84%, respectively. The impact of pretilachlor on the photosynthetic rate of *N. spongiaeforme* in basal medium was relatively more pronounced compared to *Ns*(MHR)^5, and the addition of nitrate did not enhance photosynthetic oxygen evolution. Notably, the photosynthetic pigments (Chl a and phycobiliproteins) of both test organisms in basal and nitrate medium were unaffected within the 8-hour exposure to the herbicide. These results indicate that the primary target of pretilachlor appears to be the photosynthetic processes rather than the photosynthetic pigments Figures 14a and 14b).

The observed impact of pretilachlor on the photosynthetic rate of the test organisms, the present study aimed to elucidate its specific effects on the photosynthetic machinery, focusing on Photosystem I (PS-I), Photosystem II (PS-II), and the overall photosynthetic electron transport chain. The PS-I activity in the control cultures of *N. spongiaeforme* and its mutant strain *Ns*(MHR)^5 at zero hours was measured at 50.68 and 54.68 nmol of O_2 consumed mg^{-1} Chl a min^{-1}, respectively. In basal medium supplemented with pretilachlor, the PS-I activity of *N. spongiaeforme* and *Ns*(MHR)^5 was 46.5 and 52.1 nmol of O_2 consumed mg^{-1} Chl a min^{-1}, respectively, at 8 hours (Figures 15a). This indicated a reduction of only 8.24% and 4.7% in PS-I activity for *N. spongiaeforme* and *Ns*(MHR)^5, respectively. In nitrate medium supplemented with pretilachlor, the PS-I activity of *N. spongiaeforme* and *Ns*(MHR)^5 was 47.5 and 52.8 nmol of O_2 consumed mg-1 Chl a min^{-1}, respectively, at 8 hours, showing a reduction of 6.8% and 3.4%, respectively. These results suggest that pretilachlor did not significantly impact the PS-I activity of both organisms (Figures 15b).

The PS-II activity of *N. spongiaeforme* exhibited a time-dependent sensitivity to pretilachlor in both basal and nitrate mediums. The PS-II activity of *N. spongiaeforme* decreased from 35.3 in basal medium to 26.7 nmol of O_2 evolved mg chl a min^{-1} over an 8-hour period. Conversely, the PS-II activity of *Ns*(MHR)^5 was not significantly impacted by pretilachlor in either basal or nitrate medium (Figures 16a). Notably, the PS-II activity of *Ns*(MHR)^5 in nitrate cultures slightly surpassed that of the control cultures of N. *spongiaeforme*. Furthermore, the effect of pretilachlor on the PS-II activity of *N. spongiaeforme* in nitrate medium was comparatively less pronounced than in basal medium cultures (Figures 16b).

The impact of pretilachlor on the photosynthetic whole chain activity of *N. spongiaeforme* and its mutant strain *Ns*(MHR)^5 paralleled the observed effects on PS-II activity. In basal medium, the whole chain activity of control cultures of *N. spongiaeforme* and *Ns*(MHR)^5 was measured at 27.8 and 32.2 nmol of O_2 evolved mg^{-1} Chl a min^{-1}, respectively, at zero hours. In basal medium supplemented with pretilachlor, the whole chain activity of *N. spongiaeforme* decreased in a time-dependent manner to 17.34 nmol of O_2 evolved mg^{-1} Chl a min^{-1} in 8 hours, while the whole chain activity of *Ns*(MHR)^5 remained unaffected by pretilachlor (Figures 17a). In nitrate medium supplemented with pretilachlor, the whole chain

activity of *N. spongiaeforme* decreased by 14.5% (from 31.5 to 23.4 nmol of O_2 evolved mg^{-1} Chl min^{-1}) in 8 hours, while the whole chain activity of *Ns*(MHR)^5 was not significantly affected (Figures 17b). These results further support the notion that pretilachlor exerts a notable influence on the photosynthetic processes, particularly PS-II activity, while the mutant strain *Ns*(MHR)^5 exhibits a comparatively resilient response to the herbicide.

The cultures of *N. spongiaeforme* and its mutant strain *Ns*(MHR)^5 were subjected to treatment with 15.5 ppm pretilachlor for 4 and 8 hours, and the respiratory activity, measured in terms of O_2 uptake in the dark, was determined. The addition of nitrate to the medium did not alter the respiratory rate of either *N. spongiaeforme* or *Ns*(MHR)^5. Initially, the O_2 uptake by the basal and nitrate-supplemented cultures of *N. spongiaeforme* and *Ns*(MHR)^5 was 17.0 and 21.28 nmol of O_2 uptake mg^{-1} Chl a min^{-1}, respectively, at zero hours. However, in the basal medium supplemented with pretilachlor, the O_2 uptake decreased to 13.2 and 17.3 nmol of O_2 uptake mg^{-1} Chl min^{-1} for *N. spongiaeforme* and *Ns*(MHR)^5, respectively, at 8 hours, indicating a 22.3% and 18.7% inhibition in respiratory activity Figures 18a).

The dark respiratory activity of nitrate cultures of *N. spongiaeforme* and *Ns*(MHR)^5 in the presence of pretilachlor was 13.8 and 19.3 nmol of O_2 uptake mg^{-1} Chl min^{-1}, respectively, at 8 hours (Fig. 18b). The inhibition in respiratory activity of *N. spongiaeforme* and *Ns*(MHR)^5 in the presence of pretilachlor was 18.8% and 9.3%, respectively. Additionally, it was observed that the respiratory rate of *N. spongiaeforme* in both basal and nitrate media supplemented with pretilachlor decreased from 4 to 8 hours, while in the case of *Ns*(MHR)^5, respiratory activity slightly increased at 8 hours compared to 4 hours. Moreover, nitrate medium did not induce any change in the respiratory activity of both organisms compared to basal medium.

The results indicate that pretilachlor affected the photosynthesis and respiration of *N. spongiaeforme* to a similar extent, whereas the photosynthesis and respiration of *Ns*(MHR)^5 were less severely impacted compared to N. spongiaeforme. The primary toxic effect of pretilachlor on the organisms appears to be on PS-II and the photosynthetic whole chain activity (Table 1).

Table 1: Effect of 8 hours exposure of *N. spongiaeforme* and *Ns*(MHR)^5 to pretilachlor (15.5 ppm) on photosynthesis rate, PS-I activity, PS-II activity, photosynthetic whole chain activity (PWCA) and respiration rate

Organism	Culture condition	Photosynthetic rate (1)		PS–I activity(2)		PS–II Activity(3)		PWCA (4)		Respiration rate (5)	
		+ N_2	+ NO_3	+ N_2	+NO_3	+ N_2	+NO_3	+ N_2	+NO_3	+ N_2	+NO_3
N. spongiaeforme	Control	46.7	46.7	50.7	50.7	35.3	35.3	27.8	27.8	17.0	17.0
	Pretilachlor	37.6	38.5	46.5	47.5	26.7	28.8	17.3	23.4	13.2	13.8
Ns(MHR)^5	Control	57.9	57.9	54.7	54.7	39.8	39.8	32.2	32.2	21.3	21.3
	Pretilachlor	50.6	52.2	52.1	52.8	40.1	40.6	31.1	31.5	17.3	19.3

(1): O_2 evolution (nmol mg^{-1} chl *a* min^{-1}), **(2):** nmol O_2 consumed mg^{-1} chl *a* min^{-1}), **(3):** nmol O_2 evolved mg^{-1} chl *a* min^{-1}), **(4):** nmol O_2 evolved mg^{-1} chl *a* min^{-1}), **(5):** O_2 uptake (nmol mg^{-1} chl *a* min^{-1})

DISCUSSION

Cyanobacteria play a pivotal role in rice field microbial communities, serving as vital contributors to fertility through their inherent biofertilizer capabilities (Fernandez-Valiente et al. 2000; Singh and Datta 2005, 2006, 2007). The efficacy of cyanobacterial contribution to rice growth hinges on their successful establishment in field conditions, coupled with their growth dynamics and nitrogen-fixing prowess. The impact of pesticides on microbial species may not align with their intended effects on target organisms. Within microorganisms, pesticides have demonstrated interference with crucial physiological processes, including respiration, photosynthesis, biosynthesis reactions, as well as influencing cell growth, division, and molecular composition (De Lorenzo et al. 2001). The toxicity of pesticides to cyanobacteria is predominantly contingent upon the pesticide concentration, with a notable impact on primary producers attributed to the inhibition of photosynthesis in microalgae (Kulshreshta et al. 2000, Mahapatra et al. 2003). Widespread herbicide application has been observed to adversely affect plant growth, crop productivity, soil fertility, and non-target microorganisms (Fernandez-Valiente et al. 2000). The escalating use of herbicides has raised concerns regarding their ecological ramifications in aquatic systems and on non-target organisms (Lockert et al. 2006). Precision in pesticide application for agricultural pest control is imperative, as any disturbance affecting vulnerable algae can have profound repercussions on higher trophic levels. Pretilachlor, a widely utilized herbicide in rice fields, operates as a pre-emergence herbicide targeting grasses, sedges, and broadleaf weeds in transplanted rice. Its swift uptake by germinating weed roots results in inhibited cell division and subsequent weed mortality.

The cyanobacterial strains utilized in this study, namely *N. spongiaeforme* (a rice field isolate) and its mutant strain *Ns*(MHR)^5, exhibit filamentous, heterocystous, mucilaginous, and non-branched forms. Growth assessment under varying concentrations of pretilachlor involved monitoring absorbance, protein content, and dry weight of the cultures for both *N. spongiaeforme* and *Ns*(MHR)^5. The concentration-dependent decline in growth was evident in the presence of pretilachlor, with a notable decrease in absorbance observed. Specifically, in 20 ppm pretilachlor, the absorbance decrease was 84.2% for *N. spongiaeforme* and 52.27% for *Ns*(MHR)^5 compared to control cultures over 12 days. In nitrate medium supplemented with 20 ppm pretilachlor, similar trends were observed, with absorbance reductions of 82.5% and 48.7% for *N. spongiaeforme* and *Ns*(MHR)^5, respectively. The inhibitory effect of pretilachlor extended to protein content as a growth parameter, with substantial reductions in basal medium cultures [90.95% for *N. spongiaeforme*, 74.62% for *Ns*(MHR)^5] and in nitrate medium supplemented with 20 ppm pretilachlor [83.8% for *N. spongiaeforme*, 58.34% for *Ns*(MHR)^5] over 12 days. The dry weight of cultures also exhibited a decline, with basal medium cultures in 20 ppm pretilachlor experiencing reductions of 74.6% for *N. spongiaeforme* and 69.9% for *Ns*(MHR)^5. Nitrate medium supplemented with 20 ppm pretilachlor supported 71.7% less dry weight for *N. spongiaeforme* and 67.4% for *Ns*(MHR)^5. All growth parameters like absorbance, protein content and dry weight biomass demonstrated a positive correlation at 95% confidence level. Comparisons with the study by (Galhano et al. 2009) on the differential effects of herbicides (bentazon and molinate) on *Anabaena cylindrica* underscored the concentration-dependent and pleiotropic impacts of pesticide stress on cyanobacteria. The observed adverse effects on growth, photo pigments, photosynthesis, respiration, carbohydrate and protein content emphasize the potential ecological consequences of herbicide exposure in aquatic systems.

The reported variations in cyanobacterial responses to different herbicides underscore the intricate relationship between herbicide toxicity and cyanobacterial species. The differential sensitivity of *Anabaena flos-aquae*, *Microcystis flos-aquae* and *M. aeruginosa* to seven herbicides (dicloflop, triclopyr, ametryne, simazine, prometryne, cyanazine, and simetryn) revealed distinct patterns, with *M. flos-aquae* exhibiting

the highest sensitivity, followed by *M. aeruginosa* and *A. flos-aquae* (Ma et al. 2010). Similar observations of herbicide impacts on growth have been reported, such as the effects of atrazine and DCMU on the growth of the diazotrophic cyanobacterium *Anabaena variabilis* (Singh and Sandhu 2010; Singh et al. 2011, 2012, 2013) and the toxicity of chlorpyrifos to marine cyanobacteria (Shoaid et al. 2012).

Notably, the present study investigated the response of *N. spongiaeforme* and its mutant strain *Ns*(MHR)^5 to the herbicide pretilachlor. Concentration-dependent reductions in growth were observed, with *N. spongiaeforme* showing a more pronounced effect than *Ns*(MHR)^5. The growth-promoting effect of nitrate supplementation was evident, with *Ns*(MHR)^5 exhibiting greater biomass in both basal and nitrate media compared to *N. spongiaeforme*. The determined LD50 and lethal doses of pretilachlor indicated the relatively higher resistance of *Ns*(MHR)^5 to the herbicide compared to *N. spongiaeforme*. Analysis of photosynthetic parameters, including photosynthetic rate and chlorophyll a content, revealed a concentration-dependent decrease in the growth of both organisms in the presence of pretilachlor. However, *Ns*(MHR)^5 demonstrated a lesser impact on photosynthetic rate, suggesting a protective mechanism in the mutant strain. The inhibitory effect of pretilachlor on photosynthetic whole chain activity and PS-II was more pronounced than on PS-I, indicating a primary impact on electron transport at the PS-II level. The recovery of activity with time in *Ns*(MHR)^5 suggested a degree of resilience in the mutant strain. Comparisons with existing literature highlighted the variability in cyanobacterial responses to herbicides, with different herbicides exhibiting diverse effects on photosynthetic activity. The observed concentration-dependent decreases in photosynthetic rate and chlorophyll a content align with previous studies demonstrating herbicide-induced inhibition of electron transport and photophosphorylation in cyanobacteria. Structural insights into herbicide binding to PS-II, as demonstrated by (Broser et al. 2011), underscore the complexity of herbicide-cyanobacteria interactions at the molecular level.

CONCLUSION

The study contributes valuable insights into the differential responses of cyanobacterial strains to the herbicide pretilachlor, emphasizing the importance of considering specific herbicide-organism interactions in assessing ecological consequences in aquatic ecosystems. The observed protective mechanisms in the mutant strain *Ns*(MHR)^5 want further investigation to elucidate the underlying molecular basis, providing potential avenues for the development of herbicide-resistant cyanobacterial strains.

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