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Abstract

Butachlor caused toxicity to duckweed *Spirodela polyrhiza* in a concentration dependent manner by disrupting growth, photosynthetic pigments and primary metabolism and by activating antioxidant defenses. At 25 and 30 mg L⁻¹, the frond number progressively declined after 96 h exposure (~40 and ~60% reduction). Chlorophyll a, chlorophyll b and total chlorophyll decreased by 71%, 74% and 82.1% respectively and carotenoids decreased by 43% at 30 mg L⁻¹. Activities of antioxidant enzymes showed significant increase in superoxide dismutase (+92%), catalase (+85%), peroxidase (+17%) and ascorbate peroxidase (+55.1%) as compared to control. Histochemical staining in vivo showed lipid peroxidation (MDA) was increased by 178.6%. Primary metabolites were adversely affected. Carbohydrates and total protein decreased by 77.4% and ~91% respectively, while proline accumulated up to +89.1% at 30 mg L⁻¹. These results indicate that butachlor induces oxidative stress and metabolic disturbance in *S. polyrhiza*, triggers defensive antioxidant and osmoprotective responses but results in significant growth inhibition. The species-specific sensitivities documented here inform ecotoxicological risk assessment and selection of duckweed taxa for phytoremediation of herbicide contaminated waters.

Keywords: Antioxidants, Bioindicator, Butachlor, Carbohydrate, Herbicide, Photosynthetic pigments, Phytoremediation, Protein, *Spirodela polyrhiza*.

1.Introduction

Herbicides have revolutionized modern agricultural practice by offering practical and effective methods of controlling weed infestations and enhancing crop productivity [1]. Herbicides are used in crop (agricultural) production, in forestry, for weed control in public areas and maintaining of parks, golf and other sport grounds [2]. They are often applied directly to control aquatic weeds or indirectly transported to water bodies by surface and ground waters primarily by leaching and runoff from agricultural fields [2]. Several reports on the discharge of widely used herbicides into a number of water bodies [3,4,5]. Herbicides are carried from agricultural lands and more readily reach surface and groundwater for farm ponds [6]. Widespread use of herbicides such as butachlor, bentazone, 2,4-D, glyphosate and atrazine not only endanger ecosystems, but also human health [7]. Butachlor (N (butoxymethyl)-2-chloro-N-(2,6-diethylphenyl) acetamide) is an efficacious and selective acetamide herbicide [4,8] used extensively as a pre/post emergent herbicide for weed control in rice fields. Butachlor is preferred by farmers for its effective weed management as it causes least damage to rice crop [9]. Butachlor affects different processes like lipid metabolism, synthesis of gibberellic acid and chlorophyll pigment, cell division, cell permeability, abortion, mineral distribution, and amino acid synthesis [10]. It impacts several plant metabolic functions including redox balance. Butachlor is a class K3 herbicide according to the Herbicide Resistance Action Committee (www.plantprotection.org/HRAC) [11] that inhibits the growth of susceptible plants by inhibiting the biosynthesis of very long chain fatty acids (VLCFAs) by targeting enzymes required for the biosynthesis of VLCFAs. It is extensively used for rice production due to its safety and cost-effectiveness [8]. Butachlor is toxic to many soil microbes apart from its mutagenicity [4]. Due to its persistence in soil and potential to leach into ground water [12], butachlor threatens non-target organisms living in an impacted ecosystem. Butachlor is widely used and found in terrestrial and aquatic environment Chen et al. [13], causing contamination issues.

Keeping in mind their sensitivity to a variety of herbicides, duckweeds such as *Spirodela polyrhiza* are often used for evaluation of herbicide-toxicity. Further, Butachlor is frequently used as a standard for evaluating environmental risk [14]. The present study aimed to evaluate the physiological and biochemical alterations caused by butachlor stress in two aquatic plant duckweed species *Spirodela polyrhiza*.

2. Material and methods

2.1 Plant Material

Fresh samples of *Spirodela polyrhiza* plants were collected from the pond located at Roxburgh Botanical Garden, Department of Botany, University of Allahabad, Prayagraj, Uttar Pradesh, India (25° 27' 42.2352" N ,81°5' 7.278" E). Plant samples were identified from Botanical Survey of India Central Regional Centre, Prayagraj, Uttar Pradesh, India.

After repeated washing of the plants for thorough cleansing of the roots and fronds, they were transferred to 250 ml flasks containing half-strength Hutner's medium with 10 g of sucrose. They were maintained at 25°C in a growth chamber and subjected to consistent, cold fluorescent light at 6,800 lux according to ASTM E.1415-91 [15] for 1 day (24 hours).

A stock solution of 1000 ppm butachlor purchased from sigma aldrich was made and varying concentrations (5, 10, 15, 20, 25, and 30 mg L⁻¹) of this stock were distributed into flasks.

After screening for healthy adult plants of similar sizes and shapes was carried out, the selected plants were transferred to 250 ml flasks containing varying concentrations of butachlor herbicides. Thereafter, these flasks containing the plants were placed in a plant growth chamber for 4 days.

2.2. Determination of relative growth rate

Relative growth rate (RGR), measures the rise of a quantity per unit of time in relation to its initial value [16]. It is a useful tool for understanding growth dynamics over a certain

time period. Here, the effect of butachlor stress on aquatic plant *Spirodela polyrhiza*, was evaluated by initially counting the number of fronds. Thereafter, Relative Growth Rate (RGR) was calculated based on the following formula.

$$\text{RGR (day}^{-1}\text{)} = ((\ln(N_2)) - \ln(N_1)) / (t_2 - t_1)$$

where

- N_1 = Number of fronds at the start time t_1
- N_2 = Number of fronds at the end time t_2
- $\ln$ = Natural logarithm (base e)
- $t_2 - t_1$ = Time interval (in days or hours, depending on experiment)

2.3. Photosynthetic pigments

100 mg fresh plant material (*Spirodela polyrhiza*) (treated and untreated) were chopped into very small pieces and mixed with 80% (v/v) acetone for extraction of photosynthetic pigments after 4 days of treatment with butachlor herbicide. The mixture was centrifuged at $10,000 \times g$ for about 15 min at 25 °C using eppendorf centrifuge-5920 R instrument. The supernatant was taken and the pellets were re-suspended in 80% acetone until decolorized. Spectrophotometric absorbance of the resultant solutions was recorded (Shimadzu double beam UV—Visible spectrophotometer—1700, Japan) at 663.2, 646.5 and 470 nm. Carotenoids (Car), total chlorophyll and chlorophylls a and b (Chl a and Chl b) contents were calculated according to Lichtenthaler [17].

2.4. Determination of antioxidant enzyme activity

Activity assay of antioxidant enzymes Superoxide dismutase activity (SOD; EC 1.15.1.1) was determined by measuring the inhibition in the reduction of nitro blue tetrazoliumchloride (NBT) according to the method of Giannopolitis and Ries [18]. The activity of peroxidase (POD; EC 1.11.1.7) was determined according to the method of Zhang [19] with the extinction coefficient $25.5 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit of POD activity is defined as the amount of enzyme oxidizing 1 nmol of guaiacol min^{-1} . Ascorbate

peroxidase activity was calculated using the extinction coefficient ($\epsilon=2.8 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit of APX activity is defined as the amount of enzyme required to oxidize 1 nmol of ascorbate min^{-1} [20] (Nakano and Asada). Catalase activity of the samples was determined by homogenizing 100 mg of plant material with 1 mL of 50 mM PPB (Potassium Phosphate Buffer) and 1 mM EDTA. Catalase (CAT; EC 1.11.1.6) activity was expressed according to the method of Aebi [21].

2.5. Determination of Lipid peroxidation

Measurement of lipid peroxidation was carried out using malondialdehyde (MDA) equivalents as per the procedure described by Heath and Packer [22]. MDA equivalents concentration was calculated using the extinction coefficient $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as nmol g^{-1} FW (fresh weight).

2.5.1. In—vivo localization of lipid peroxidation

The in—vivo localization of lipid peroxidation (MDA equivalents) in each sample was performed according to the method of Pompella et al. [23]

2.6. Estimation of biochemical content

Carbohydrate content of each sample was measured by the method of Loewus [24], proline content was measured using toluene as a blank [26]. The protein content was calculated using Bradford's method [25].

2.7. Statistical analysis

The statistical analysis was performed using SPSS 16 and Sigma Plot 10.

3. Result

Butachlor herbicide had a concentration-dependent impact on *Spirodela polyrhiza*. Moreover, the impact varied with the type of plant, suggesting a species-dependent response.

3.1. Relative growth rate (RGR)

After 96 h of exposure, there was a decline in the number of fronds with increase in butachlor concentration in *Spirodela polyrhiza* (Fig. 1) (Table 1). In *Spirodela polyrhiza* 25 and 30 mgL⁻¹ butachlor concentration showed 40 and 60% reduction in fronds of *Spirodela polyrhiza* respectively (Fig. 2).

Table 1

Parameter	0 mg/L	5 mg/L	10 mg/L	15 mg/L	20 mg/L
RGR (Relative Growth rate)	2.078 ±0.01	1.782 ±0.01	1.765 ±0.02	1.603 ±0.03	1.563 ±0.02
Chl a (mg g⁻¹ FW)	0.525 ±0.00	0.466 ±0.00	0.360 ±0.00	0.334 ±0.01	0.229 ±0.00
Chl b (mg g⁻¹ FW)	0.349 ±0.00	0.300 ±0.01	0.344 ±0.00	0.295 ±0.01	0.331 ±0.00
Total Chl (mg g⁻¹ FW)	0.874 ±0.00	0.766 ±0.00	0.704 ±0.00	0.629 ±0.00	0.560 ±0.00
Carotenoids (mg g⁻¹ FW)	0.341 ±0.00	0.391 ±0.000	0.341 ±0.000	0.294 ±0.000	0.275 ±0.00
Superoxide dismutase (SOD) (Units mg⁻¹ FW)	0.035 ±0.00	0.042 ±0.00	0.047 ±0.00	0.051 ±0.00	0.057 ±0.00
Catalase (CAT) Activity (Units g⁻¹ FW)	2473.77 ±16.14	2825.72 ±15.03	3407.78 ±10.29	3945.85 ±16.14	4025.38 ±30.87
Peroxidase (POD) Activity (Units g⁻¹ FW)	2635.29 ±3.01	2647.5 ±3.14	2687.58 ±6.91	2724.18 ±7.54	2813.07 ±6.57
Ascorbate Peroxidase (APX) Activity (nmol H₂O₂ g⁻¹ FW min⁻¹ ×10³)	16.30 ±0.22	17.12 ±0.23	23.76 ±0.19	23.80 ±0.42	24.17 ±0.35
Malondialdehyde (MDA) (nmol g⁻¹ FW)	60.2 ±4.3	70.95 ±3.72	90.3 ±3.72	103.2 ±14.09	146.2 ±4.3
Total Carbohydrate (µg mg⁻¹ FW)	136.25 ±0.37	116.75 ±0.57	108.92 ±0.64	86.21 ±0.19	59.96 ±0.95
Proline (µmol g⁻¹ FW)	0.1312 ±0.00	0.1412 ±0.00	0.186 ±0.00	0.204 ±0.00	0.205 ±0.00
Total Protein (µg mg⁻¹ FW)	4.342 ±0.01	1.947 ±0.01	1.326 ±0.01	1.068 ±0.02	0.75 ±0.01

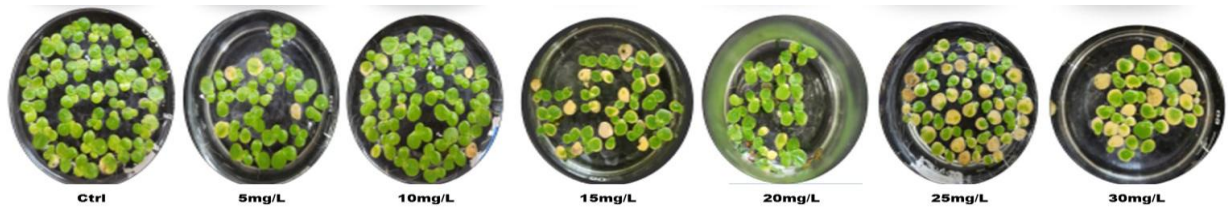


Fig.1

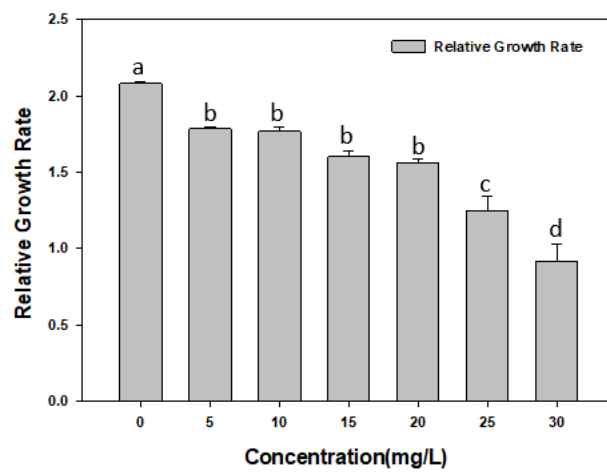


Fig. 2

3.2. Photosynthetic pigment content

A decline in the pigment content *Spirodela polyrhiza* (Fig. 3) on exposure to various butachlor concentration 0 to 30 mg L⁻¹ is shown in Table 1. *Spirodela polyrhiza* showed reduction in Chl a content in presence of 30 mg L⁻¹ butachlor 71%; further, the Chl b content in *Spirodela polyrhiza* is reduced by 74%. On exposure to 30 mg L⁻¹ butachlor, the total chlorophyll content (Chl a + Chl b) of *Spirodela polyrhiza* it was reduced by 82.1%; furthermore, the carotenoid content declined by 43% in *Spirodela polyrhiza*.

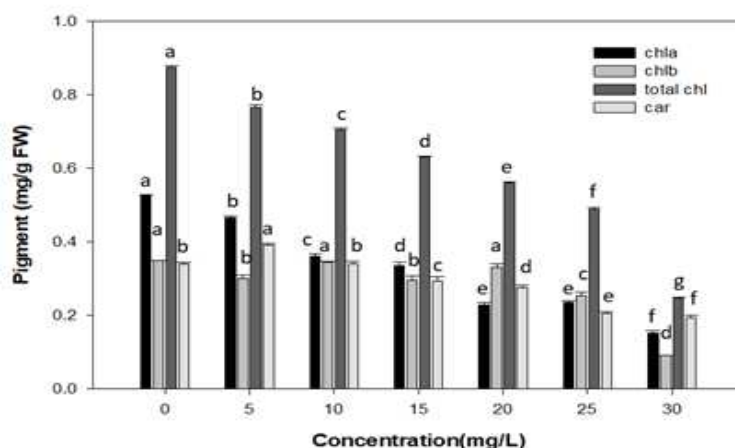


Fig.3

3.3. Antioxidant assays

SOD activity increased with increasing concentration of butachlor in the species tested (Table 1). An increase of 92% in SOD activity (with respect to the control) was observed in *Spirodela polyrhiza* at 30 mg L⁻¹ (Fig. 4a). The CAT activity of the duckweed species increased with increasing butachlor concentration (Table 1). In *Spirodela polyrhiza*, 85% increase in CAT activity (as compared to the control) was observed at 30 mg L⁻¹ (Fig. 4b). The increased concentration of butachlor also increased POD activity (peroxidase) of both the duckweed species (Table 1). In presence of 30 mg L⁻¹ butachlor, POD activity in *Spirodela polyrhiza* increased by 17 % over the control (Fig 4c). The APX activity increased with the increase of butachlor concentration in the duckweed species

(*S. polyrhiza*) (Table 1). Butachlor concentrations of 30 mg L⁻¹ caused an increase of 55.1% in the APX activity of *Spirodela polyrhiza* (Fig 4d).

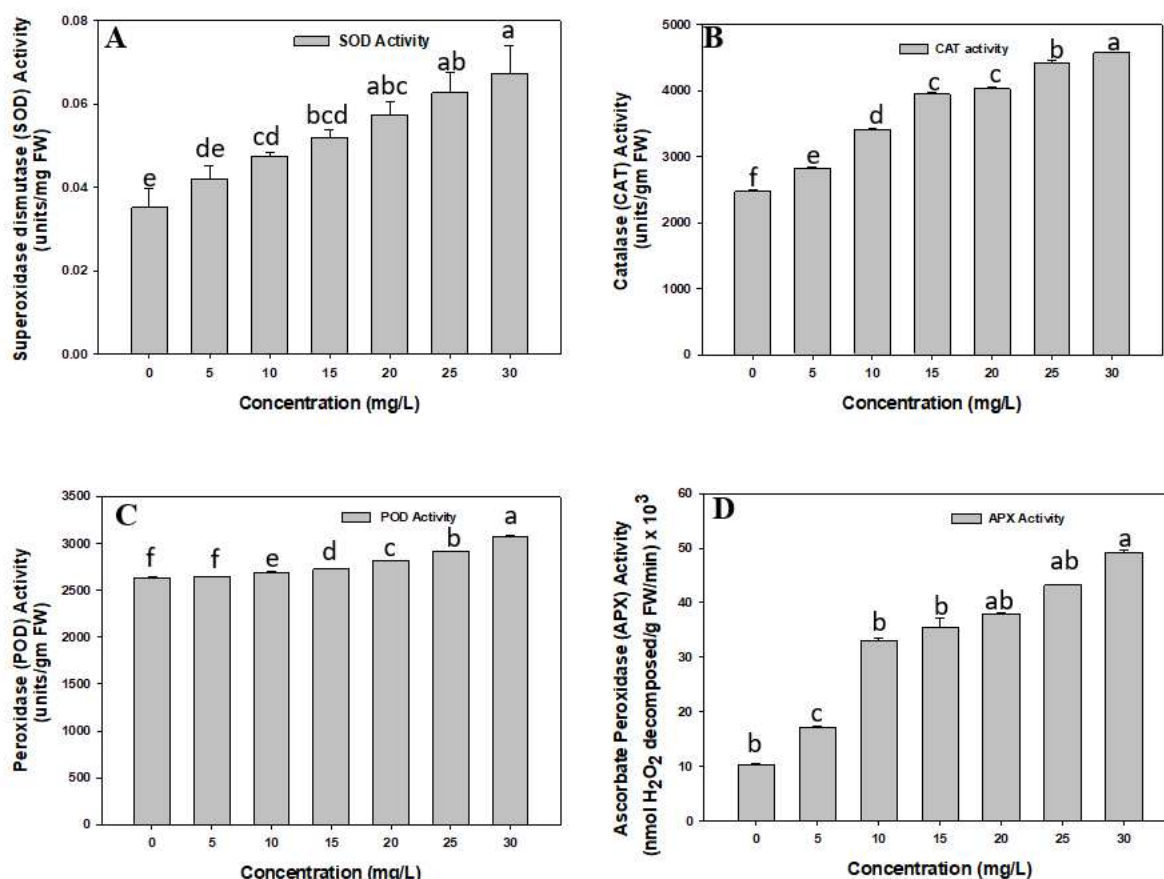


Fig. 4

3.4. Impact on biochemical components

3.4.1. Lipid peroxidation (MDA) assay

Oxidative degradation of lipids was measured using the lipid peroxidation assay and expressed in terms of malondialdehyde (MDA) equivalents content in the duckweed (Table 1). The MDA equivalent content shows increasing trend on increasing concentration of herbicides butachlor (0, 5, 10, 15, 20, 25 and 30mg L⁻¹) in *Spirodela*

polyrhiza (Fig 5a). A significant increasing trend for MDA equivalents 178.6% was observed in *Spirodela polyrhiza*, compared to control values.

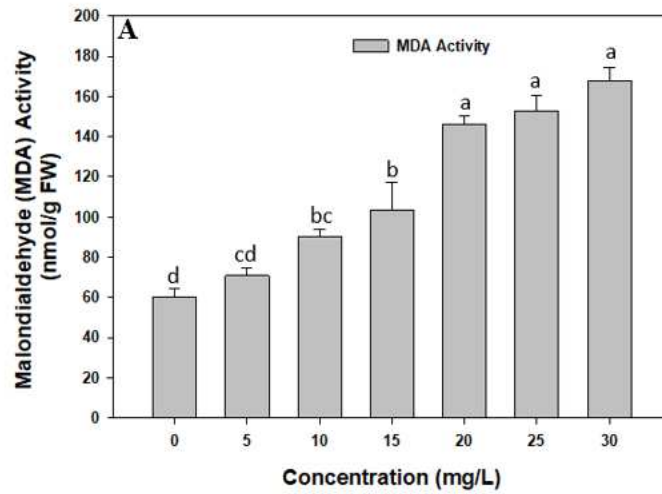


Fig.5 a

3.4.2. In—vivo localization of lipid peroxidation

Roots were bleached by immersing in boiling ethanol to visualize pink stain and photographs were taken by light microscope (Fig. 5b).

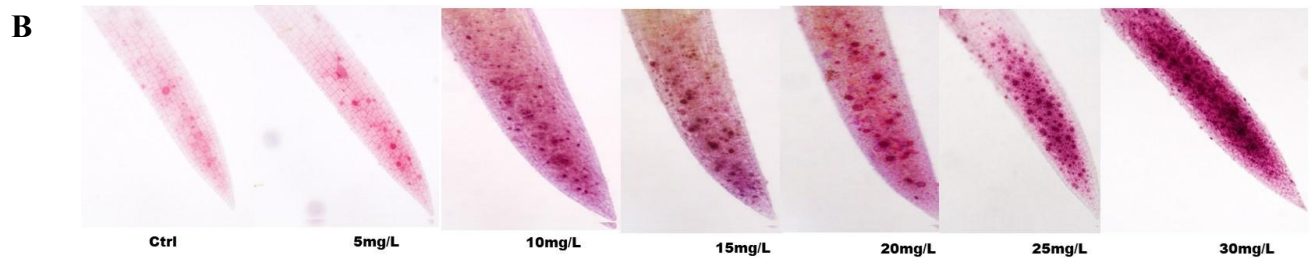


Fig. 5 b

3.5. Estimation of biochemical components

Table 1 shows a decline in carbohydrate content in response to increase in butachlor concentration in the duckweed species (*Spirodela polyrhiza*). At 30 mg L⁻¹ butachlor concentration, there was a 77.43% decrease in carbohydrate content of *Spirodela polyrhiza* (Fig 6a). Proline content increased with an increase in butachlor concentration (Table 1). In *Spirodela polyrhiza*, at butachlor concentration of 10, 15, 20, 25 and 30 mg L⁻¹ an increase of 42.06%, 55.46%, 56.29%, 83.30% and 89.08% respectively was observed as compared to control; in *Spirodela polyrhiza* (Fig. 6b), respectively after 96 h of treatment. Total protein content decreased with an increase in butachlor concentration in the duckweed species (Table 1). Presence of 30 mg L⁻¹ butachlor, caused 91% decrease in protein content of *Spirodela polyrhiza* (Fig. 6c).

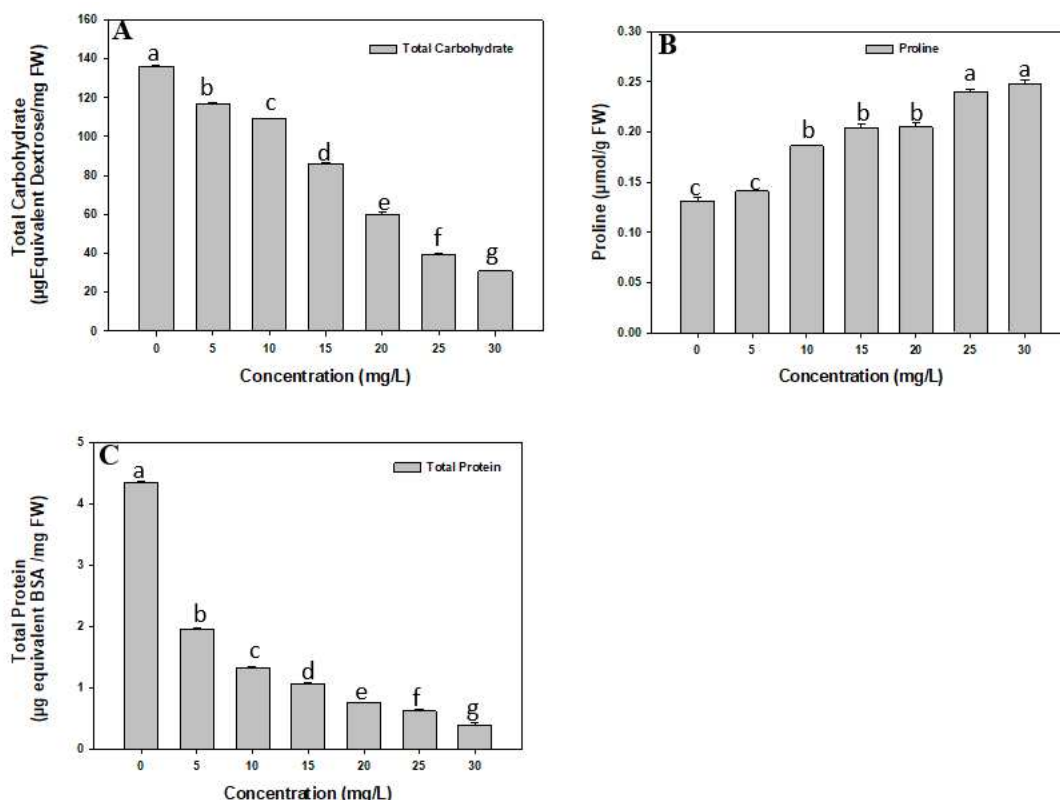


Fig. 6

4. Discussion

Butachlor is a herbicide used for pre-emergence control of grasses and weeds. It is also known to inhibit the growth and reduce the photosynthesis of aquatic plants (by causing a decreased chlorophyll content and chlorophyll fluorescence). The effect is, however, species-specific. In our study, species dependent effect of butachlor were observed on *S. polyrhiza*. The increase in concentration of butachlor resulted in a decline in the growth rate of the duckweed. Highest reduction in growth rate were observed in the presence of 25 and 30 mg L⁻¹ butachlor in *Spirodela polyrhiza* (40 and 60%). This is in line with Wang et al. [27] who suggest that butachlor interferes with physiological functions of aquatic macrophytes, especially those belonging to the Lemnaceae family. This comprehensive investigation demonstrated the concentration-based effect of

butachlor toxicity on *Spirodela polyrhiza*. Parameters such as growth rate, photosynthetic pigment content, oxidative degradation, enzymatic activity and primary metabolites such as carbohydrate and protein clearly show the same. Chlorophyll a and Chlorophyll b, pigments that absorb and transmit light, are important for photosynthesis [28]. A dose-dependent decrease in photosynthetic pigments such as carotenoid, chlorophyll a and chlorophyll b were observed in duckweeds (*Spirodela polyrhiza*). Photosynthetic ability of these plants was significantly hampered at higher concentrations of butachlor (30 mg L⁻¹). However, butachlor stress inhibits PSII activity and damages chlorophyll pigments, leading to a reduction in photosynthesis [29]. Sule et al. [30] think that SODs provide first defense line against oxygen radicals, especially superoxide anion (O₂•-) the main ROS generated by mitochondrial respiration and other metabolic pathways. This reaction demonstrates an important activation of the oxidative stress. The significant increase of SOD in *Spirodela polyrhiza* species can verify its role as a sensitive biomarker for herbicide toxicity [31]. SOD activity was gradually increased (20%–91.19%) with the increase of butachlor concentrations (5–30 mg L⁻¹). This is in line with studies reporting that chloroacetamide herbicides induce excess ROS [32]. The persistent SOD augmentation indicates long-term oxidative damage that could lead to a reduction of duckweed photosynthesis, growth and reproductive capacity [33]. The enzyme catalase dismutases catalyze the breakdown of hydrogen peroxide (H₂O₂) into water and molecular oxygen (O₂) which is an important antioxidant and part of the antioxidant defense system [34]. Catalase is an important first line antioxidant for removal or stopping the production of reactive oxygen species in cells and the rapid inactivation of cell damaging chemicals [35]. The present study showed that the activity of CAT was enhanced by *Spirodela polyrhiza* after 96 h of butachlor exposure, although the quantity and pattern depended on the concentration of the herbicide. The increased CAT activity observed is a conventional defense mechanism of plants that detoxifies hydrogen peroxide, a byproduct of ROS, protecting cellular structures from oxidative damage as per Tagun [36]. The activity of CAT in duckweed species was gradually increased after butachlor exposure. In our study, CAT

activity was not only dose-dependent on butachlor but also species-dependent. Previous results suggest that the effect of butachlor may be different in terms of plant species, plant parts, phenological period, exposure dose and duration [37]. An increase in POD (peroxidase) activity is associated with self-defense and stress tolerance. The activity of peroxidase enzyme is upregulated during stress to increase the rate of respiration [38]. Here, the POD activity of *Spirodela polyrhiza* was increased by 11% and 17% respectively at 25 and 30 mg L⁻¹ butachlor concentrations. One key antioxidant enzyme in these scavenging systems is ascorbate peroxidase (APX). It catalyzes the reduction of H₂O₂ to H₂O with ascorbate as electron donor. Saurabh et al. [39] reported that the expression of APX is differentially regulated during normal plant growth and development and in response to environmental stressors. Cell stress quickly disrupts the redox equilibrium and results in an accumulation of ROS [40]. The extent of APX expression is directly related to the duration and severity of applied stress and also to the diversity of stresses [41]. The activity of APX in the duckweed species studied was significantly increased after the exposure to butachlor. A marginally greater increase in the activity of *Spirodela polyrhiza* was observed at the highest doses at 30 mg L⁻¹ (55%). Butachlor and other herbicides are known to enhance antioxidant defenses of *M. aquaticum* and *C. demersum* [42]. Exposure to herbicides often leads to the production of reactive oxygen species (ROS) within plant cells [43], which helps them to decrease the oxidative stress induced by the herbicide. Another marker for studying the stress is the oxidative degradation of lipids. It was observed that there was a dose-dependent enhancement of lipid peroxidation in *Spirodela polyrhiza* which was apparent with the increase in malondialdehyde (MDA) content. Increased MDA, in addition to being a marker of membrane damage, can also have negative effects such as reduced cellular activity, growth inhibition and plant mortality in case of overwhelmed antioxidant defenses [44]. MDA is a well-established marker of oxidative stress, being a major and harmful consequence of polyunsaturated fatty acid breakdown during ROS-mediated lipid peroxidation [44]. Butachlor has a specific impact on pathways related to carbohydrate metabolism, thus disrupting the distribution of starch and sucrose. This

interference can affect growth and physiological processes and may change the energy storage and utilization of the plant [45]. 30 mg L⁻¹ butachlor (highest concentration tested) caused a reduction in the carbohydrate content of *Spirodela polyrhiza* by 77.43%. The plant carbohydrate balance is not restricted to one single pathway but is a wide disruption on exposure to herbicide which damages several carbohydrate metabolic pathways [45]. Proline is one of the vital amino acids that plants acquire under unfavorable environmental conditions. In the species (*Spirodela polyrhiza*), higher doses of butachlor increased in the proline content at all butachlor concentrations. Under these conditions, the protective role of proline has been correlated with its ability to maintain redox balance and enhance the antioxidant enzyme activity [46]. It has been shown that stressed plants accumulate proline up to 100 times more than in normal conditions [47]. The higher accumulation of proline under the butachlor stress conditions can be related to the alleviation of ROS-induced oxidative stress and stress tolerance [48, 49]. Since proline can serve as a signaling molecule capable of modulating mitochondrial functions, affecting cell proliferation or cell death and inducing specific gene expression, all of which are critical for plant recovery from stress. Butachlor influences seed germination, lipid metabolism, chlorophyll pigments and gibberellic acid biosynthesis, cell division, cell permeability, abortion and distribution of minerals and amino acid synthesis [50]. Moreover, butachlor disturbs different metabolic processes and redox homeostasis in plants. This leads to a significant reduction in protein content, as observed in duckweed species (*Spirodela polyrhiza*). The greater reduction in protein content under Butachlor treatment than other herbicide indicates that Butachlor may have a stronger inhibitory effect on protein biosynthesis in duckweed. This is also corroborated by studies on other herbicides with a similar mode of action, which report a significant decrease of protein and pigment content in aquatic plants [50].

5. Conclusion

The herbicide butachlor induces a reduction in growth rate, photosynthetic pigments, carbohydrate levels, and protein content in *Spirodela polyrhiza*, with effects varying by dosage. Antioxidant activities associated with reactive oxygen species and oxidative

lipid breakdown escalate due to the defensive mechanisms in duckweed species (*Spirodela polyrhiza*). *Spirodela polyrhiza* shown significant resilience to butachlor. The responsiveness of *Spirodela polyrhiza*, a species of duckweed, to butachlor underscores its potential as a bioindicator for identifying herbicide pollution in aquatic ecosystems.

Author Contributions

Harshita Mishra and K. Suresh Kumar contributed to the study conception and design. Material preparation, data collection and analysis were performed by Harshita Mishra, Vandana, Swati Mishra and Ashutosh Pathak. The first draft of the manuscript was written by Harshita Mishra and K. Suresh Kumar made the necessary changes to the manuscript. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in this article

Ethics approval and consent to participate

Not required.

Consent for publication

Not applicable.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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Table 1: Effects of increasing butachlor concentration on growth, pigment content, antioxidant enzyme activities, and biochemical parameters in *Spirodela polyrhiza*.

Fig. 1 Relative growth rate (RGR) of *Spirodela polyrhiza* after 96h of incubation at 0, 5, 10, 15, 20, 25 and 30 mg L⁻¹ concentrations of butachlor

Fig. 2 Effects of increasing butachlor concentrations (0, 5, 10, 15, 20, 25 and 30 mg L⁻¹ in *Spirodela polyrhiza* on Relative Growth Rate (RGR]. Data are means $\pm$ SE. Different letters above bars indicate significant differences among treatments ($p < 0.05$)

Fig. 3 Effects of increasing butachlor concentrations (0, 5, 10, 15, 20, 25 and 30 mg L⁻¹) on pigment content (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids in *Spirodela polyrhiza*. Data are means $\pm$ SE. Different letters above bars indicate significant differences among treatments ($p < 0.05$)

Fig. 4 Effects of increasing butachlor concentrations (0, 5, 10, 15, 20, 25 and 30 mg L⁻¹) on Enzymatic activity in *Spirodela polyrhiza* (a, b, c, d). **(a)** superoxide dismutase (SOD) activity; **(b)** catalase (CAT) activity; **(c)** Guaiacol Peroxidase (GPOD) Activity; **(d)** Ascorbate peroxidase (APX) Activity. Data are means $\pm$ SE. Different letters above bars indicate significant differences among treatments ($p < 0.05$).

Fig. 5 (a) Changes in malondialdehyde (MDA) content (nmol g⁻¹ FW), indicating lipid peroxidation, in *Spirodela polyrhiza* exposed to increasing concentrations of butachlor

(0, 5, 10, 15, 20, 25 and 30 mg L⁻¹) for 96 h. Data are means $\pm$ SE. Different letters above bars indicate significant differences among treatments ($p < 0.05$)

Fig.5 (b) In vivo detection of lipid peroxidation observed in *Spirodela polyrhiza* root exposed to butachlor (0, 5, 10, 15, 20, 25 and 30 mg L⁻¹) by Schiff's reagent staining. Increased magenta staining with increasing butachlor concentration indicates enhanced malondialdehyde (MDA) accumulation and membrane lipid peroxidation under herbicide-induced oxidative stress

Fig. 6 Effects of increasing butachlor concentrations (0mg/L, 5mg/L, 10mg/L, 15mg/L, 20mg/L, 25mg/L, 30 mg/L) on biochemical parameters in *Spirodela polyrhiza*. (a) Carbohydrate content; (b) Proline content; (c) Protein content. Data are means $\pm$ SE. Different letters above bars indicate significant differences among treatments ($p < 0.05$)