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Toxicological Effects of Chlorpyrifos on Growth, Chlorophyll a Synthesis and Enzyme Activity of a Cyanobacterium *Spirulina* (*Arthrospira*) *platensis*

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A B S T R A C T

Chlorpyrifos, is one of the most widely used organophosphorus insecticides for agricultural activities, and it is highly toxic to non-target organisms. This paper aims to acquire the experimental data on the eco-toxicological effects of Chlorpyrifos and the data can support the assessment of toxicity on the phytoplankton. The microalgae *S. platensis* was employed to evaluate toxicity of Chlorpyrifos by means of measuring the specific growth rate, generation time, percent growth inhibition, the pigment content of chlorophyll a and carotenoid and the antioxidant enzyme super oxide dismutase. In this study, the results showed that EC_{50} values was found to be 33.65 mg L^{-1}, indicating the Chlorpyrifos had a relatively limited growth of algae during the acute toxicity experimental period. The growth of the microalgae was significantly affected at 40mg L^{-1} of Chlorpyrifos, showing growth inhibition after 72h of exposure. Biochemical properties, including carotenoid, chlorophyll and antioxidant enzymes of C. vulgaris were influenced by Chlorpyrifos at relatively high concentrations. Moreover, when algae were exposed to Chlorpyrifos, SOD activity was significantly enhanced compared to control.

Introduction

India is primarily an agriculture-based country with more than 60-70 per cent of its population dependent on agriculture. India's fast growing population is projected to cross 1.3 billion by 2020 (Kanekar *et al.,* 2004).

In the current agricultural practices, a wide range of pesticides are often extensively used with the aim of increased production. Such pesticides are toxic to humans, plants and animals (Ghosh and Philip 2006). The quantum of organophosphorous insecticide has increased as it serves as an alternative to organochlorine and carbamate pesticides because of their efficiency and relatively lower persistence (Shreekumar *et al.,* 2017). These organophosphorus insecticides can contaminate surface waters through unintentional drift of aerial spraying, surface run off and wet deposition (Sabater and Carrasco, 2001). Environmental contamination due to the excessive use of

pesticides has become a great concern to the public and to environmental regulatory authorities.

Among the organophosphorous insecticide, one of the widely used insecticide is Chlorpyrifos [0, 0-diethyl 0-3, 5, 6-trichloro - 2-pyridinyl-phosphorothioate] (Cho *et al.,* 2002). It is effective against a broad spectrum of insect pests on a variety of crops like cotton, vegetables, fruits, sugarcane, golf turf grass and residential pest control.In India, chlorpyrifos was the second most used agricultural insecticide during 2013 - 2014,with a production of 9540 tons (Shreekumar *et al.,* 2017. It has low water solubility, 2mgL^{-1}, but it is highly soluble in many organic solvents. Chlorpyrifos has high soil sorption co-efficient *(K_d* = 13.4 to 1862 mL/g) depending on the soil type with a half-life of 10 to 120 days in different soil. (Pandey and Singh, 2004)· Like other organophosphorous pesticides, its insecticidal action is due to the inhibition of the enzyme acetylcholinesterase, resulting in the accumulation of the neurotransmitter, acetylcholine, at nerve endings (Kanekar *et al.,* 2004), this results in the excessive transmission of nerve impulses, which causes a potential risk to the humans and other organisms.

Freshwater phytoplankton species show a variable sensitivity to pesticides. Generally, photosynthesis and growth of phytoplankton are negatively affected byexposure to pesticides (Shoaib *et al.,* 2011). It is estimated that these microalgae may account for 40 to 45% of oceanic production and are considered as more productive than all the worlds' rainforests (Mann, 1999) and any negative impact caused on phytoplankton would have deleterious effect.

These pesticides are often toxic to freshwater organisms found in the environment. Due attention is required to study the impact of organophosphorous insecticide onnontarget organisms in the aquatic environment. Micro-algae need special attention considering the ecological position in the food chain. They are at the base of aquatic food web as primary producers. They play a significant role in nutrient cycle and oxygen production (Asselborn *et al.,* 2015). Few reports are available on the effects of chlorpyrifos on nontarget aquatic organisms (Asselborn *et al.,* 2015). Studies conducted reveals that some algae can bioaccumulate pesticide (Subashchandrabose *et al.,* 2013) and hence can play a key role in the transport of this organic contaminants through the food chain to higher trophic levels (Wang and Wang, 2005).

Cyanobacteria, *Spirulina platensis*a multicellular, filamentous micro algae, with high nutritional value due to rich protein, carbohydrates, essential fatty acids, vitamins, minerals, carotenes, chlorophyll a and phycocyanin, is used as a food supplement for humans and animals. This photosynthetic prokaryotes, which plays a key role in photosynthetic fixation of energy and its transfer to higher trophic levels (Lee *et al.,* 2001).

Researchers have reported that cyanobacterial photosynthesis, growth and heterocyst differentiation is significantly reduced or inhibited by herbicides and pesticides (Shoaib *et al.,* 2012). Due to nutritional, ecological and economic properties *Spirulina platensis* has been the area of research. (Ali and Saleh, 2012).

The aim of this study was to evaluate the effects of different concentrations of the organophosphorus insecticide chlorpyrifos on the growth, pigment and protein content of the cyanobacterium, *Spirulina (Arthrospira) platensis.*

Materials and Methods

The indoor culture of microalgae

The indoor batch cultivation of *S. platensis* was carried out in Erlenmeyer flasks (250, 500 and 1000 mL). The indoor culture was maintained in plant growth chamber with an illumination of 3500 ± 100 lux using compact fluorescent lamps (Philips, 23 W). The light intensity was measured using lux meter (LX-103, Taiwan). The photoperiod was fixed at 12:12 hour light and dark periods.

The temperature was maintained at 24 ± 2^{o}C. The cultures were shaken twice in a day to ensure the proper mixing of the algal suspension. A closed airlift indoor culture unit of 20 L capacity was used for the continuous culture of algae and the cultures were aerated using an air injection device which supplied air at the bottom of the aspirator bottle, and the air-flow was adjusted to a level that ensured proper mixing of the culture.

Selection of Growth Medium

The pure culture of *S. platensis* was subcultured in modified Nallayam Research Centre medium (Bhuvaneswari G.R. *et al.,* 2014) under specified photoautotrophic conditionsin indoor airlift cultures. The composition of the growth medium isconsist - 5 g NaCl, 2.5 g $NaNO_3$, 0.01 g $FeSO_4.7H_2O$, 0.5 g K_2SO_4, 0.16 g $MgSO_4.7H_2O$, 8 g $NaHCO_3$, and 0.5 g K_2HPO_4 per litre.

Preparation of stock and working test solution of Chlorpyrifos (CP)

Chlorpyrifos (purity ≥99% Chlorpyrifos), purchased from Sigma Aldrich, the USA was used for the experiments. A stock solution of CP (2000mg L^{-1}) was prepared freshly prior to the experiment by dissolving required amount of CP in Acetone.

Toxicity assay

Toxicity studies were carried out in various concentrations viz. 10, 20, 40, 60, 80 and 100 mg L^{-1} of CP solution. These concentrations were obtained by the appropriate dilution of the stock solution of CP in respective media. Simultaneously controls were also prepared for each concentration by adding the same amount of acetone to that of test solutions, without CP in the algal medium.

Algal species and culture conditions

Toxicity experiment was conducted according to OECD guidelines 201 (OECD, 2006), with certain modifications when necessary. The inoculum of *S. platensis* was prepared in mNRC medium for the experiment, two days before the test to ensure that the algal cells exposed to CP are in exponential phase.

The exponentially growing algal culture was harvested by centrifugation and resuspended in CP solution of graded concentrations in the medium. The culture density for all the experiments was maintained at 3 x 10^5 cells mL^{-1}.

Three replicates at each test concentration including control were incubated for 72 hrs under the following photoautotrophic conditions, specified earlier. The cultures were manually shaken twice a day, i.e. in morning and evening to resuspend any settled cells. Samples were analyzed at every 24 hrs time interval by measuring the direct optical density at 750 nm to calculate the specific growth rate and generation time, SOD activity and protein content using a double beam UV-visible spectrophotometer (MOTRAS Scientific, New Delhi).

The number of algal cells was counted using Sedgewick Rafter cell counter (Partex Products, Mumbai) using a light microscope.

Analytical procedures

Specific growth rate and generation time

The specific growth rate (K) of the alga was calculated by using the formula given by Kratz and Myers (1955):

$$K\ (day^{-1}) = \frac{2.303\ \log N_t - \log N_0}{(T_t - T_0)}$$

Where N_0 is the initial optical density at 750 nm at time T_0 and

N_t is the final optical density at time T_t when culture is in exponential phase

The generation time (G) was calculated by using the formula:

$$G\ (days) = \frac{0.693}{K}$$

Where K is the specific growth rate

Test endpoint

The test endpoint was measured in terms of inhibition of growth, expressed as the logarithmic increase in biomass (in terms of cell counts) during the exposure period. Percent inhibition (in terms of cell counts) was calculated as:

$$\%I = \frac{(\mu c - \mu t)}{\mu c} \times 100$$

Where:

%I = Percent inhibition in cell counts;

μc = mean value of cell counts in the control group;

μt = mean value of cell counts of the treatment groups

Median effective concentration (EC_{50}) of CP for microalgae S. *platensis*

72-h EC_{50} of CP for *S. platensis* was calculated using probit analysis (Finney, 1971). EC_{50} of CP is the concentration of the test substance that results in 50% reduction in growth or algal cells within the stated exposure period.

Extraction and analysis of chlorophyll *a* and carotenoid

Algal cultures from all controls and treatments of volume 15 mL were taken after 72 h exposure with various concentrations of CP used for toxicity experiment. The cultures were centrifuged (Eltek Microprocessor High-speed Research refrigerated centrifuge, MP 400 R, India) at 7700 g for 10 minutes at 4°C.

The supernatants were discarded, and 15 mL of N, N-dimethyl formamide (DMF) was added to the remaining pellets and kept for 24-h for incubation at the room temperature. After the incubation, it was centrifuged at 7700 g for 10 minutes. The supernatants were collected in separate tubes, and optical densities were measured at prescribed wavelengths (664, 647 and 461 nm). The pigments (chlorophyll-a (Moran, 1982) and carotenoid (Chamovitz *et al.*, 1993)) present in *S. platensis* was calculated as follows:

$$\text{Chlorophyll-a}\ (\mu g\ mL^{-1}) = OD_{664} \times 11.92$$

$$\text{Carotenoid}\ (\mu g\ mL^{-1}) = [OD_{461} - (0.046 \times OD_{664})] \times 4$$

Enzyme assay

Assessment of antioxidant enzyme is necessary to estimate the microalgal cell's

tolerance and response to CP. For this purpose, 5mL of the microalgal suspension was withdrawn from the culture at a regular time interval and centrifuged at 4500 rpm for 10 min at 4^{o}C. The biomass pellet was washed with distilled water to remove unnecessary traces of the medium and the centrifuged again.

The recovered cell pellet was resuspended in 0.1 M Tris HCl (pH 7.4), sonicated for 5 min at 4^{o}C and centrifuged at 10,000 rpm for 10 min. The cell lysate supernatant collected after centrifugation was used to determine the activities of SOD. The amount of enzyme that caused a 50%decrease in the nitroblue tetrazolium reduction is referred as one unit of SOD activity (Kurade *et al.*, 2016).

Data analysis

The 72-h median effective concentration EC_{50} of CP for *S. platensis* was calculated using probit analysis, SPSS 21.0.

Further, the toxicity experiments were statistically analysed using SPSS 21.0 in which data were subjected to one-way analysis of variance (ANOVA) and when differences observed were significant, the means were compared by Duncan multiple range tests, at a level of significance of 0.05 ($p < 0.05$).

Results and Discussion

Influence of CP on growth rates and generation time

CP could suppress the growth of *S. platensis* in a concentration-dependent manner during 72 h exposure reaction period. Compared with control groups, CP at all studied concentrations can significantly inhibit the growth of the algae. The specific growth rate of alga was decreased up to 40 mg L^{-1}, while no growth detected at exposure to higher concentration. Generation time (G) presented a similar response pattern to the specific growth rate (Table 1).

Percent growth inhibition

Based on the number of cells in the controls and treatments, percent inhibition of growth was calculated post 72-h of the experiment. A significant difference ($p < 0.05$) in percent growth inhibition of *S. platensis* was observed among the various concentrations of CP exposed groups. Low concentrations (10 mg L^{-1}) of CP had minor effects on the growth of *S. platensis.* The highest percent inhibition (54.64%) occurred at 40 mg L^{-1} CP concentration (Figure 1).

The EC_{50} for the % growth inhibition at the end of the bioassay was calculated and was found as 33.65 mg L^{-1} of CP with a confidence interval (95%).

Effect of CP on chlorophyll-*a* and carotenoid content

The content and composition of pigment were measured after 72-h of exposure to CP. The pigments, chlorophyll-*a*, and carotenoids were measured and found to have significant ($p<0.05$) reduction in level in all the CP exposed groups when compared with control. The chlorophyll-*a* was 9.37 µgmL^{-1} in control (Table 2) wherein it was reduced to 6.53 µgmL^{-1} in highest of CP i.e. 40 mg L^{-1}. A 32.2 % decrease in the Chlorophyll-*a* content was recorded in 40mg L^{-1}(Fig 2) CP exposed group in comparison to control. The carotenoids content ranged from 3.10 to1.66 µgmL^{-1} in control and treatments. The carotenoids content was significantly reduced in comparison to control and among treatment, and also significant between treatments was found in a dose-dependent manner. The carotenoid content was reduced by 48.33% in exposure to 40 mgL^{-1} concentration.

Table.1 Growth rates of *S. platensis* after 72 h exposure to various concentration of CP

Concentration of CP (mg L^{-1})	SGR (day^{-1})	Generation Time (days)
0	0.24^{b}±0.02	2.88^{a}±0.19
10	0.23^{b}±0.01	3.01^{a}±0.03
20	0.13^{a}±0.01	5.21^{b}±0.07
40	0.10^{a}±0.01	6.84^{c}±0.67

Table.2 Effect of various concentrations of CP on the pigment composition of *S.platensis*

Concentration of CP (mgL^{-1})	Chl a (μgmL^{-1})	Carotenoids (μgmL^{-1})
0 (control)	9.37^{b}±0.27	3.10^{c}±0.11
10	8.88^{b}±0.64	2.90^{c}±0.01
20	8.40^{b}±0.09	2.38^{b}±0.23
40	6.53^{a}±0.16	1.66^{a}±0.05

Data are represented in mean ± SE, n=3. The data labels represent the significant difference ($p<0.05$).

Fig.1 Percent inhibition of growth in *S. platensis* exposed to different concentration of CP

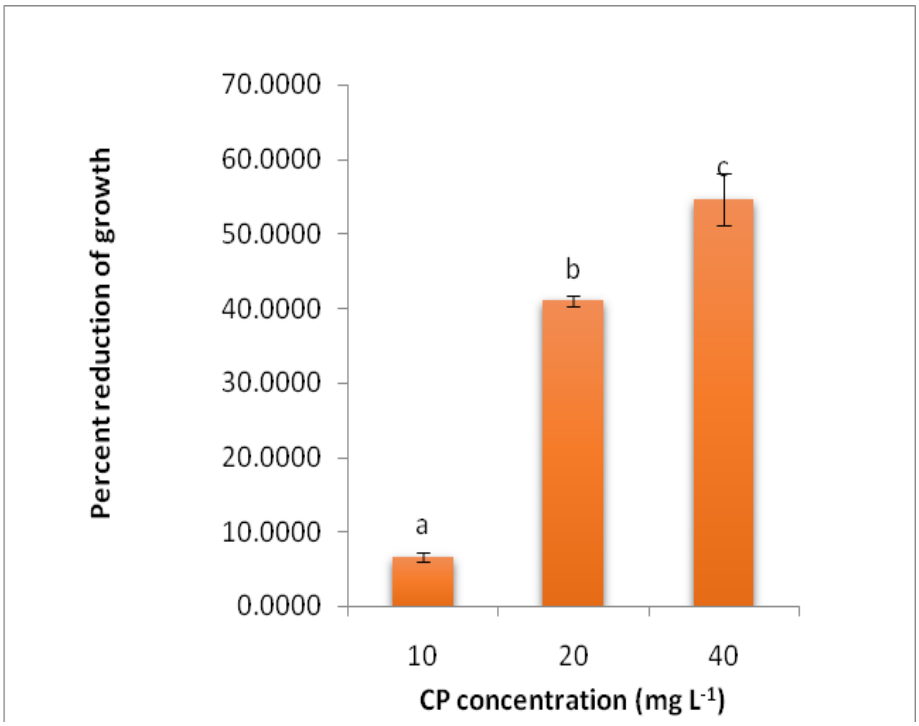


Fig.2 Effect of various concentrations of CP on the pigment composition of *S. platensis*. Data are represented in mean ± SE, n=3. The data labels represent the significant difference ($p<0.05$)

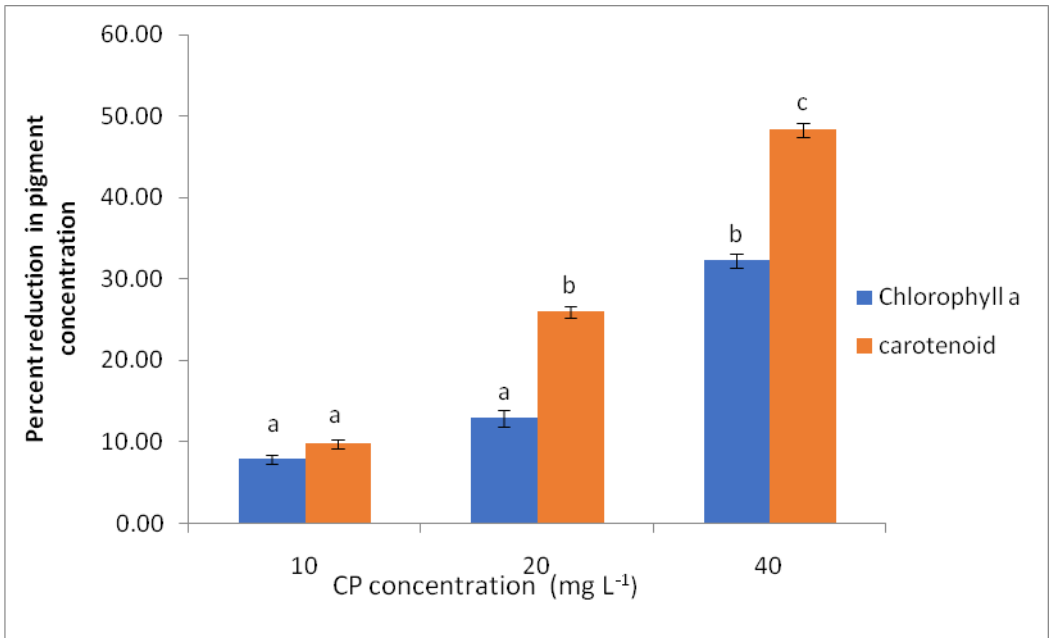
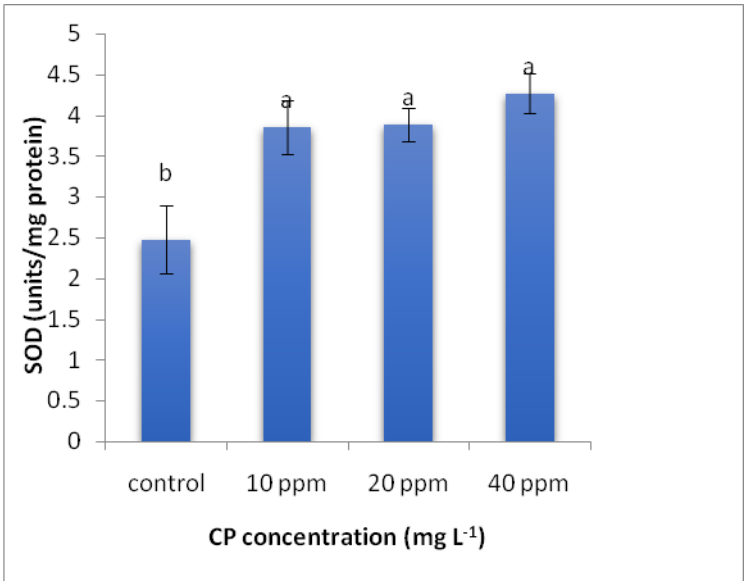


Fig.3 Effect of Chlorpyrifos on SOD Enzyme



Effect on Antioxidant Enzyme –SOD

The SOD activity in *S.platensis* cells in the presence of CP was enhanced due to the exposure to various concentration over the untreated cells. There was significant ($p<0.05$) increase in SOD enzyme activity between the control and treatment (Fig 3). However, there was no significant difference was found between the treatments.

The microalgae, which are the primary producers and in the base of the aquatic food chain, plays a key role in the structure and

function of an ecosystem. Among the aquatic organisms, it is reported that sensitivity of algae and cyanobacteria are high. They are very important indicators used to assess the toxicity of chemicals released to the aquatic environment (Burkiewicz K., 2005). To understand the toxicity of a compound, algal toxicity tests are widely used based on assessing the growth inhibition of the microalgae.

In the present study, the toxicity of CP to *S. Platensis* was evaluated by phytotoxicity tests based on growth inhibition, the percent reduction in the growth of algae with pesticide culture compared with control cultures without the pesticide as reported by Oliveira *et al.,* (2007). At higher concentration of pesticide, there was a highly significant reduction of Specific growth rate and proportionate increase in percent inhibition of growth was observed. This could be because of the change in the proportion of pesticide concentration and the existing number of algal cells (Oterler *et al.,* 2016). This dose-dependent reduction in growth was observed by many researchers (Asselborn *et al.,* 2015; Wang and Wang, 2016).

The commission of the European Communities (1996) classified different toxic classes based on the EC_{50}value of the toxicant. Based on that CP is found to be harmful to *S.platensis* because the EC_{50} value is 33.65 mgL^{-1} which is in the classified range (10-100 mgL^{-1}). Wang and Wang (2016) found the EC_{50} value ranging from 27.80 mg/L (24 h) to 25.80 mg/L (72 h) in a cyanobacterial species (*Merismopedia sp*) against chlorpyrifos. Sun *et al.,* (2015) found the EC50 value of a cyanobacterium which is 21.13 µ mol L^{-1}, which is too low compared to our results.

Chlorophyll is considered as the sensitive biomarker when exposed to toxicants because of their role in photosynthetic electron transport activity (Huang *et al.,* 2012). Organophosphorus insecticides are known to affect the photosynthetic process of microalgae by interfering with the synthesis of chlorophyll a (Caceres *et al.,* 2008). The growth, synthesis of chlorophyll *a* and the photosynthetic process of the microalgal cells was significantly inhibited at high concentrations of CP (Wong and Chang, 1988). Our results are in agreement with Oterler, (2016) as he also found a negative correlation of Chlorophyll content with increase in pesticide concentration. A similar pattern of drop-in Chlorophyll_a content and decreased synthesis in the cells causing decreased protein content was found in the results of the studies conducted by Ou *et al.,* (2003) and Xia *et al.,* (2007). Chlorophyll content is directly related to the biomass. Hence, logically, the reduction in biomass obviously leads to decrease in Chlorophyll concentration.

Carotenoid also serves as sensitive biomarkers for monitoring aquatic contaminants. Its role is to deactivate the excited chlorophyll to avoid the stress-induced damage of the photosynthetic system triggered by the formation of reactive oxygen species (ROS) with exposure to toxicants (Xiong *et al.,* 2016). Like, the reduction in chlorophyll pigments, carotenoids content also was greatly inhibited at relatively high concentrations of CP indicating CP is toxic to S. *Platensis* metabolism which is supported by the results of the study by Asselborn *et al.,* (2015). Our results shows that % decrease in carotenoid content is higher than the % decrease in chlorophyll-*a* content, revealing that carotenoid is more sensitive to CP than chlorophyll-*a*. The decrease in carotenoid contents might be associated with the lipid peroxidation along with the potassium leakage at high concentrations of organophosphorus pesticide as reported by

Chen *et al.,* (2011) and Kurade *et al.,* (2016). Singh *et al.,* (2013) found that the herbicide Anilofos caused inhibitory effects on photosynthetic pigments of the test organism in a dose-dependent manner. The organism exhibited 60, 89, 96, 85 and 79% decrease in chlorophyll a, carotenoids, phycocyanin, allophycocyanin and phycoerythrin, respectively, in 20 mg L^{-1} anilofos on day six. Their findings support our results well.

The SOD activity in *S. platensis* cells in the presence of CP was enhanced due to the exposure to various concentration of CP overuntreated cells. This result is in agreement with various researchers, as they have found significant and progressive increase in SOD activity to the increasing concentration of pesticide (Asselborn *et al.,* 2015; Kurade *et al.,* 2016). Many researchers had proved that organic pollutants tend to stimulate overproduction and accumulation of reactive oxygen species (ROS), including superoxide anions ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Torres *et al.,* 2008). Once the microalgal cells are exposed to pollutants, the cellular detoxification system is initiated by synthesis of SOD to put an end to the toxic stress caused due to ROS (Li *et al.,* 2009).

Superoxide dismutase (SOD) serve as sensitive biomarkers, which can be used as early warnings of pollution. It the first line of defense system of the cell against ROS, catalyses the disproportionation of superoxide anions O2 •−, to produce H_2O_2 and O_2. This is followed by the action of catalase which disintegrate hydrogen peroxide into water and oxygen. If these enzymes SOD and catalase fails to catalyse the process of disintegration of ROS it may lead to programmed cell death (Torres *et al.,* 2008). An increase in the SOD levels of microalgae as a response to oxidative stress induced by pesticides has been reported earlier (Singh *et al.,* 2013; Kurade *et al.,* 2016).

The present study demonstrates that the exposure of microalgae to the insecticide Chlorpyrifosposes negative impact. The growth of *S. platensis* was inhibited after the exposure to different concentrations of CP with the specific growth rate decreasing progressively upon the increase in the pesticide concentration. There was also a significant difference between the control and treatment in parameters like percent growth reduction, Chlorophyll *a*, carotenoid and antioxidant enzymes. Thus, it reveals that effects of Chlorpyrifos are not only restricted to target organisms but also causes an adverse impact on non-target organisms especially phytoplankton, which plays an important role in the functioning of aquatic ecosystems as sole primary producers.

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