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Oxidative stress in *Arthrospira platensis* by two organophosphate pesticides

HATİCE TUNCA, ALİ DOĞRU, FERAY KÖÇKAR, HEDİYE E. KILIÇ & TUĞBA O. SEVİNDİK

Abstract: Although it is known that organophosphate insecticides are harmfull to aquatic ecosystems, oxidative damages caused by Dimethoate and Chlorpyrifos are not studied on *Arthrospira platensis* Gomont. In this study, various Chlorpyrifos (0-150 µg mL^{-1}) and Dimethoate (0-250 µg mL^{-1}) concentrations were added to the culture medium in laboratory to evaulate growth rate, chlorophyll-*a* content and antioxidant parameters of *A. platensis*. Optical Density (OD560) and chlorophyll-*a* decreased compared to the control for seven days in both pesticide applications. Superoxide dismutase (SOD) activity increased at 50 µg mL^{-1} Chlorpyrifos concentration but it decreased at all concentrations. Although Ascorbate peroxidase (APX) and glutathione reductase (GR) activities increased with Chlorpyrifos application, they did not change with Dimethoate application. Malondialdehyde (MDA) amount decreased at 150 µg mL^{-1} Chlorpyrifos concentration but it increased in Dimethoate application. The H$_2$O$_2$ content were increased in both applications. Proline decreased in 50 and 75 µg mL^{-1} Chlorpyrifos concentrations and increased at 150 µg mL^{-1} concentration, while it increased at 25 µg mL^{-1} Dimethoate concentration. The results were tested at 0.05 significance level. These pesticides inhibit *A. platensis* growth and chlorophyll-*a* production and cause oxidative stress. The excessive use may affect the phytoplankton and have negative consequences in the aquatic ecosystem.

Key words: *Arthrospira platensis*, chlorpyrifos, dimethoate, antioxidants, oxidative stress.

INTRODUCTION

Organochlorinated pesticides have been replaced by organophosphate compounds due to their toxicities and being preference for faster degradable active substances in the environment (Larson et al. 1997, Hansen et al. 1983, Coats et al. 1989, Sreekumar & Devatha 2017). These compounds are often preferred due to their wide range of activities and low cost (Shetty et al. 2000). Organophosphorus insecticides can enter the aquatic ecosystem through basin drainage, accidental spillage or spraying into the air (Sabater & Carrasco 2001). Organophosphate compounds that interfere with the aquatic ecosystem can be metabolized by cyanobacteria and algae, and they can be used as additional nutrients by these organisms since they contain carbon, nitrogen, and phosphorus. The phosphorus addition in the aquatic systems as Organophosphate can alter the phytoplankton composition and, as in plants, is likely to cause damage to the photosynthetic pigments and the membrane, reduce photosynthetic activity, accumulate active oxygen forms and increase enzymatic and non-enzymatic antioxidants (Cáceres et al. 2008, Thengodkar & Sivakami 2010).

Chlorpyrifos (O, O-diethyl O- (3,5,6-trichloro-2-pyridinyl) phosphorothioate) is one of the common insecticides used in agriculture and urban applications and is effective in controlling plant and soil insects on various food products (Larson et al. 1997, Schiff et al. 2002). Although Chlorpyrifos are in the banned pesticides in worldwide and Turkey, they are continuing to be use in many major countries like USA and it is used over 1000 lbs at only Minnesota in 2018 (TOB 2020, MDA 2020). The water solubility is low (2 mg L^{-1}) but it is highly soluble in many organic solvents. When processed in moist soils, the volatility half-life of Chlorpyrifos is 45-163 hours, 62-89% of the applied Chlorpyrifos remain in the soil after 36 hours (Racke 1992). Although Chlorpyrifos bioaccumulation is thought to be less like other organophosphate compounds because it can not stay in the environment for a long time, 14 C-labeled studies have shown that this pesticide is found in lake and marine sediments (Lacorte et al. 1995, Green et al. 1996, Carvalho et al. 2002, Jantunen et al. 2008).

Dimethoate, also an organophosphate pesticide (O, O-dimethyl S-methylcarbamoylmethylphos-phoroditioate) is a common dithiophosphate pesticide applied worldwide to insects and mites in agricultural products and ornamental plants (Hayes & Laws 1991, Doğan & Can 2011). Dimethote was banned in EU countries but it is still used in other countries and it is reported that the used active ingredient is 2,959,621 lbs per year (NIH 2020). Dimethoate is low soluble (1.4 mg mL^{-1}) in water and highly soluble in most organic solvents and has low volatility (MacBean 2010, PPDB 2020). Dimethoate exerts acute toxic effects as a cholinesterase inhibitor in these pesticidal insects that show contact and systemic effects (Tomlin 1997). The entrance of Dimethoate into the aquatic ecosystem causes to affect non-target organisms so that one step of the food chain will be damaged, it will affect the entire food web and the ecosystem balances deteriorate (Guo et al. 2012). While the effects of Dimethoate and Chlorypyrifos were investigated as regards of food chain interactions in the aquatic ecosystem (Kramarz & Laskowski 1999, Varo et al. 2002), some researches focused on only the harmful effects of non-target aquatic organisms (Wijngaarden et al. 1993, Moore al. 1998, Doğan & Can 2011, Tripathi & Singh 2003, Lundebye et al. 1997, Andersen et al. 2006).

Cyanobacteria, one of the photosynthetic prokaryotes involved in ecological processes such as nitrogen fixation and carbon production, can accumulate pesticides in the environment and are effective in transporting organic pollutants to higher trophic levels along the food chain (Lee et al. 2001, Bashan et al. 1998, Wang & Wang 2005). Cyanobacteria are exposed to various natural stresses such as nutrient restriction, pollution, drought, salinity, temperature, pH, light intensity and quality, as well as pesticides that are indispensable for modern agricultural practice. Insecticides interact with biological systems around them, causing environmental problems (Thompson et al. 1993, Berard et al. 1999). Cyanobacteria display a variable sensitivity to these compounds. In general, the rate of photosynthesis and growth of phytoplankton are adversely affected by pesticide exposure (Shoaib et al. 2011).

Arthrospira platensis is suitable for animal diet due to its high nutritional content (Belay et al. 1996). Since it is a cyanobacterium, it is the primary step of the food network (Vonshak & Tomaselli 2000). The high ecological features of *A. platensis* have attracted the attention of scientists and most scientific studies have focused on it (Ciferri 1983, Ali & Saleh 2012). It is reported that *A. platensis* able to biodegrade and tolerate pesticides (Thengodkar & Sivakami 2010). So *A. platensis* is important for determining

the maximum limits of pesticide concentrations which is withstood by organisms. In addition, this strain can be easily produced in a laboratory environment (Vonshak & Tomaselli 2000).

Studies investigating the effects of Chlorpyrifos and Dimethoate are limited to their effects on algal and cyanobacterial growth and development. Kumar et al. (2014) specified that the reduction in growth rate arose from Chlorpyrifos application may be related to inhibition of pigment synthesis in *Chroococcus turgidus* NTMS12 cells. Asselborn et al. (2015) investigated the effects of Chlorpyrifos on the growth of *Ankistrodesmus gracilis* and reported that algae growth significantly decreased compared to control. Some studies show that Dimethoate has a growth inhibitory effect on algae and cyanobacteria and decreases the amount of chlorophyll-*a* (Wong & Chan 1988, Perona et al. 1991, Mohapatra & Mohanty 1992, Shizhong et al. 1997, Mei & Zan 1998). Yadav (2015) found that Dimethoate reduced the amount and growth rate of chlorophyll-*a* in *Spirulina platensis*.

Antioxidants are compounds that reduce the harmful effects of oxidation via inhibiting free oxygen formation or eliminating formed free radicals (Baublis et al. 2000, Sivritepe 2000). The antioxidant enzymes related to each other in Halliwey-Asada pathway. Superoxide dismutase (SOD: EC 1.15.1.1) is a metalloenzyme that converts superoxide anions to oxygen and hydrogen peroxide (H_2O_2) (Valentine et al. 1998). Ascorbate peroxidase (APX: EC 1.11.1.11) uses ascorbate as an electron donor and catalyzes the conversion of hydrogen peroxide to water (Noctor & Foyer 1998, Chew et al. 2003). Glutathione reductase (GR: EC 1.6.4.2) reduces oxidized glutathione (GSH) to reduced glutathione together with oxidation of NADPH and provides the substrate for Halliwey-Asada pathway (Contour-Ansel et al. 2006, Anjum et al. 2010). Malondialdehyde

is a peroxidation metabolite that forms from lipids contain three or more than double bonds (Altınışık 2000). Some studies display that proline content increases under various stress factors (Bassi & Sharma 1993, Delauney & Verma 1993) and this increase maintains the integrity of cell parts and cell contents such as enzymes, membranes and polyribosomes (Arakawa & Timasheff 1985, Kandpal & Rao 1985, Rudolph et al. 1986).

Despite the arrangements, Chlorpyrifos and Dimethoate consumption continues to contaminate the aquatic ecosystems (Nag et al. 2020, Dereumeaux et al. 2020). Bhuvaneswari et al. (2018) examined the effects of Chlorpyrifos on *A. platensis* antioxidant enzymes, but this study was limited only with SOD. More detailed investigation of the Chlorpyrifos and Dimethoate effects on the antioxidant enzymes are important for assessing the oxidative stress caused by organophosphate pesticides. Because, even if toxic doses are high for a living thing, cellular responses to these substances may contain lower concentrations (Stacey & Kappus 1982). Antioxidant systems play an important role in understanding intracellular levels of toxicity (Regoli & Giuliani 2014). For this reason, the aim of the study is to evaluate the effects of Chlorpyrifos and Dimethoate on the growth rate, inhibition of chlorophyll-*a* production, antioxidant enzymes, and non-enzyme antioxidant parameters for *A. platensis*.

MATERIALS AND METHODS
Algae culture and treatment

A. platensis-M2 was obtained from the Soley Microalgae Institute (California, USA) (Culture collection No: SLSP01). Algae were grown in Spirulina Medium (Aiba & Ogawa 1977) under axenic conditions. 20 mL algal cultures were inoculated to 180 mL culture medium in 250

mL Erlenmeyer flask and were allowed to grow under the conditions of 93 µmol photons m^{-2} s^{-1} photosynthetically available radiation in 12:12 h light/dark cycle at 30±1 °C for 10 days. At the end of 10 days, cultures were renewed and, all the flasks contained 50 mL algal culture. The commercial formulation of Chlorpyrifos and Dimethoate (480 g L^{-1} and 400 g L^{-1}, respectively, EC, Sakarya, Turkey) were used in all bioassay and prepared in distilled water. Various Chlorpyrifos (0-150 µg mL^{-1}) and Dimethoate (0-250 µg mL^{-1}) concentrations were added to the culture medium. The range of concentrations was determined with preliminary range-finding bioassays according to EC50 value for growth parameters and IC50 value for enzyme activity assays.

Cell growth and chlorophyll-*a* assay

Optic density (OD) of microalgae were measured spectrophotometrically over a period of 7 days under control and stressed conditions taking absorbance at 560. OD measurement used for microalgal growth by means of density and live microalgal cultures and OD 560 was selected because chlorophylls show low absorbance at this wavelength. Chlorophyll-*a* content was estimated by methanol extraction in the ratio of (1/10) and measured spectrophotometrically every 24 h (MacKinney 1941). Experiments were conducted for 7 days according to the chart of growth rate. Three replicate cultures were used for each treatment.

Antioxidant enzyme activities

On the 7th day of the study, 2 mL culture solutions from the control and treated samples were centrifuged at 14.000 rpm for 20 min at 4°C and resulting pellets were kept at -20 °C until enzyme activity measurements. Pellets were grounded with liquid nitrogen and suspended in specific buffers with proper pH values for each

enzyme. The protein concentrations of algal cell extracts were determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

The SOD activity was determined by the method of Beyer & Fridovich (1987), based on the photoreduction of NBT (nitroblue tetrazolium). Extraction of pellets (0.2 g) was performed in 1.5 mL homogenization buffer containing 100 mM K_2HPO_4 buffer (pH 7.0), 2% PVP and 1 mM Na_2EDTA. After centrifugation at 14.000 rpm for 20 min at 4°C, the resulting supernatants were used to measure SOD activity. The reaction mixture consisted of 100 mM K_2HPO_4 buffer (pH 7.8) containing 9.9 x 10^{-3} M methionine, 5.7 x 10-5 M NBT, %1 Triton X-100 and enzyme extract. The reaction was started by the addition of 0.9 µM riboflavin and the mixture was exposed to light with an intensity of 375 µmole m^{-2} s^{-1}. After 15 min, the reaction was stopped by switching off the light and absorbance was read at 560 nm. The SOD activity was calculated by a standard graphic and expressed as unit mg^{-1} protein.

The GR activity was measured with the method of Sgherri et al. (1994). Extraction was performed in 1.5 mL of suspension solution containing 100 mM K_2HPO_4 buffer (pH 7.0), 1 mM Na_2EDTA, and 2% PVP. The reaction mixture (total volume of 1 mL) contained 100 mM K_2HPO_4 buffer (pH 7.8), 2 mM Na_2EDTA, 0.5 mM oxidised glutathione (GSSG), 0.2 mM NADPH and enzyme extract containing 100 µg protein. The decrease in absorbance at 340 nm was recorded. The correction was made for the non-enzymatic oxidation of NADPH by recording the decrease at 340 nm without adding GSSG to assay mixture. The enzyme activity was calculated from the initial rate of the reaction after subtracting the non-enzymatic oxidation using the extinction coefficient of NADPH (E = 6.2 mM cm^{-1} at 340 nm) (nmol $NADPH^{-1}$ min^{-1} mg protein $^{-1}$).

The APX activity was determined according to Wang et al. (1991) by estimating the decreasing rate of ascorbate oxidation at 290 nm. APX extraction was performed in 50 mM Tris–HCl (pH 7.2), 2% PVP, 1 mM Na_2EDTA, and 2 mM ascorbate. The reaction mixture consisted of 50 mM K_2HPO_4 buffer (pH 6.6), 2.5 mM ascorbate, 10 mM H_2O_2, and enzyme-containing 100 μg protein in a final volume of 1 mL. The enzyme activity was calculated from the initial rate of the reaction using the extinction coefficient of ascorbate (E = 2.8 mM cm^{-1} at 290 nm) ($nmol^{-1}AA^{-1}min^{-1}$ mg $protein^{-1}$).

Determination of malondialdehyde and hydrogen peroxide

The malondialdehyde content was determined by the method of Heath & Packer (1968). 0.2 g of pellet was homogenized in 3 mL of 0.1% TCA (4°C) and centrifuged at 4100 rpm for 15 min and the supernatant was used in the subsequent determination. 0.5 mL of 0.1 M Tris–HCl pH 7.6 and 1 mL of TCA–TBA–HCl reagent (15% w/v) (trichloroacetic acid–0.375% w/v thiobarbituric acid–0.25 N hydrochloric acid) were added into the 0.5 ml of the supernatant. The mixture was heated at 95°C for 30 min and then quickly cooled in the ice bath. To remove suspended turbidity, the mixture centrifugated at 4100 rpm for 15 min, then the absorbance of the supernatant at 532 nm was recorded. Non-specific absorbance at 600 nm was measured and subtracted from the readings recorded at 532 nm. The MDA content was calculated using its extinction coefficient of 155 $mM^{-1} cm^{-1}$. For determination of the hydrogen peroxide content, 0.5 mL of 0.1 M Tris–HCl (pH 7.6) and 1 mL of 1 M KI were added to 0.5 mL of supernatant. After 90 min, the absorbance was recorded at 390 nm.

The proline content determination

The proline content was determined by the method of Weimberg et al. (1982). 0.1 g of pellet was homogenized in 10 mL of 3% aqueous sulphosalicylic acid and the homogenates were incubated in the hot water bath at 95 °C for 30 minutes. The samples were cooled and centrifuged at 4100 rpm for 10 min. 2 mL of the extract reacted with 2 mL of acid–ninhydrine and 2 mL of glacial acetic acid for 1 h at 100°C. The reaction mixture was extracted with 4 mL toluene. The chromophore containing toluene was separated and the absorbance was recorded at 520 nm.

Statistical analysis

The differences between the control and treated samples were analyzed by one-way ANOVA, taking $p<0.05$ as significant according to LSD analysis.

The difference was determined using the following formula:

LSD: $\sqrt{2} * \sqrt{Sx/n} * t_{(n+n-2)}$. Sx is the error term of mean square. N is the replication number of application. t is the distribution value in the t table.

Three replicate cultures were used for each treatment and standart error (SE) was used for assessment of statistical analysis because this value shows that how close the sample mean is to the mean and it describes bounds for a random sampling process. When comparing means from different groups, it is important to report the sensitivity of estimates for different means. The mean values ± SE were given in Figures.

Results

Both of pesticide applications decreased significantly OD 560 absorbance and chlorophyll-*a* compared to the control during seven days depending on the dose ($p<0.05$)
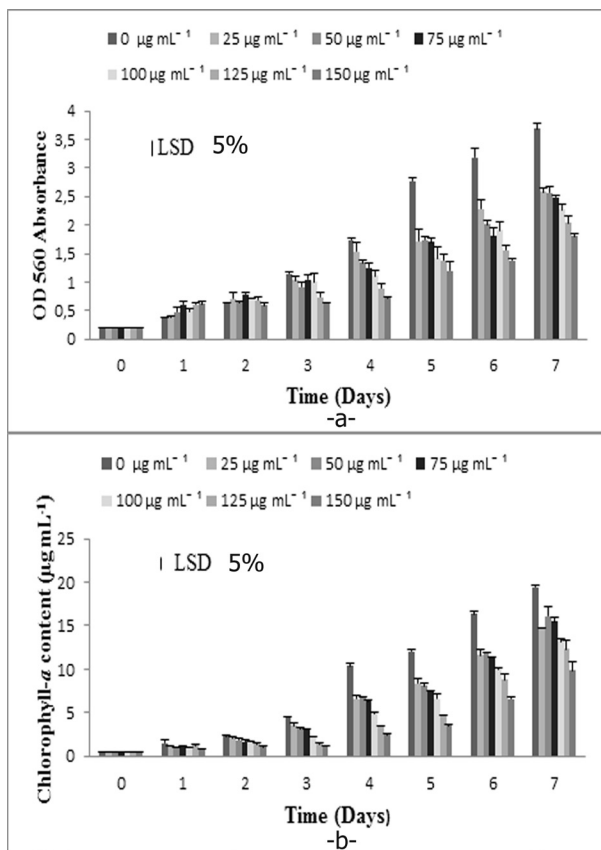
Figure 1. Biomass values (a) and (b) chlorophyll-*a* content of *Arthrospira platensis* supplemented with 0-150 µg mL $^{-1}$ Chlorpyrifos concentrations during 7 days. Data are the means ± SE of three replicates.
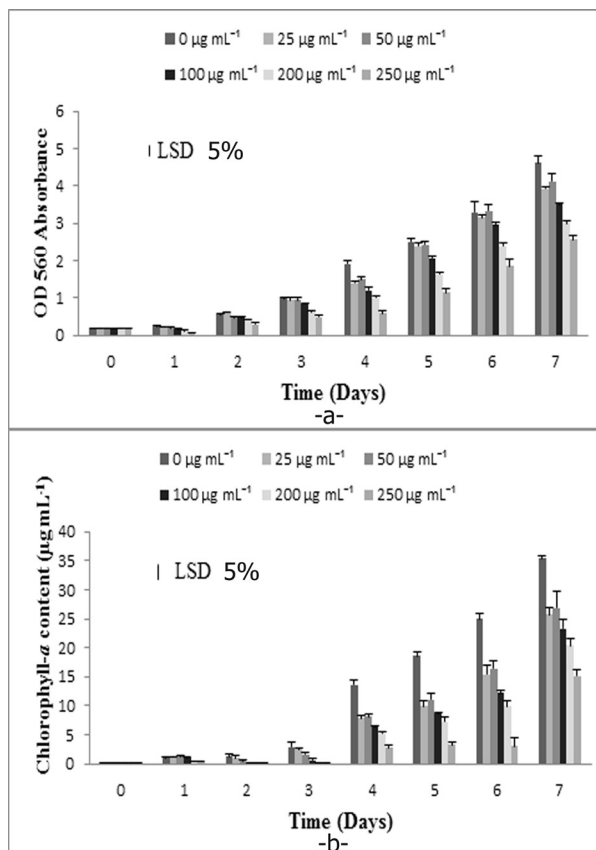

Figure 2. Biomass values(a) and (b) chlorophyll-*a* content of *Arthrospira platensis* supplemented with 0-250 µg mL^{-1} Dimethoate concentrations during 7 days. Data are the means ± SE of three replicates.

(Figure 1 - 2). In Chlorpyrifos application, OD 560 absorbance showed differences at 125 and 150 µg mL^{-1} concentrations compared to control, following to the 3rd day. The other doses except 25 µg mL^{-1} concentration differed from control in Chlorophyll-*a* parameter. In Dimethoate application, OD560 absorbance and chlorophyll-*a* amount differed at 200 and 250 µg mL^{-1} concentrations compared to the control, after the 3rd day.

Total SOD activity of *A. platensis* cultures exposed to Chlorpyrifos showed a significant increase at 50 µg mL^{-1} concentration compared to control ($p<0.05$) (Fig 3a). In Dimethoate application, SOD activity decreased at 20, 40, 60, 80 and 100 µg mL^{-1} concentrations ($p<0.05$) (Fig 4a). The GR enzyme activity increased

compared to control at 25, 50, 75, 100, 125 and 150 µg mL^{-1} concentrations in the Chlorpyrifos applications ($p <0.05$)(Fig 3b), but there was no significant change at 25, 50, 100, 200 and 250 µg mL^{-1} Dimethoate concentrations ($p>0.05$)(Fig 4b). Although APX activity displayed increases at 50, 100, 125 and 150 µg mL^{-1} Chlorpyrifos concentrations ($p <0.05$) (Fig 3c), Dimethoate application did not show a significant change at 20, 40, 60, 80 and 100 µg mL^{-1} concentrations compared to control ($p>0.05$) (Fig 4c). The MDA amount decreased at 150g mL^{-1} Chlorpyrifos application ($p<0.05$) (Fig 5a). However increased at 100, 200 and 250 mLg mL^{-1} Dimethoate concentrations ($p<0.05$) (Fig 6a). The H_2O_2 content increased at 100 µg mL^{-1} Chlorpyrifos application (Fig 5b) and at 50, 100, 200 ve 250 µg
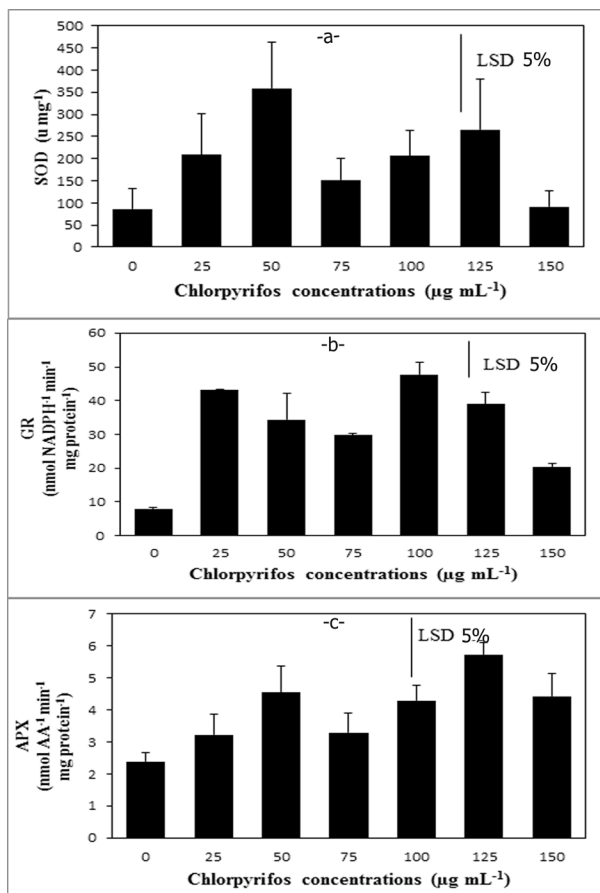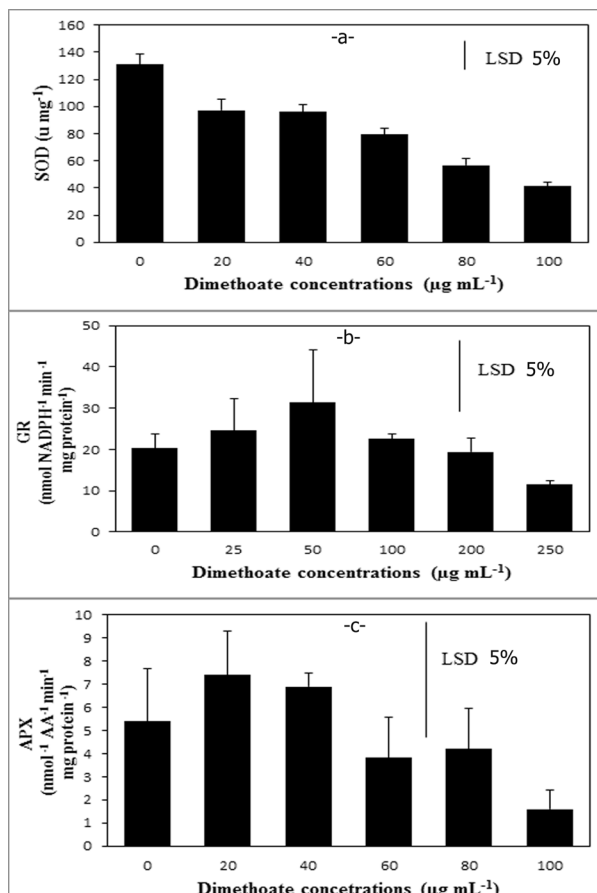
Figure 3. Total superoxide dismutase (SOD) (a), glutathione reductase (GR) (b), and ascorbate peroxidase (APX) (c) activities of *A. platensis* supplemented with Chlorpyrifos concentrations. Data are the means ± SE of three replicates. Mean values in columns are significantly different at the 5% level according to the least significant differences (LSD) Test.

Figure 4. Total superoxide dismutase (SOD) (a), glutathione reductase (GR) (b), and ascorbate peroxidase (APX) (c) activities of *A. platensis* supplemented with Dimethoate concentrations. Data are the means ± SE of three replicates. Mean values in columns are significantly different at the 5% level according to the least significant differences (LSD) Test.

mL^{-1} Dimethoate concentrations($p<0.05$) (Fig 6b). The amount of free proline showed a significant decrease in 50 and 75 µg mL^{-1} Chlorpyrifos concentrations and it increased at 150 µg mL^{-1} concentration ($p<0.05$) (Fig 5c), while Dimethoate application displayed a significant increase at 25 µg mL^{-1} application ($p<0.05$) (Fig 6c).

Discussion

In this study, the effects of Chlorpyrifos and Dimethoate were investigated on *A. platensis* and for this purpose, OD560, chlorophyll-*a* amount, the activity of superoxide dismutase, ascorbate peroxidase and glutathione reductase, H$_2$O$_2$, malondialdehyde and proline parameters were evaluated.

Some studies have supported the growth inhibitory effect of Chlorpyrifos and Dimethoate on some algae and cyanobacteria (Wong & Chan 1988, Perona et al. 1991, Mohapatra & Mohanty 1992, DeLorenzo & Serrano 2003, Shizhong et al. 1997, Mei & Zan 1998, Kumar et. al. 2014, Bhuvaneswari et al. 2018). Asselborn et al. (2015) investigated the effect of Chlorpyrifos on the growth of *Ankistrodesmus gracilis* at 9.37, 18.75, 37.5, 75 and 150 µg mL^{-1}
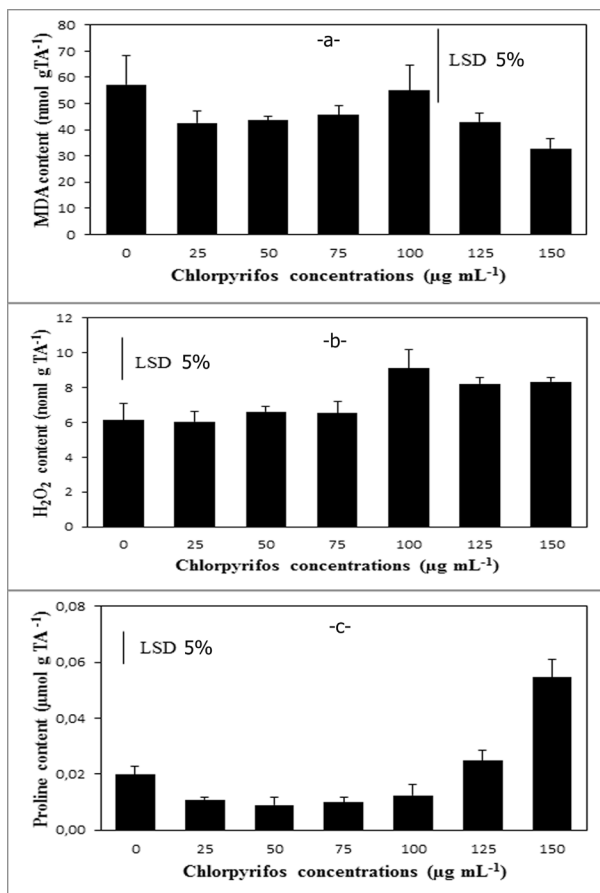
Figure 5. Malondialdehyde (a), hydrogen peroxide (b) and proline (c) contents of *A. platensis* supplemented with Chlorpyrifos concentrations. Data are the means ± SE of three replicates. Mean values in columns are significantly different at the 5% level according to the least significant differences (LSD) Test.
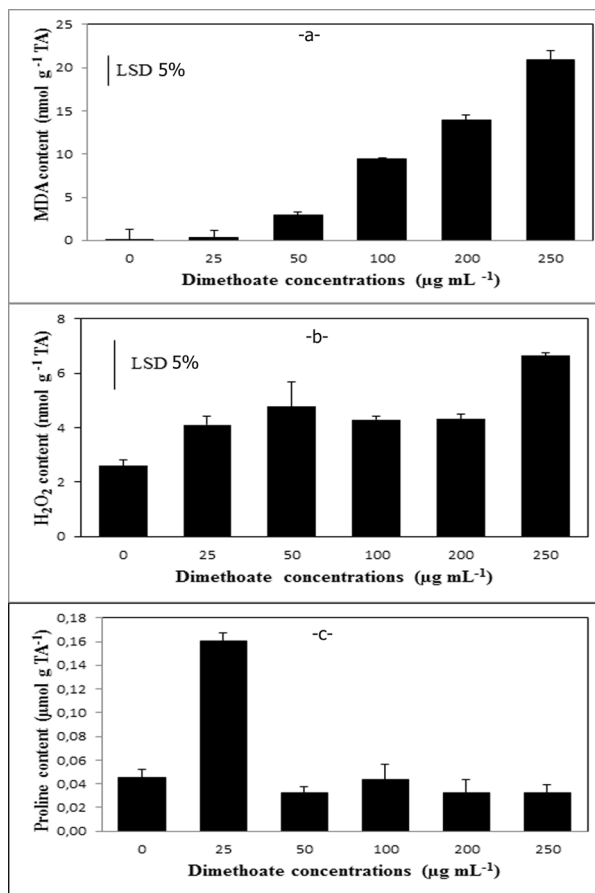
Figure 6. Malondialdehyde (a), hydrogen peroxide (b) and proline (c) contents of *A. platensis* supplemented with Dimethoate concentrations. Data are the means ± SE of three replicates. Mean values in columns are significantly different at the 5% level according to the least significant differences (LSD) Test.

concentrations and reported that algae growth significantly decreased compared to control. The concentrations used by Asselborn et al. (2015) are close to the concentrations used in Chlorpyrifos application (0-150 µg mL^{-1}). Yadav (2015) found that 0.5, 1, 5, 10 and 15 µg mL^{-1} Dimethoate concentrations reduced the amount of growth rate of chlorophyll-*a* in *Spirulina platensis*. Yadav (2015) is closest to our study but the used concentrations are lower than the concentrations used in Dimethoate application (0-250 µg mL^{-1}). However, these studies are important to understand the destructive effects of organophosphates on phytoplankton growth

rate and chlorophyll-*a*. Kumar et al. (2014) specified that the reduction in growth rate may be related to inhibition of pigment synthesis. They emphasized that chloroplasts are a potential target for peroxidation due to complex membrane systems rich in polyunsaturated fatty acid and it caused to inhibition of chlorophyll synthesis (Kumar et al. 2014). Although cyanobacteria do not have a chloroplast structure because they are prokaryotic, they have thylakoid membrane occurring chlorophyll synthesis and photosynthesis at a high rate and these structures are targets for toxic compounds

SOD is an antioxidant enzyme responsible for detoxifying superoxide radicals produced in cells under stress conditions (Elstner et al. 1988). Kong et al. (1999) showed that SOD enzyme is a key enzyme that eliminates active oxygen in algae cells. The total SOD activity of *A. platensis* cultures treated with Dimethoate decreased at 20, 40, 60, 80 and 100 µg mL^{-1} concentrations compared to the control. Lee & Shin (2003) found that cadmium applications reduce the activity of SOD enzyme in *Nannochloropsis oculata*. They reported that this decrease was due to the inactivation of enzymes by H_2O_2 produced in different compartments. (Vitoria et al. 2001). In our study, Lee & Shin (2003) cadmium application showed a similar mechanism with Dimethoate application and it increased the amount of H_2O_2. Therefore, excessive accumulation of H_2O_2 may inactivate the enzyme. Cao et al. (2011) suggested that manganese deficiency in *Amphidinium* sp. reduced the SOD enzyme activity, which may be caused by a decrease in active oxygen production, loss of photosynthetic functions and oxygen release. Hollnagel et al. (1996) studied the effect of light perception on *Gonyaulax polyedra* and observed that the activity of SOD decreased 2-3 times in parallel to lack of photosynthesis during the night phase. Because photosynthetic organisms increase the formation of ROS species and superoxide anion due to energy and electron transfer under high light (Pospisil 2016). Loss of photosynthetic metabolism may result in significant reductions in superoxide anion and SOD enzyme activity. In our study, it was observed that Dimethoate caused significant decreases in the amount of chlorophyll-*a* and SOD activity

The total SOD activity showed a statistically significant increase only at 50 µg mL^{-1} concentration compared to the control in *A. platensis* cultures treated with Chlorpyrifos, so the decrease and increase fluctuations are not statistically significant in other concentrations. Wang et al. (2012) applied Cypermethrin to *Scenedesmus obliquus* and determined that this pesticide increases SOD activity at low concentrations but inhibits it at higher concentrations. They attributed that Cr directly may overexpress the SOD gene or may indirectly increase the O_2^- level. Bhuvaneswari et al. (2018) showed that SOD activity increases in *A. platensis* at 10, 20 and 40 ppm Chlorpyrifos concentrations. The results of these studies are similar to those obtained from Chlorpyrifos application in our study.

In Chlorpyrifos application, GR enzyme activity was significantly increased at all concentrations (25, 50, 75, 100, 125, 150 µg mL^{-1}). Increases in GR activity may have occurred to neutralize oxidative stress products (Gamble & Burke 1984, Gillham & Dodge 1987, Bowler et al. 1992). It is concluded that GR enzyme activity changed according to SOD and APX activity alterations and they decreased by 75 µg mL^{-1} in all analyzes, this may be related to the substrate content in the glutathione pool. Foster & Hess (1980) stated that the activity of glutathione reductase enzyme has an important role in defense of cells against oxidative damage in response to high oxygen concentrations. Many studies have reported GR activity increased due to environmental stress factors (Gamble & Burke 1984, Gillham & Dodge 1987, Dhindsa 1991). Xia et al. (2009) suggested that the GR activity increase in *Cucumis sativus* as a result of Chlorpyrifos application was due to its main role in oxidative stress and in detoxification of pesticide. GR enzyme activity increased with copper sulfate application in *S. obliquus* (Dewez et al. 2005). Moreover, copper, lead and cadmium heavy metal applications showed a dose-dependent increase in GR enzyme activity of *C. vulgaris* (Bajguz 2010).

There was no change in GR activity as a result of Dimethoate applications. Dewez et al. (2005) applied fludioxonil on *S. obliquus*, and they found that GR activity was not significantly affected. It explained that the oxidized glutathione pool could be used by other enzymes.

APX uses ascorbic acid as an electron donor to eliminate harmful H_2O_2 (Verma & Dubey 2003). The absence of changes in GR activity supports the absence of changes in the amount of APX enzyme at similar concentrations in Dimethoate application since the ascorbate pool is balanced by GR. It has been reported in previous studies that GR activity is associated with APX activity (Teisseire & Vernet 2001, Mallick & Rai 1998). Similarly, the increase in APX activity can be explained by the increase in GR activity reduced to ascorbic acid at the Chlorpyrifos application. The alterations of GR enzyme activity are compatible with SOD enzyme activity results in Chlorpyrifos application because SOD enzyme provides H_2O_2 to the environment.

The MDA content increased significantly at 100, 200 and 250 µg mL^{-1} concentrations in Dimethoate application. The MDA content results are similar to H_2O_2 content results for Dimethoate. The increase of H_2O_2 content leads to ·OH radical formation by Haber-Weis reaction, ·OH radical attacks to membrane lipids, and thus MDA occurs in cell membrane as a result of lipid peroxidation (Bowler et al. 1992, Goel & Sheoran 2003). In addition, non-functional superoxide dismutase caused the accumulation of O^{2-} in the cells. It is known that lipid peroxidation is associated with the O^{2-} amount in the medium (Choudhary et al. 2007). Kumar et al. (2008) reported that the MDA content, an indicator of lipid peroxidation, increases due to Endosulfan application. Wang et al. (2011) stated that Cypermethrin has the effects of increasing the MDA content on *Skeletonema costatum*, *Scrippsiella trochoidea,* and *Chattonella marina*.

For Chlorpyrifos, the MDA content decreased at 150 µg mL^{-1} concentration. The H_2O_2 content did not change compared to the control because enzymes such as GR and APX, which reduce the H_2O_2 content from the medium via the Halliwel-Asada pathway, are active. Thus, lipid peroxidation may be reduced.

The H_2O_2 content increased significantly at 50, 100, 200 and 250 µg mL^{-1} concentrations with Dimethoate application. As a result of Dimethoate application, SOD activity decreased but H_2O_2 amount increased. The H_2O_2 content may be increased due to increased activity of oxidases such as glycolate oxidase, glucose oxidase, amino acid oxidase and sulfite oxidase found in plants (Asada & Takahashi 1987, Asada 1999). Also, decreased or unchanged APX enzyme activity that detoxifies H_2O_2 from the cells causes this molecule to accumulate (Morita et al. 1999). The H_2O_2 content increased at 100 µg mL^{-1} concentration, but it did not change at lower or higher concentrations compared to control in Chlorpyrifos application. H_2O_2 content was not affected at the high concentrations (125 and 150 µg mL^{-1}), which may be due to the significant increase in APX activity at these concentrations. Mallick & Mohn (2000) supported this point that the H_2O_2 content was not possibly changed due to the increase of the APX enzyme consuming H_2O_2 content.

The free proline content increased significantly at 150 µg mL^{-1} concentrations in Chlorpyrifos application. The free proline content statistically increased compared to the control at 25 µg mL^{-1} concentration in Dimethoate application. Proline deposition has been reported in the study about plants exposed to heavy metal stress (Saradhi 1991, Bassi & Sharma 1993). Proline also plays a role in the capture of free radicals (Smirnoff & Cumbes 1989, Hare & Cress 1997, Jain et al. 2001). It is an effective singlet oxygen scavenger (Alia et al. 2001) and regulates

cellular redox potential (Saradhi 1991). Proline interferes with cellular macromolecules such as DNA, proteins, and membranes as the tracer of the OH-radical and stabilizes the function and structure of these molecules (Saradhi 1991, Kavir et al. 2005). Proline reduces free radical production under stress conditions (Alia 1993). Some studies reported that an increase in the proline content may be an adaptive response to lipid peroxidation occurring under stress conditions. (Fatma et al. 2007, Kumar et al. 2014). There are also studies showing that various pesticides increase proline accumulation. (Fatma et al. 2007, Kumar et al. 2008, Choudhary et al. 2007). Chlorpyrifos increased the proline content at a concentration of 6 mg L^{-1} on NTMS12 *Chroococcus turgidus* (Kumar et al. 2014).

The free proline content decreased significantly at 50, 100, 200 and 250 µg mL^{-1} concentrations in Dimethoate application. Most of the studies have suggested that the proline content increases with stress conditions. However, some studies have shown that proline decreases under stress conditions. Ewald & Shclee (1983) found that sulfide reduces the free proline content on *Trebouxia* sp. because it inhibits proline synthesis. Similarly, in our study, Dimethoate may inhibit proline synthesis or disrupt the proline structure at higher concentrations. Furthermore, the decrease may be due to the use of free proline by free radicals in Chlorpyrifos application at intermediate concentrations.

As a result, the decrease in biomass and chlorophyll-*a* was found to be due to Chlorpyrifos and Dimethoate treatments. The alterations in antioxidant enzyme activities and other parameters showed differences according to pesticide type and the used concentrations This difference arises from the ability of the applied pesticide to produce ROS in different ratios.

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HATİCE TUNCA[1]
https://orcid.org/0000-0003-3724-5215

ALİ DOĞRU[1]
https://orcid.org/0000-0003-0060-4691

FERAY KÖÇKAR[2]
https://orcid.org/0000-0003-2572-8391

HEDİYE E. KILIÇ[1]
https://orcid.org/0000-0002-8257-6331

TUĞBA O. SEVİNDİK[1]
https://orcid.org/0000-0001-7682-0142

[1]Sakarya University, Science Faculty, Biology Department, Esentepe Campus, Serdivan, 54187, Sakarya, Turkiye

[2]Balıkesir University, Arts and Science Faculty, Molecular Biology and Genetics Department, Cagil Campus, 10145, Balıkesir, Turkiye

Correspondence to: **Hatice Tunca**
E-mail: htunca@sakarya.edu.tr

Author Contributions

Hatice TUNCA: Taking responsibility in the construction of the whole or body of the manuscript. Ali DOĞRU: Taking responsibility in logical interpretation and presentation of the results. Feray KÖÇKAR: Organising and supervising the course of the project or the article and taking the responsibility. Hediye Elif KILIÇ: Taking responsibility in execution of the experiments, patient follow-up, data management and reporting. Tuğba ONGUN SEVİNDİK: Organising and supervising the course of the project or the article and taking the responsibility.