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Oxidative Damages of Two Neonicotinoid Pesticides to *Arthrospira platensis* (Gomont)

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ABSTRACT

In this study, chlorophyll-*a* amount, OD 560 and antioxidant parameters (total SOD, APX, GR, MDA, H_2O_2 and Proline) were determined in order to understand the effects of Thiacloprid and Imidacloprid on *Arthrospira platensis* Gomont. Both Imidacloprid and Thiacloprid applications showed significant reductions in growth rate and chlorophyll-*a* content of *A. platensis* cultures with dose-dependent manner when the days and concentrations were compared each other. SOD activity significantly decreased in the Imidacloprid application while Thiacloprid caused a significant increase only at 75 µg mL^{-1} concentration. APX activity significantly increased in the Imidacloprid and Thiacloprid applications at 50 µg mL^{-1} and 35 µg mL^{-1} concentrations, respectively. Imidacloprid treatment increased GR activity at 20 and 30 µg mL^{-1} concentrations while GR activity increased at 15, 25 and 35 µg mL^{-1} Thiacloprid concentrations. The MDA content of *A. platensis* cultures did not change with Imidacloprid or Thiacloprid applications. The H_2O_2 content did not change at all different Imidacloprid concentrations. However, the H_2O_2 content decreased at 15 µg mL^{-1} and increased at 45 and 75 µg mL^{-1} Thiacloprid concentrations. Free proline content increased in the Imidacloprid and Thiacloprid applications at 100 µg mL^{-1} and 75 µg mL^{-1} concentrations, respectively. These neonicotinoid pesticides cause oxidative stress in *A. platensis* cells.

Keywords: *Arthrospira platensis*, oxidative stress, antioxidant, thiacloprid, imidacloprid

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Neonikotinoid Pestisitin *Arthrospira platensis*'e (Gomont) Oksidatif Zararları

Öz: Bu çalışmada Thiacloprid ve Imidacloprid'in *Arthrospira platensis* Gomont üzerindeki etkilerini anlamak için klorofil-*a* miktarı, OD 560 ve antioksidan parametreler (toplam SOD, APX, GR, MDA, H_2O_2 ve Prolin) belirlenmiştir. Hem İmidacloprid hem de Thiacloprid uygulamaları, *A. platensis* kültürlerinin büyüme hızında ve klorofil-*a* içeriğinde doza bağlı olarak günler ve konsantrasyonlar karşılaştırıldığında önemli azalmalar göstermiştir. Imidacloprid uygulamasında SOD aktivitesi önemli ölçüde azalırken, Thiacloprid sadece 75 µg mL^{-1} konsantrasyonunda önemli bir artışa neden olmuştur. APX aktivitesi, sırasıyla 50 µg mL^{-1} ve 35 µg mL^{-1} konsantrasyonlarında İmidacloprid ve Thiacloprid uygulamalarında önemli ölçüde artmıştır. İmidakloprid uygulaması GR aktivitesini 20 ve 30 µg mL^{-1} konsantrasyonlarında artırırken, GR aktivitesini 15, 25 ve 35 µg mL^{-1} Thiacloprid konsantrasyonlarında arttırmıştır. *A. platensis* kültürlerinin MDA içeriği, İmidacloprid veya Thiacloprid uygulamaları ile değişmemiştir. H_2O_2 içeriği herhangi bir imidakloprid konsantrasyonunda değişmemiştir. Ancak H_2O_2 içeriği 15 µg mL^{-1}'de azalmış, 45 ve 75 µg mL^{-1} Thiacloprid konsantrasyonlarında ise artmıştır. Serbest prolin içerikleri sırasıyla 100 µg mL^{-1} ve 75 µg mL^{-1} konsantrasyonlarında Imidacloprid ve Thiacloprid uygulamalarında artmıştır. Bu neonikotinoid pestisitler, *A. platensis* hücrelerinde oksidatif strese neden olmaktadır.

Anahtar kelimeler: *Arthrospira platensis*, oxidative stress, antioxidant, thiacloprid, imidacloprid

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Introduction

The usage of pesticides is still the most preferred method of agricultural struggle against diseases and pests despite having long-lasting destructive effects on environmet, non-target organisms, and humans (Karahan et al. 2018). Pesticides can be transported

by atmospheric precipitation, farmland, sewage in various centers, hazardous waste disposal waters, even over long distances by air (Tankiewicz et al. 2010). This situation is caused by the contamination of the water resources by pesticides (Malato et al. 2001). When pesticides enter the aquatic environment, they continuously reduce the quality of groundwater and surface water being essential drinking water resources for the majority of the world population (Tankiewicz et al. 2010). This critical problem has attracted the interest of scientists due to its considerable toxicity in physical and biochemical processes. The accumulation of this contamination and toxicity to organisms can lead to irreversible changes and hazards (Daneshvar et al. 2007; Tankiewicz et al. 2010)

Neonicotinoids are the most important class of synthetic insecticides produced in recent years, and these insecticides are nicotine derivatives (Bolboaca and Jaentschi 2005; Jeschke et al. 2011, Casida and Durkin 2013). These water-soluble compounds, the most widely used insecticides in the world, can be taken up by plants and consumed by non-target organisms (Morrissey et al. 2015; Wood and Goulson 2017). Aquatic organisms, such as terrestrial organisms, may be susceptible to these compounds, although they attract significantly less attention (Wood and Goulson, 2017). The neonicotinoid group insecticides are classified as N-nitroguanidines (like as imidacloprid) and N-cyanoaminides (like as thiacloprid) (Bolboaca and Jaentschi 2005).

Imidacloprid (1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine) is the first and most widely used neonicotinoid insecticide developed as a neurotoxin affecting the central nervous system of the insects (Akbaş et al. 2014). Imidacloprid is used for leaf treatment of plants such as grain, cotton, wheat, legumes, potatoes, some fruits, grass and vegetables, and it is also applied against harmful insects in soil, seeds, trees, and animals (BCPC 2004). It can interfere in the irrigation system (Franco et al. 2009). Its toxicity is high due to some features such as the ability of transport from agricultural areas to surface water, widespread use, and persistence in water (US EPA 2008; Starner and Goh 2012).

Thiacloprid [3-(6-chloro-3-pyridinylmethyl)-2-thiazolidinylidene] cyanamide is a neonicotinoid insecticide that affects the nervous system of insects (Matsuda et al. 2005; Matsuda et al. 2009; Kocaman et al. 2014). Thiacloprid is widely used in orchards and vegetables to combat aphids, as well as seed coating in corn and some crops (Schuld and Schmuck 2000; Schmuck et al. 2001). Like other neonicotinoids, Thiacloprid is highly soluble in water and potentially contaminates surface water following precipitation events (water solubility is 185 mg L^{-1})(EPA 2003). According to some scientific studies, Thiacloprid is also harmful to freshwater invertebrates (Beketov and Liess 2008; Beketov et al. 2008; Morrissey et al. 2015). Therefore, it is important to evaluate the concentrations of which these chemicals are toxic to aquatic organisms (Tisler et al. 2009)

A lot of physical, chemical, and biological environmental factors cause oxidative stress by affecting the production of reactive oxygen species (ROS) in plants and cyanobacteria (Smirnoff 1993; Hendry 1994; Bartosz 1997, Olga et al. 2003). Under normal conditions, concentrations of oxygen radicals are kept at low levels by the activity of antioxidants (Asada 1984). The imbalance between the production of ROS and the activity of antioxidants leads to oxidative damage (Del-Rio et al. 1991; Del Vos et al. 1992; Smirnoff 1993). These active oxygen species are inactivated by enzymes such as superoxide dismutase, catalase and peroxidase and by antioxidant molecules such as organic chemicals proline, ascorbate, and carotenoids in cellular systems. Free radicals can damage the major cellular components such as lipids, proteins, carbohydrates and nucleic acids (Olga et al. 2003). In addition, another group of pesticides, such as herbicides, has been shown to produce singlet oxygen and other active oxygen species in various regions of the photosynthetic electron transport chain and cause oxidative stress in cells (Halliwell 1987).

The aim of this study is to explore the changes in the growth parameters of *Arthrospira platensis* being a phototrophic primary producer and to evaluate the aspects of the oxidative stress by neonicotinoids.

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 and Ogawa 1977) under axenic conditions. 20 mL algal cultures were inoculated to 180 mL culture medium in 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 ºC during 10 days. At the end of 10 days, cultures were renewed and, all the flasks contained 50 mL algal culture. The commercial formulation of Thiacloprid and Imidacloprid (240 g L^{-1}, 350 g L^{-1}; respectively, EC, Sakarya, Turkey) were used in all bioassays and prepared in distilled water. Various concentrations of Imidacloprid and Thiacloprid were added to the culture medium. The range of concentrations was determined with preliminary range-finding bioassays according to the EC50 value (the concentration of a drug that gives half-maximal response) for growth parameters (Sebaugh 2011).

Cell growth and chlorophyll-*a* assay

Optic density (OD) of microalgae was measured spectrophotometrically over a period of 7 days under control and stressed conditions taking absorbance at 560. To determine the growth rate, OD560 absorbances were measured spectrophotometrically for 7 days and EC50 values were calculated with OriginPro 8.5 programme. Chlorophyll-*a* contents were estimated by methanol extraction and were measured spectrophotometrically for 7 days (MacKinney, 1941).

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 obtained pellets were kept at -20 °C until enzyme activity measurements. Pellets were crushed 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 superoxide dismutase (SOD) activity was determined by the method of Beyer and Fridovich (1987), based on the photoreduction of NBT (nitroblue tetrazolium). The pellets (0.2 g) were extracted 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 $9.9x10^{-3}$ M methionine, $5.7x10^{-5}$ M NBT, 1% Triton X-100 and enzyme extract. The reactions were 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 reactions were stopped by switching off the light and absorbance was read at 560 nm. The SOD activities were calculated by a standard graphic and expressed as unit mg^{-1} protein.

The ascorbate peroxidase (APX) activity was determined according to Wang et al. (1991) by estimating the decreasing rate of ascorbate oxidation at 290 nm. APX extractions were performed in 50 mM Tris–HCl (pH 7.2), 2% PVP, 1 mM Na_2EDTA, and 2 mM ascorbate. The reaction mixtures 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 activities were calculated from the initial rate of the reaction using the extinction coefficient of ascorbate (E = 2.8 mM cm^{-1} at 290 nm).

The glutathione reductase (GR) activity was measured with the method of Sgherri et al. (1994). Extractions were 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 mixtures (total volume of 1 mL) contained 100 mM K_2HPO_4 buffer (pH 7.8), 2 mM Na_2EDTA, 0.5 mM oxidize glutathione (GSSG), 0.2 mM NADPH and enzyme extract containing 100 µg protein. The decrease in absorbances at 340 nm were recorded. The corrections were made for the non-enzymatic oxidation of NADPH by recording the decrease at 340 nm without adding GSSG to the mixture. The enzyme activities were 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).

Determination of malondialdehyde and hydrogen peroxide

The malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content were determined by the method of Heath and Packer (1968). 0.2 g pellets were homogenized in 3 mL of 0.1% TCA (4°C), and centrifuged at 4100 rpm for 15 min, and the supernatants were 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) (TCA–0.375% w/v, TBA–0.25 N HCl) were added into the 0.5 ml of the supernatant. The mixtures were heated at 95°C for 30 min and then quickly cooled in the ice bath. To remove suspended turbidity, the mixtures were centrifuged at 4100 rpm for 15 min, then the absorbances of supernatant at 532 nm were recorded. Non-specific absorbances at 600 nm were measured and subtracted from the readings recorded at 532 nm. The MDA contents were calculated using its extinction coefficient of 155 mM^{-1} cm^{-1}. Hydrogen peroxide content was determined in the assay mixture consisting of 0.5 mL of 0.1 M Tris–HCl (pH 7.6). 1 mL of 1 M KI were added to 0.5 mL of supernatant. After 90 min, the absorbances were recorded at 390 nm.

The proline content determination

The proline content was determined by the method of Weimberg et al. (1987). 0.1 g pellets were 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. Samples were cooled and centrifuged at 4100 rpm for 10 min. Two milliliters 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 mixtures were extracted with 4 mL toluene. The chromophores containing toluene were separated, and the absorbances were 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. Three replicate cultures were used for each treatment. The mean values ± SE were given in Figures.

Results

Imidacloprid and Thiacloprid applications showed significant reductions in OD560 and chlorophyll-*a* content *of A. platensis* cultures with dose-dependent manner when the days and concentrations were compared each other (Fig1 and 2).

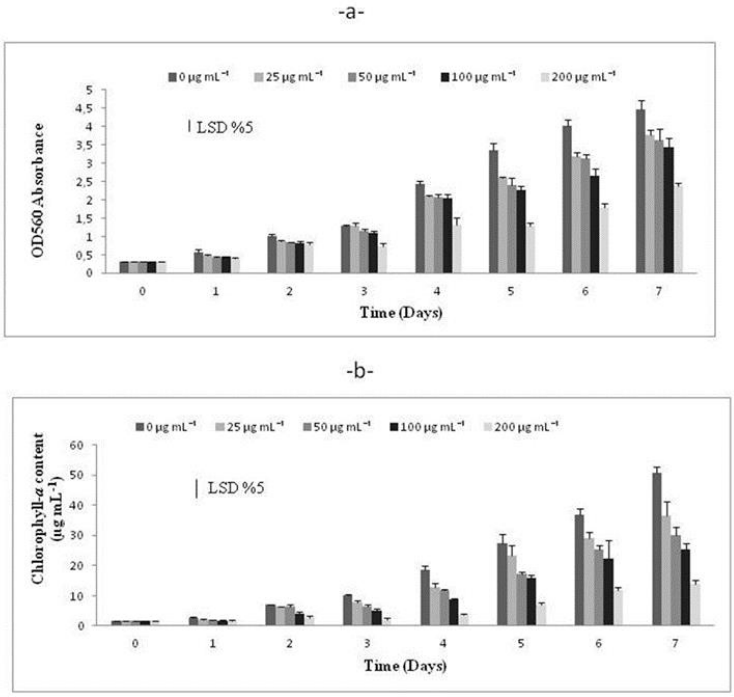

Figure 1. Biomass values (a) and (b) chlorophyll-a content of Arthrospira platensis supplemented with 0-200 µg mL $^{-1}$ Imidacloprid concentrations during 7 days. Data are the means ± SE of three replicates.

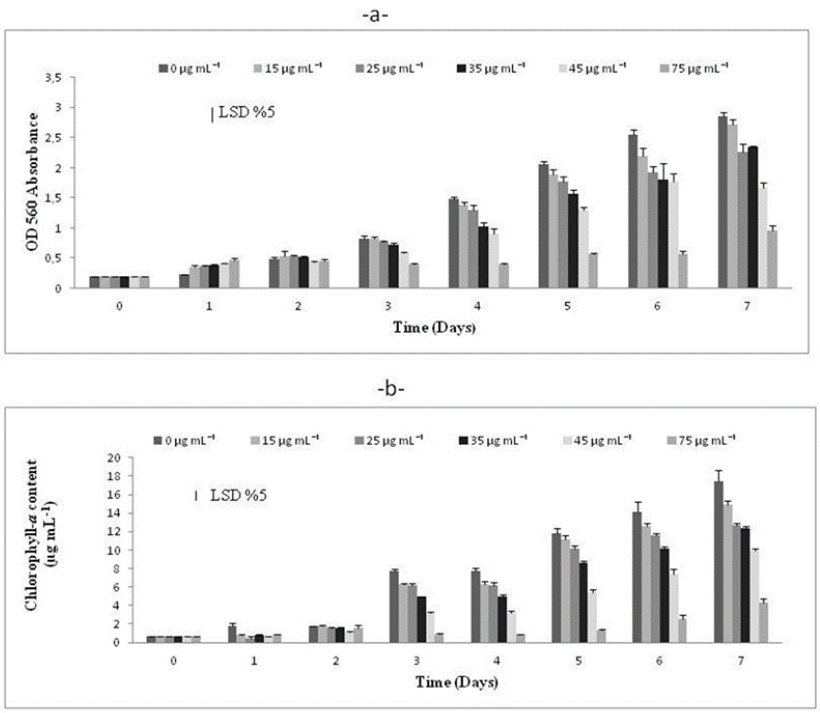

Figure 2. Biomass values(a) and (b) chlorophyll-*a* content of *Arthrospira platensis* supplemented with 0-75 µg mL^{-1} Thiacloprid concentrations during 7 days. Data are the means ± SE of three replicates.

SOD activity significantly decreased in the Imidacloprid application at all concentrations (10, 20, 30, 40, and 50 µg mL^{-1}) (Fig 3a) ($p < 0.05$), while Thiacloprid caused a significant increase in only 75 µg mL^{-1} concentrations (Fig 4a) ($p < 0.05$). APX activity significantly increased in the Imidacloprid and Thiacloprid applications at 50 µg mL^{-1} (Fig 3b) and 35 µg mL^{-1} (Fig 4b) concentrations, respectively ($p < 0.05$). Imidacloprid treatment increased GR activity at 20 and 30 µg mL^{-1} concentrations (Fig 3c) ($p < 0.05$), while Thiacloprid application increased statistically at 15, 25 and 35 µg mL^{-1} concentrations compared to control (Fig 4c) ($p < 0.05$).

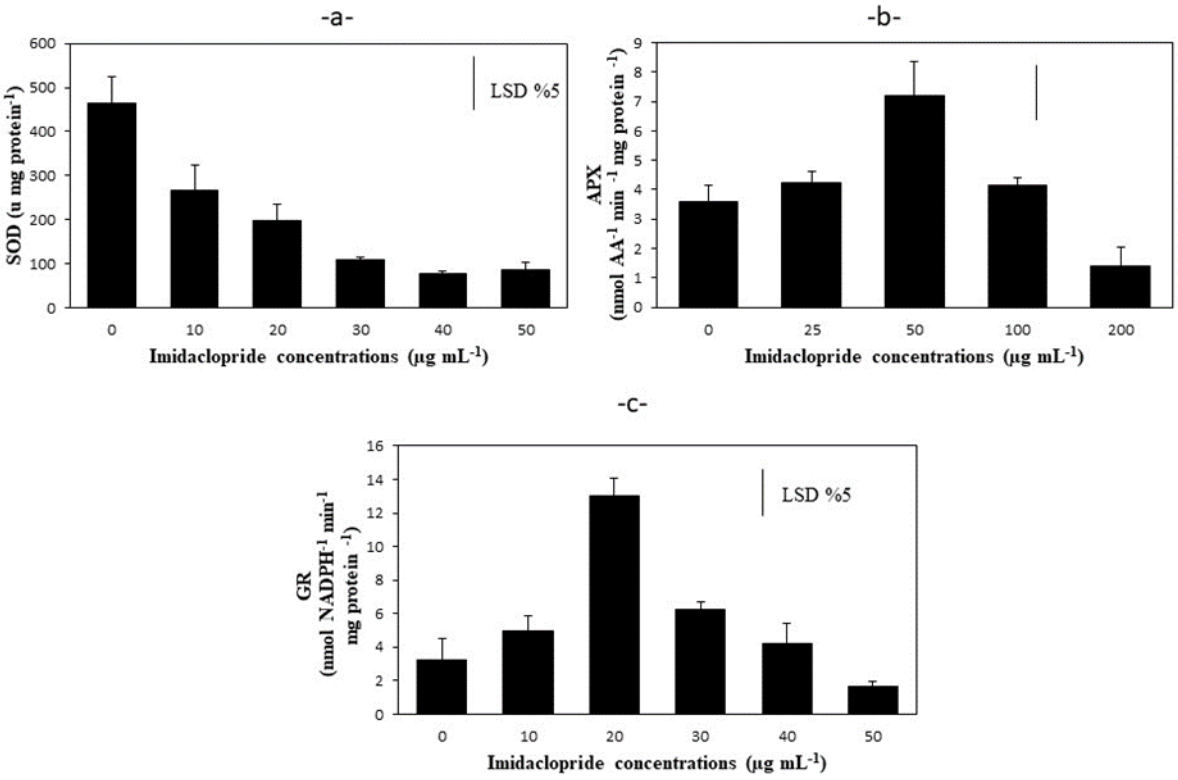

Figure 3. Total superoxide dismutase (SOD) (a), ascorbate peroxidase (APX) (b) and glutathione reductase (GR) (c) activities of *A. platensis* supplemented with Imidacloprid concentrations. Data are the means ± SE of three replicates. Mean values in columns are significantly different at the 5% level according to the least significant differences (LSD) Test.

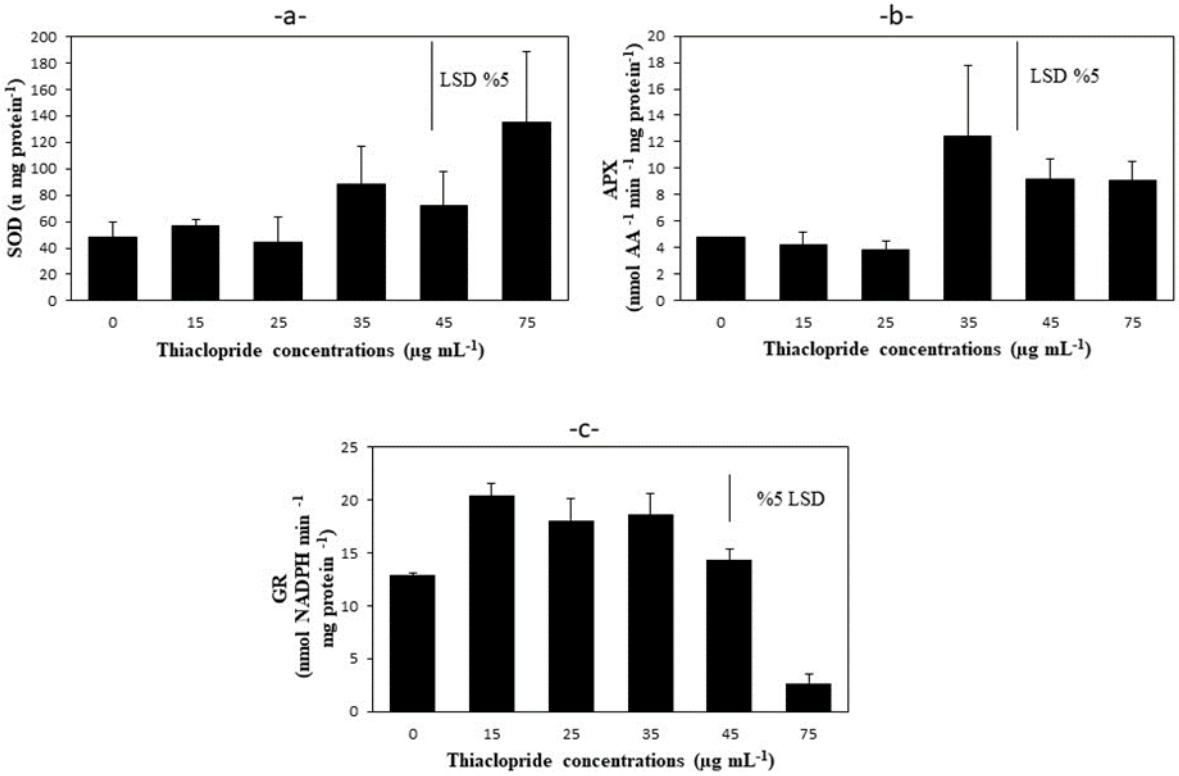

Figure 4. Total superoxide dismutase (SOD) (a), ascorbate peroxidase (APX) (b) and glutathione reductase (GR) (c) activities of *A. platensis* supplemented with Thiacloprid concentrations. Data are the means ± SE of the three replicates. Mean values in columns are significantly different at the 5% level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

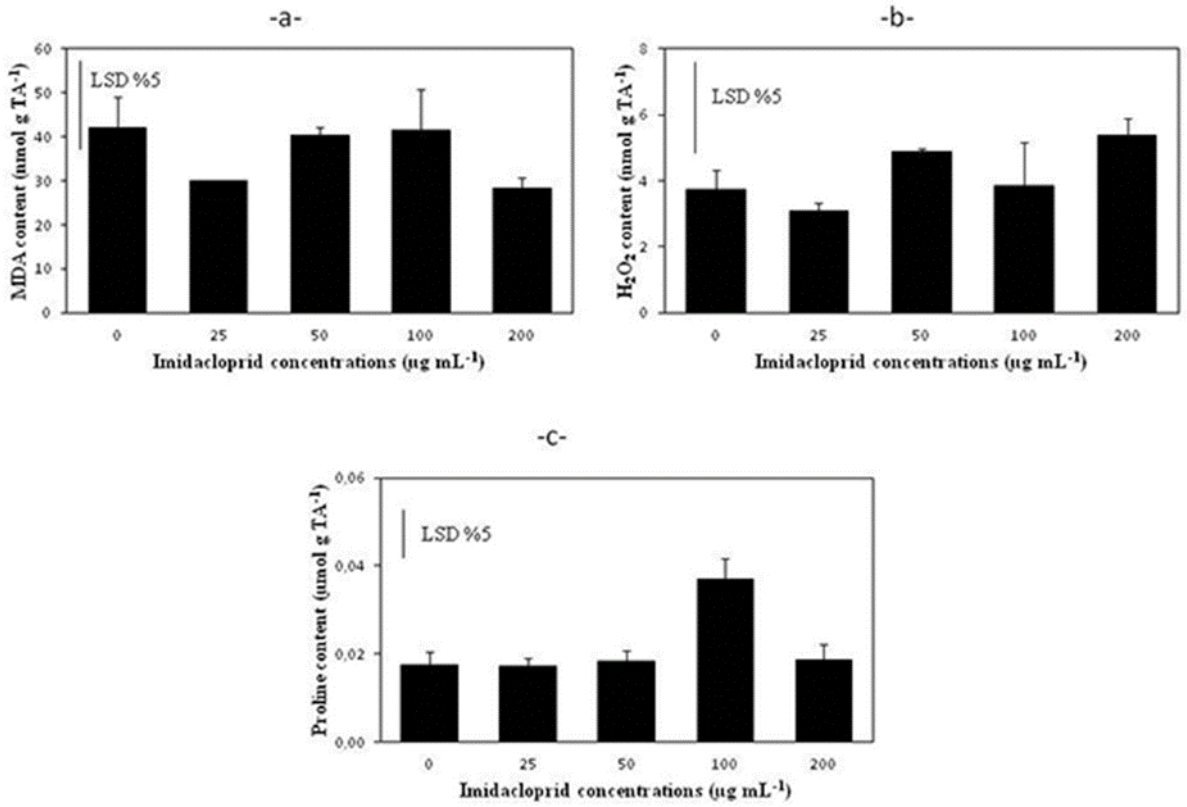

Figure 5. Malondialdehyde (a), hydrogen peroxide (b) and proline (c) contents of *A. platensis* supplemented with Imidacloprid concentrations. Data are the means ± SE of the three replicates. Mean values in columns are significantly different at the 5% level according to the least significant differences (LSD) Test.

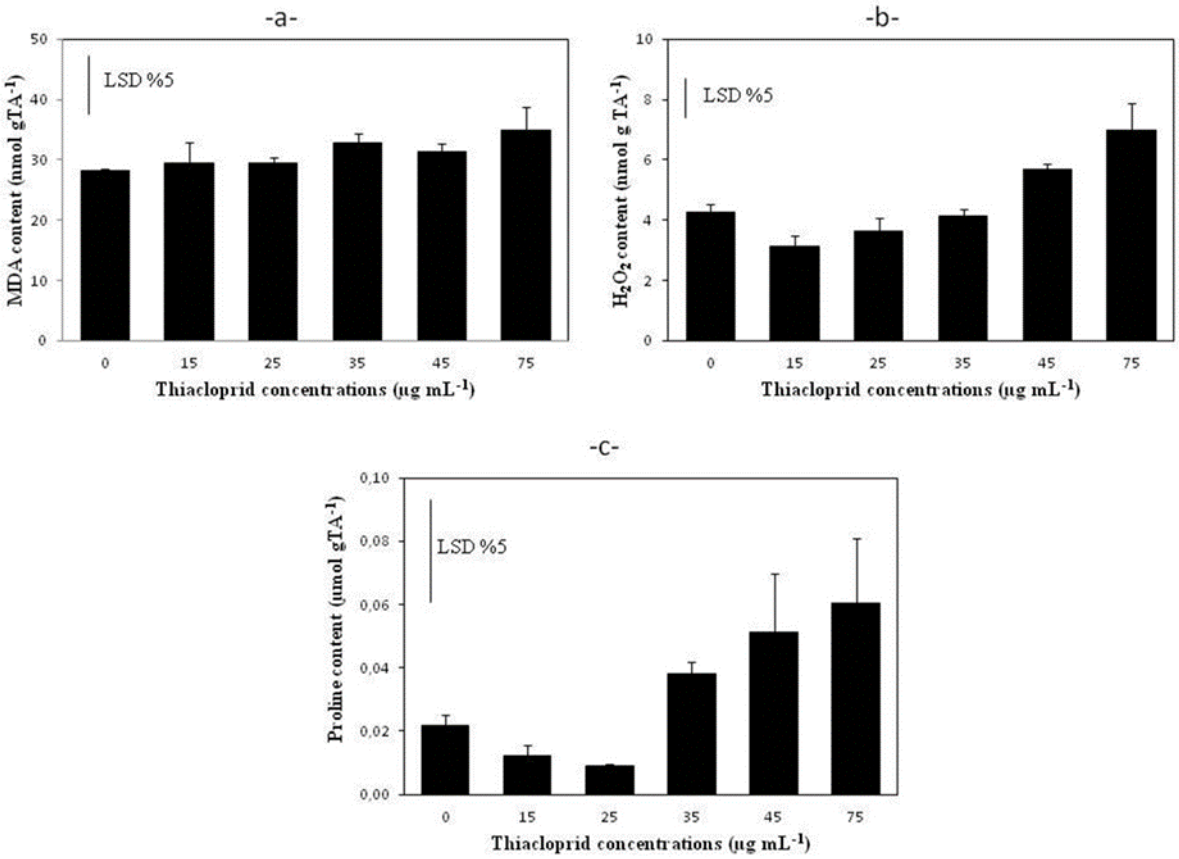

Figure 6. Malondialdehyde (a), hydrogen peroxide (b) and proline (c) contents of *A. platensis* supplemented with Thiacloprid concentrations. Data are the means ± SE of three replicates. Mean values in columns are significantly different at the 5% level according to the least significant differences (LSD) Test.

Discussion

In this study, Imidacloprid and Thiacloprid effects were investigated with some parameters such as OD560, chlorophyll-*a* content, some antioxidant enzyme activities (superoxide dismutase, ascorbate peroxidase and glutathione reductase) and H_2O_2, MDA and proline on *A. platensis*. Malev et al. (2012) evaluated the toxicity of Imidacloprid on *Desmodesmus subspicatus* and specified that Imidacloprid has harmful effects on non-target microorganisms. They reported that algal inhibition was significant at 127.8 and 255.6 mg L^{-1} concentrations compared to the control group. The data obtained by Malev et al. (2012) are similar to the effects of Imidacloprid on *A. platensis* in our study. The studies about Thiacloprid on algal toxicity are limited in the literature. Therefore, our work will fill the blank in the literature.

SOD is an antioxidant enzyme being responsible from detoxifying superoxide radicals produced in algal cells under stress conditions (Tunca 2020). When the pollution increases in the environment, the cellular detoxification system is stimulated and SOD synthesis rate and / or activity increases. These changes occur faster in molecular cell levels than growth and reproduction process (Rabinowich and Fridovich 1985). Therefore, changes in SOD activity are sensitive biomarkers to environmental pollution (Li et al. 2005). In our study, the total SOD activity significantly increased at the highest Thiacloprid concentration (75 µg mL^{-1}) on *A. platensis*. SOD activity have been investigated in many algae due to evaluation of various pesticide effects (Prasad et al. 2005; Galhano et al. 2010; Qian et al. 2008; Qian et al. 2009; Salman et al. 2016; Li et al. 2005; Kumar et al. 2014). According to the previous studies, SOD activity increased by the pesticide application, which increased the production of $\cdot O_2^-$ and other free radicals.

In our study it was observed that Imidacloprid caused significant reductions in chlorophyll-*a*. The loss of photosynthetic metabolism may have caused significant reductions in SOD enzyme activity or vice versa. Liu et al. (2015) reported that Azoxystrobin inhibits the SOD activity (19-300 µg L^{-1}) on *Chlorella vulgaris*. They suggested that degradation of SOD enzyme structure is caused by Azoxystrobin, and thus algal growth may be inhibited.

GR is effective in the detoxification of H_2O_2 in plant cells due to functions in the Haliwell-Asada pathway (Bray et al. 2000). GR catalyzes the last step

of the ascorbate-glutathione pathway. GR enzyme activity was significantly increased at 20 and 30 µg mL^{-1} concentrations in Imidacloprid treatment, when GR enzyme activity was significantly increased at 15, 25 and 35 µg mL^{-1} Thiacloprid concentrations. Mofeed and Mosleh (2013) observed that GR activity increased with fenhexamid and atrazine application on *Scenedesmus obliquus*. It is concluded that this enzyme displayed an essential role in the detoxification of these pesticides. The increases in GR activity may have occurred to neutralize the $ROS^{\cdot}$ APX uses ascorbic acid as an electron donor to eliminate harmful H_2O_2 (Verma and Dubey 2003). In our study, Imidacloprid treatment significantly increased APX activity at 50 µg mL^{-1} but decreased at 200 µg mL^{-1}. Previous studies have reported that APX activity was induced due to increased oxidative stress (Weckx and Clijsters 1996). The high concentrations of salicylic acid and 2,6 dichloroisoniconitic acid have been reported to inhibit APX activity (Durner and Klessig 1995). According to the results of these studies, Imidacloprid pesticide increases APX enzyme activity by causing oxidative stress at low concentrations, but it inhibits APX enzyme at higher concentrations due to breaking down the enzyme structure.

Thiacloprid treatment showed a statistically significant increase in APX activity at 35 µg mL^{-1}, whereas it did not change at other concentrations (15, 25, 45 and 75 µg mL^{-1}). The increase in APX enzyme activity can be explained by the increase in GR enzyme activity at a similar concentration.

There was no significant change in the MDA content by Imidacloprid applications, and this situation was parallel the results of H_2O_2 assay. Chen (2020) explained that MDA and H_2O_2 content may have been prevented by other antioxidant responses caused by sulfonamides- induced oxidative stress in *Chlorella vulgaris*. In addition, proline may inhibit cell membrane damage and MDA amount. Siripornadulsil et al. (2002) reported that Cadmium treatment did not change the MDA content of transgenic *Chlamydomonas reinhardtii* strain producing proline at high concentrations and they reported that proline could prevent free radical damage by acting as an antioxidant.

Thiacloprid pesticide application showed no significant change in MDA content. Jiménez et al. (1998) reported that the MDA content did not change during the senescence, but the H_2O_2 content increased in *Pisum sativium*. They attributed this situation to MDA metabolization in mitochondria. According to Thaicloprid application, the increase in the proline content may have contributed to the unchanged MDA content.

The proline content of *A. platensis* cultures increased statistically at 75 µg mL^{-1} Thiacloprid concentration when it was increased statistically in at Imidaclopride concentration of 100 µg mL^{-1} compared to the control. Proline may have increased according to pesticide accumulation (Fatma et al. 2007; Duval et al. 1999; Galhano et al. 2011; Kumar et al. 2008; Choudhary et al. 2007; Kumar et al. 2014).

In conclusion, the changes in antioxidant enzyme activities and other parameters varied according to the pesticide type and used concentrations. This difference arises from the ability of the applied pesticide to produce ROS in different ratios. Neonicotonoid pesticides have irreversible damages on prokaryotic primary producers. Therefore, these pesticides should be used with caution.

Acknowledgemet

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Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Conflict Of Interest

The authors confirm that there is no conflict of interest in the present study.

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