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RESEARCH ARTICLE

The effects of Bentagran on the development and antioxidant parameters of *Arthrospira platensis* Gomont and *Chlorella vulgaris* Beyerinck (Beijerinck)

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Abstract – The aim of the study is to determine the effects of Bentagran on growth and oxidative effects to *Chlorella vulgaris* and *Arthrospira platensis* and to evaluate the herbicide toxicity on primary producers of aquatic ecosystems. The decrease in both biomass accumulation and chlorophyll-*a* content in a dose-dependent manner were observed in both organisms exposed to different Bentagran concentrations (for *C. vulgaris* 60–960 $\mu g\,mL^{-1}$; for *A. platensis* 100–800 $\mu g\,mL^{-1}$) during 7 days. SOD activity increases significantly in *Chlorella vulgaris* and *Arthrospira platensis* at concentrations of 480 and 200 ug mL^{-1}, respectively. Although there was no significant change in APX (ascorbate peroxidase) activity in *C. vulgaris*, the APX activity decreased at 400 and 600 $\mu g\,mL^{-1}$ concentrations in *A. platensis*. While the GR (glutathione reductase) activity increased at 960 $\mu g\,mL^{-1}$ concentration in *C. vulgaris,* it also showed increases at 100, 200 and 400 $\mu g\,mL^{-1}$ concentrations, but it decreased at 600 $\mu g\,mL^{-1}$ concentration in *A. platensis*. MDA (malondialdehyde) and proline amounts decreased only at the concentration of 960 $\mu g\,mL^{-1}$, while H_2O_2 didn't change compared to control. Total MDA, H_2O_2 (hydrogen peroxide) and proline amounts did not show significant change compared to control. It is found that the effects of Bentagran on growth and antioxidant parameters are diverse at different concentrations and species, and this can be attributed to the different reactive oxygen species (ROS) production ability in these species.

Keywords: Microbial ecotoxicology / microalgae / cyanobacteria / herbicide / bentazone / antioxidant enzyme

1 Introduction

The effects of pesticide derivatives on animal and plant biodiversity reach undesirable levels in aquatic ecosystems as well as in terrestrial environments (Mahmood *et al.*, 2016). Being pesticide derivatives used in the applications against unwanted plants, herbicides may enter into aquatic ecosystems when their heavy usage in agriculture and horticulture (DeLorenzo *et al.*, 2001; Ma *et al.*, 2010; Silva *et al.*, 2019). Algae are important inhabitants of aquatic ecosystems, with their ability to accumulate, detoxify, or metabolize herbicide (DeLorenzo *et al.*, 2001; Sehrawat *et al.*, 2021). *Chlorella vulgaris,* an eukaryotic algae with great biodiversity, attracts attention with its properties such as the purification of heavy metals, nitrogen, and phosphorus compounds (Znad *et al.*, 2018). It is also produced for commercial food supply. *Arthrospira platensis*, a gram-negative, prokaryotic, non-toxic cyanobacterium and it has widespread usage in the commercial world (Tunca *et al.*, 2021). Species of *Arthrospira* have also been determined in the flora of Turkey (Aysel, 2005).

When aquatic producer organisms are exposed to different kinds of stresses like herbicides, light, or limited nutrients, they use defense mechanisms according to natural conditions. These kinds of different stress factors have the potential to increase the production of ROS. This production displays the oxidative stress levels in organisms (Arora *et al.*, 2002).

Antioxidant defense system (ADS) responses are important to determine the stress responses of an organism to physical and chemical stimuli and to evaluate the organism's tolerance to these stress conditions. Organisms developed the ADS to control the formation of free radicals and to prevent the harmful effects of these molecules. ADS include chemicals such as tocopherols, ascorbic acid, glutathione, which neutralize free radicals or enzymes such as superoxide dismutase (SOD; E.C 1.15.1.1: the code in enzyme nomenclature), ascorbate peroxidase (APX; E.C 1.11.1.11: the code in enzyme nomenclature), and glutathione reductase

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(GR; E.C 1.8.1.7: the code in enzyme nomenclature). SOD is the first and major detoxification enzyme. It is the most powerful antioxidant and works as a radical scavenger in cells (Ighodaro and Akinloye, 2018). SOD is involved in the prevention of lipid peroxidation (Altınışık, 2000) By catalyzing the reaction that forms hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) from superoxide radicals (Doğru and Demirtaş, 2021), it makes the superoxide anion less harmful (Ighodaro and Akinloye, 2018). On the other hand, still toxic H_2O_2 is present and should be transformed to H_2O in subsequent reactions. The series of reactions to remove H_2O_2 are known as the Ascorbate-Glutathione cycle or the Halliwell-Asada pathway (Avashthi *et al.*, 2018). APX plays a role to scavenge the H_2O_2 by using ascorbate as the electron donor (Bajguz, 2010). GR, which is a member of flavoenzyme family, catalyzes the NADPH-dependent reduction of glutathione disulphide (GSSG) to glutathione (GSH) (Verma and Dubey, 2003). The key factor for the oxidative balance of the cell is the ratio of GSSG/GSH produced in the cell. Moreover, high levels of the reduced glutathione and a low level of the oxidized glutathione disulfide are also important for maintaining the cell. Therefore, ascorbate and glutathione are not consumed; and the net electron flow is from NADPH to H_2O_2 (Noctor and Foyer, 1998; Doğru, 2021). MDA is an aldehyde metabolite that constitutes the last product in lipid peroxidation and it shows the level of oxidative damage (Urso and Clarkson, 2003). At the same time, increasing MDA concentration indicates an increase in the capacity of free radical production in the microorganism (Choudhary *et al.*, 2007). Another defense amino acid produced by plants against lipid peroxidation under stress conditions is proline. There is evidence that proline is very important in capturing ROS against stress-induced oxidative stress caused by abiotic factors (Kaul *et al.*, 2008). There are some studies in the literature about oxidative stress caused by herbicide in algae. Wang *et al.* (2021) reported that glyphosate caused oxidaitive stress on *Microcystis aeruginosa* at concentrations between 0 and 5 mg L^{-1}. Majewska *et al.* (2021) found that treatment with atrazine caused an increase in the ascorbate peroxidase levels but Diclofenac treatment drastically reduced in *Chlamydomonas reinhardtii*. Machado and Soares (2021) observed that metolachlor caused ROS overproduction and decline of the antioxidant system *Pseudokirchneriella subcapitata* at 0–35 μg mL^{-1}.

The Bentagran, and its active ingredient ‘Bentazone’ which is a non-selective and recently emerging broad-spectrum herbicide, is used to control many broadleaf weeds and reeds in soybeans, alfalfa, pepper, asparagus, sorghum, grass, rice, corn, peanuts, mint, dried beans, and fleshy lima beans (Galhano *et al.*, 2011). Researches have shown that bentazone, which has a very high solubility in water, is generally not easily absorbed by the soil and therefore enters adjacent freshwater ecosystems and eventually moves towards the river and sea waters (Boesten and Pas, 2000). Therefore, it attracts attention as a pollutant in underground and surface waters in Portugal and other European countries (Galhano *et al.*, 2011). Moreover, it is used in most parts of the world, including North Africa, New Zealand, India, the Philippines, North America, Australia, and Europe (Galhano *et al.*, 2011). Although it is found in groundwater and it shows a high affinity for water components, it does not accumulate in nature and the risk of exposure due to food is low. In the pesticide risk index assessment performed by Solomon (1997), bentazone was classified as a less harmful pesticide (Macedo *et al.*, 2008). Marques *et al.* (2011) reported that the level of chlorophyll-*a* content reduced by Bentagran toxicity in *P. subcapitata*. Tomé (1996) showed that high Bentagran caused toxic effects to *Nostoc* sp. Munkegaard *et al.* (2008) studied with bentazone combination with other herbicides on *Pseudokirchneriella subcapitata* and they observed the toxic effects on algae. Galhano *et al.* (2010), Galhano *et al.* (2011), and Bagchi *et al.* (2012) reported that Bentagran caused the oxidative stress on *Anabaena cylindrica* and *Nostoc muscorum, Synechococcus elongatus* PCC7942, respectively.

The object of the present study was to describe herbicide toxicity to aquatic microorganisms. Being herbicide nature, Bentagran can exhibit similar properties in unicellular organisms as plants containing chlorophyll due to its photosynthetic inhibition mechanisms and hydrogen peroxide accumulation potential. However, the preservation of the chlorophyll structure and the levels of oxidative stress production can vary according to organism. In the literature, certain algae species have been studied with bentazone and Bentagran, but there is no comparison between algae species. For this reason, the hypothesis of the study is that Bentagran herbicide have different effects on growth and oxidative stress due to different cell structure and detoxification mechanisms in prokaryotic and unicellular eukaryotic algae. In this study, the effects of Bentagran on *C. vulgaris* and *A. platensis* were evaluated in terms of the growth rate (OD750; Optic Density 750), chlorophyll-*a* content, and enzymatic (SOD, APX, and GR) and non-enzymatic (MDA, proline, and H_2O_2 amounts) parameters.

2 Materials and methods

2.1 Algae culture and treatment

A. platensis-M2 was obtained from the Soley Microalgae Institute (California, USA) (Culture collection No: SLSP01) and *C. vulgaris* was obtained from Çukurova University, Faculty of Fisheries. The Bentagran herbicide [3–(1–methyl ethyl) – 1H – 2,1,3 – benzothiadiazine – 4 (3H)- one 2,2–dioxide] was used in this study (CAS No: 25057-89-0, 480 g/L, ≥95% purity, Bentazone EC, İstanbul, Turkey). *A. platensis* was grown in Spirulina Medium (18 g Na_2CO_3, 1 g $NaCO_3$, 1 g K_2HPO_4, 2g $NaNO_3$, 1 g KSO_4, 1 g NaCl, 0.4 g $MgSO_4.7H_2O$, 0.02 g $FeSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$ in L; Sigma-Aldrich) (Aiba and Ogawa, 1977), while *C. vulgaris* was grown in BG11 (1.5 g $NaNO_3$, 1 g $NaHCO_3$, 0.2 mL, K_2HPO_4 1 M, 10 mL $BG11_c$; Sigma-Aldrich) (Rippka *et al.*, 1979) under axenic conditions. 20 mL algal culture was inoculated to 180 mL culture medium in an Erlenmeyer flask and was allowed to grow for 10 days 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 *A. platensis* and 25 ± 1 °C for *C. vulgaris* using the growth chamber (Biobase BJPX-A25011) At the end of 10 days, cultures were renewed and all the flasks contained 50 mL algal culture. Various concentrations of Bentagran compound for *C. vulgaris* (60, 120, 240, 480, 960 μg mL^{-1}) and *A. platensis* (100, 200, 400, 600, and 800 μg mL^{-1}) were added to the

culture medium. The range of concentrations was determined with preliminary range-finding bioassays according to EC50 value for growth parameters.

2.2 Cell growth assay

Optic density (OD) of microalgae was measured spectrophotometrically over 7 days under control and stressed conditions taking absorbance at 750 nm. Chlorophyll-*a* contents were estimated by methanol extraction and measured spectrophotometrically (Shimadzu UVmini 1240 spectrophotometer) taking absorbance at 665 nm for 7 days (MacKinney, 1941).

2.3 Antioxidant enzyme activities

After 7 days, 2 mL culture solutions from the control and each Bentagran-exposed algae medium was centrifuged (Centrution Scientific K3 series) at 15 000 rpm for 20 min. at 4 °C and resulting pellets were kept at −20 °C until enzyme activity measurements. The protein concentration of the algal cell extracts was determined according to Bradford (1976), using BSA (bovine serum albumin; Sigma-Aldrich) as a standard.

SOD activity was determined by the assay of Beyer and Fridovich (1987) but it was modified according to Kılıç *et al.* (2019). 0.2 g of pellet was suspended in homogenization buffer (1.5 mL) containing 100 mM K_2HPO_4 buffer (pH 7.0), 1 mM Na_2EDTA (Sigma-Aldrich), and 2% PVP (Sigma-Aldrich). The enzyme extract was obtained after centrifugation at 18 800 g for 20 min at 4 °C and it was used in the reaction mixture included of 100 mM K_2HPO_4 buffer (pH 7.8) containing 9.9×10^{-3} M methionine (Applichem), 5.7×10^{-5} M NBT (Applichem), %1 triton X-100 (Sigma-Aldrich). The reaction was started by the addition of 0.9 μM riboflavin (MP Biomedicals) and the mixture was exposed to light with an intensity of 375 $\mu mol\ m^{-2}\ s^{-1}$. After 15 min, the absorbance was read at 560 nm. SOD activity was calculated by a standard graphic and expressed as unit mg^{-1} protein.

Glutathione reductase activity was measured with the method of Sgherri *et al.* (1994) but it was modified according to Kılıç *et al.* (2019). 0.2g of pellet was suspended in homogenization buffer (1.5 mL) containing 100 mM KH_2PO_4 buffer (pH 7.0), 1 mM Na_2EDTA, and 2% PVP. The enzyme extract was obtained after centrifugation at 18 800 g for 20 min at 4 °C and enzyme extract containing 100 μg protein was added to the reaction mixture included 100 mM KH_2PO_4 buffer (pH 7.8), 2 mM Na_2EDTA, 0.5 mM oxidized glutathione (GSSG; Applichem), 0.2 mM NADPH (Sigma-Aldrich) and total volume was 1 mL. 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).

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined with the method of Wang *et al.* (1991) but it was modified according to Kılıç et al., 2019. APX extraction was conducted in 50 mM Tris–HCl (pH 7.2; Sigma-Aldrich), 2% PVP, 1 mM Na_2EDTA, and 2 mM ascorbate. The enzyme extract was obtained after centrifugation at 18 800 g for 20 min at 4 °C and enzyme extract containing 100 μg protein was used in the reaction mixture included of 50 mM KH_2PO_4 buffer (pH 6.6), 2.5 mM ascorbate, and 10 mM H_2O_2 in a final volume of 1 mL. The enzyme activity was calculated from initial rate of the reaction using the extinction coefficient of ascorbate ($E = 2.8\ mM\ cm^{-1}$ at 290 nm).

2.4 Non enzymatic parameters

The amount of malondialdehyde was determined according to the method used by Heath and Packer (1968). 0.2 g of the pellet was homogenized in 3 mL of 0.1% Trichloroacetic acid (TCA) (4 °C) and was 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, Sigma-Aldrich; Thiobarbituric acid, Sigma-Aldrich; 0.25 N Hydrochloric acid, Merck) 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 an ice bath. To remove suspended turbidity, the mixture was centrifuged at 4100 rpm for 15 min, and then the absorbance of the supernatant at 532 nm was recorded. Non-specific absorbance at 600 nm was measured and subtracted from absorbance at 532 nm. The MDA content was calculated using the extinction coefficient of 155 $mM^{-1}\ cm^{-1}$. For determination of H_2O_2 content, 0.5 mL of 0.1 M Tris-HCl (pH 7.6) and 1 mL of 1 M Potassium iodide (KI, Sigma-Aldrich) were added to 0.5 mL of supernatant. After 90 min., the absorbance was recorded at 390 nm. To determine the amount of proline, the method of Weimberg (1987) was followed. 0.1 g of the pellet was homogenized with 10 mL of 3% aqueous sulphosalicylic acid and the homogenates were incubated in a hot water bath at 95 °C for 30 min. The samples were then 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 (Merck) for 1 h at 100 °C. The reaction mixture was extracted with 4 mL toluene. The chromophore containing toluene (Sigma-Aldrich) was separated and the absorbance was recorded at 520 nm.

2.5 Statistical analysis

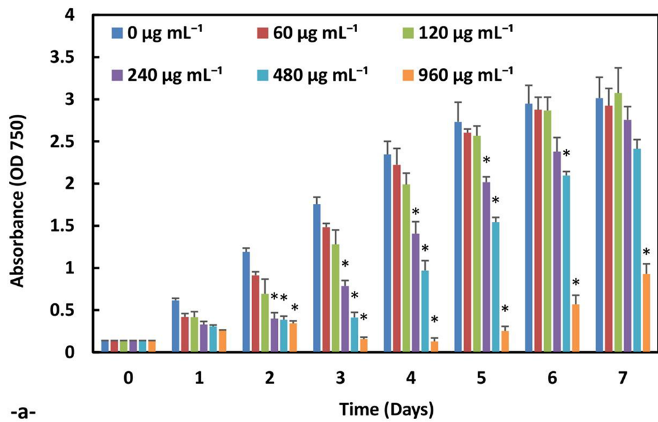
The experimental design was performed within three independent replicates for each treatment. The EC50 value was calculated with Origin Pro 8.5 program. The homogeneity test was performed on the data with IBM SPSS Statistics 20 program. The differences between the control and treated samples were analyzed by one-way ANOVA at a 95% confidence level, according to Tukey using the SPSS 20.0 statistical program for Windows.

The mean values ± SE were given in Figures.

3 Results

3.1 Biomass and chlorophyll-α content

OD750 (Optical Density at 750 nm) measurements and chlorophyll-α content in *C. vulgaris* and *A. platensis* cultures



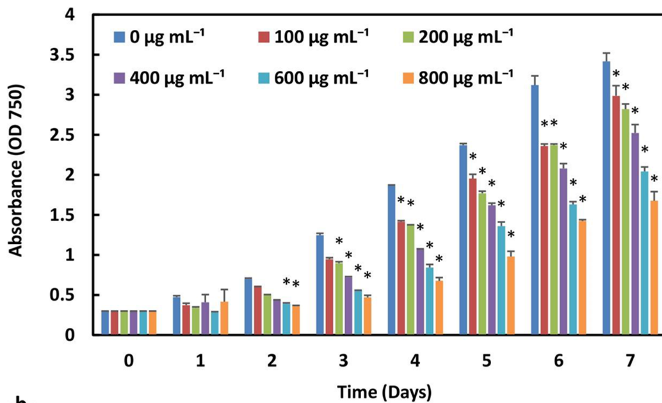
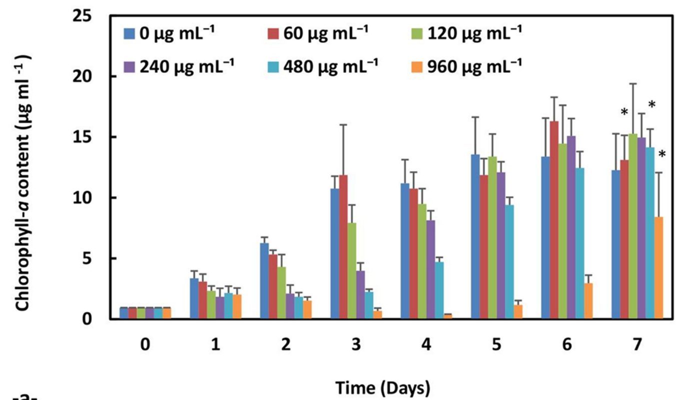


Fig. 1. Changes of OD 750 values in (a) *C. vulgaris* and (b) *A. platensis* algae by adding different concentrations of Bentagran herbicide for 7 days. Data are means ±SD of three replicates. Mean values in columns with asterisk are significantly different at the 5% level according to least significant differences (Tukey) Test.



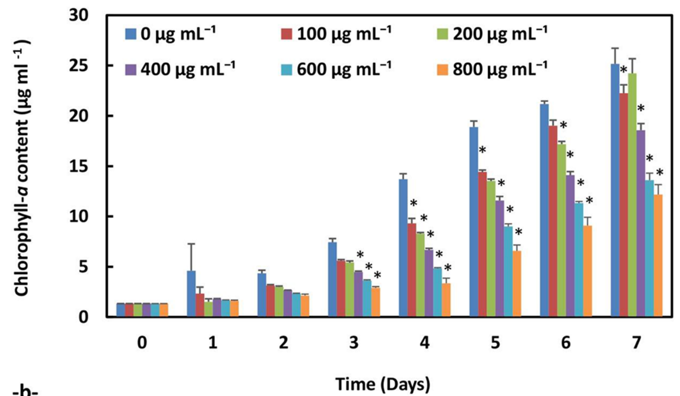


Fig. 2. Changes in the amounts of chlorophyll-*a* in (a) *C. vulgaris* and (b) *A. platensis* algae by adding different concentrations of Bentagran herbicide for 7 days. Data are means ±SD of three replicates. Mean values in columns with asterisk are significantly different at the 5% level according to least significant differences (Tukey) Test.

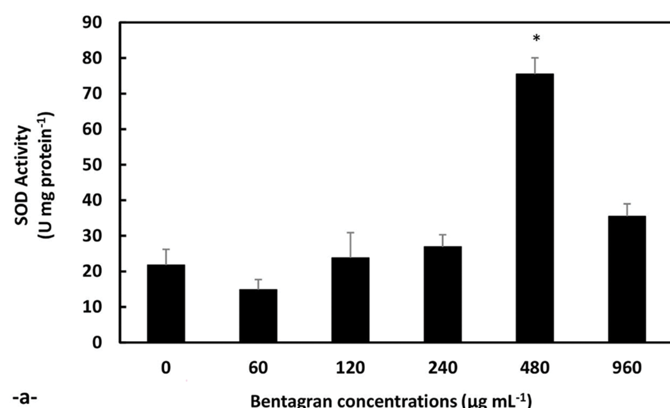
treated with different Bentagran concentrations are presented in Figures 1 and 2, respectively. Bentagran added to the culture medium for 7 days caused a significant decrease in both biomass accumulation and chlorophyll-*a* content in *C. vulgaris* and *A. platensis* cells in a dose-dependent manner. It has been found that the highest Bentagran concentrations (960 $\mu g\,mL^{-1}$ for *C. vulgaris* and 800 $\mu g\,mL^{-1}$ for *A. platensis*) lead to the most significant reduction in biomass accumulation and chlorophyll-*a* content. Besides, it was found that the biomass accumulation in *A. platensis* cells increased progressively after 2nd day; chlorophyll-*a* content in *A. platensis* cells increased after 3rd day in a time-dependent manner. However, the biomass accumulation in *C. vulgaris* were significantly reduced by Bentagran concentrations on the 2nd day of the application, chlorophyll-*a* content in *C. vulgaris* cells reduced by Bentagran concentrations on the 7th day of the application. The half-maximal effective concentration (EC50) values were 854.94 $\mu g\,mL^{-1}$ for *Arthrospira platensis* and 739.44 $\mu g\,mL^{-1}$ for *Chlorella vulgaris* on 7th day.

3.2 Antioxidant enzyme activities

The activity of SOD significantly increased (674.68%) at 480 $\mu g\,mL^{-1}$ concentration in *C. vulgaris* cultures, while increased (166.39%) at 200 $\mu g\,mL^{-1}$ concentrations in *A. platensis* cultures ($p < 0.05$) (Fig. 3). APX activity of *C. vulgaris* cultures did not show a significant change compared to control. However, it decreased at 400 (28.74%) and 600 (31.97%) $\mu g\ mL^{-1}$ concentrations in *A. platensis* cultures ($p < 0.05$) (Fig. 4). The activity of GR increased (118.15%) at 960 $\mu g\,mL^{-1}$ in *C. vulgaris* cultures, while it increased at 100 (47.61%), 200 (15.50%), and 400 (31.34%) $\mu g\ mL^{-1}$ concentrations in *A. platensis* cultures ($p < 0.05$). On the other hand, GR activity decreased (15.05%) at 600 $\mu g\,mL^{-1}$ concentrations in *A. platensis* cultures (Fig. 5).

3.3 Non-enzymatic parameters

C. vulgaris and *A. platensis* cultures did not show a significant change compared to control in the amount of H_2O_2 (Fig. 6). The amount of MDA decreased (73.71%) significantly at 960 $\mu g\,mL^{-1}$ concentration in *C. vulgaris* cultures ($p < 0.05$), while *A. platensis* cultures did not show a significant change (Fig. 7). On the other hand, free proline content also decreased (85.59%) significantly at 960 $\mu g\,mL^{-1}$ concentration in *C. vulgaris* cultures ($p < 0.05$). Furthermore, the free proline content of *A. platensis* cultures did not show a significant change compared to control ($p < 0.05$) (Fig. 8).



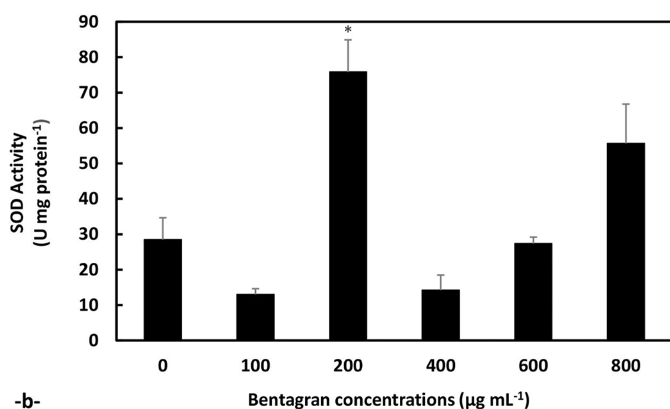
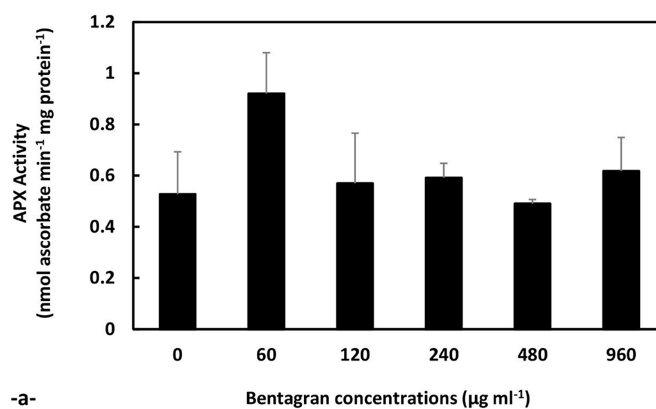


Fig. 3. Changes in SOD activities of (a) *C. vulgaris* and (b) *A. platensis* by adding different concentrations of Bentagran herbicide. Data are the means ± SD of three replicates. Mean values in columns with asterisk are significantly different at the 5% level according to least significant differences (Tukey) Test.



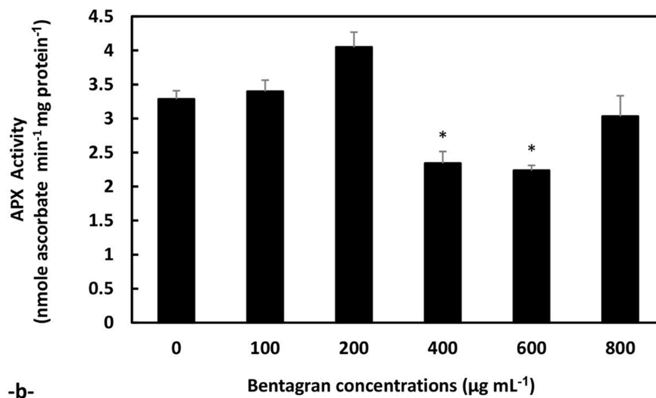


Fig. 4. Changes in APX activities of (a) *C. vulgaris* and (b) *A. platensis* by adding different concentrations of Bentagran herbicide. Data are the means ± SD of three replicates. Mean values in columns with asterisk are significantly different at the 5% level according to least significant differences (Tukey) Test.

4 Discussion

In the study, Bentagran herbicide significantly affects the growth rate for both algae, for 7 days. The EC50 values were 854.94 $\mu g\,mL^{-1}$ for *Arthrospira platensis* and 739.44 $\mu g\,mL^{-1}$ for *Chlorella vulgaris* according to growth rate. Cedergreen and Streibig (2005) and Munkegaard *et al.* (2008) measured the EC50 value of bentazone in *Pseudokirchneriella subcapitata* as 13.6 $mg\,L^{-1}$ and 51 $mg\,L^{-1}$, respectively. Macedo *et al.* (2008) calculated the EC_{50} value as 13.0 $mg\,L^{-1}$ for *Skeletonema costatum*. Hourmant *et al.* (2009) found the value as 150 $mg\,L^{-1}$ for *Chaetoceros gracilis.* When these studies are compared, even the closest dose is much lower than in our study results. The reason may arise from since the selected algae are in different divisions. In addition, the recent study in Ebro River Delta has reported the content of bentazone as 180 $\mu g\,mL^{-1}$ in aquatic ecosystems (Barbieri *et al.,* 2021). Because of the excessive herbicide usage, it is thought that there will be serious damage to the environment in the future and the algal growths are threatened in such systems.

The reductions in the growth rate are more than the decrease of the chlorophyll-*a* production, especially in *Chlorella vulgaris*. Pesticides affect mitotic division stages by prevention of macromolecule synthesis and microtubule formation, thus they inhibit the algal growth rates (Cedergreen and Streibig, 2005). It is predicted that these processes occur more rapidly than disruption of chlorophyll-*a* synthesis and the inhibition of photosynthetic reactions. Bentagran acts also as an inhibitor in photosynthesis reactions by blocking the electron transfer flow in photosystem II (PS II) and CO_2 fixation. In the presence of light, blocking of PS II induced by Bentagran leads to secondary reactions such as interruption of energy flow and formation of various ROS like superoxide radicals (Macedo *et al.*, 2008). Marques *et al.* (2011) examined the effects of herbicide pollution on microalgae *Pseudokirch-neriella subcapitata* in drainage channels of Portuguese rice fields. They reported a reduced level of chlorophyll-*a* content in *P. subcapitata* due to Bentagran toxicity. Moreover, Tomé (1996) showed that high Bentagran concentrations caused a reduction in the chlorophyll-*a* content of cyanobacteria *Nostoc* sp. Following these studies, our research has demonstrated that the chlorophyll-*a* content in *C. vulgaris* and *A. platensis* cells were reduced in a dose-dependent manner as a result of Bentagran toxicity. Similar to other toxic matter (Prasad and Strzałka, 1999; Surosz and Palinska, 2004; Schoefs and Bertrand, 2005; Günsel *et al.*, 2018), this herbicide can be effective in the enzymatic inhibition of chlorophyll

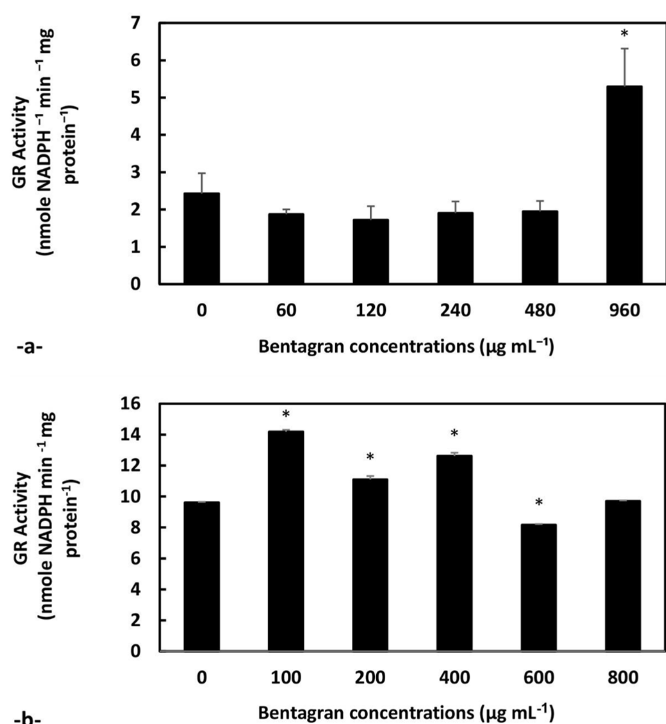


Fig. 5. Changes in GR activities of (a) *C. vulgaris* and (b) *A. platensis* by adding different concentrations of Bentagran herbicide. Data are the means ± SD of three replicates. Mean values in columns with asterisk are significantly different at the 5% level according to least significant differences (Tukey) Test.

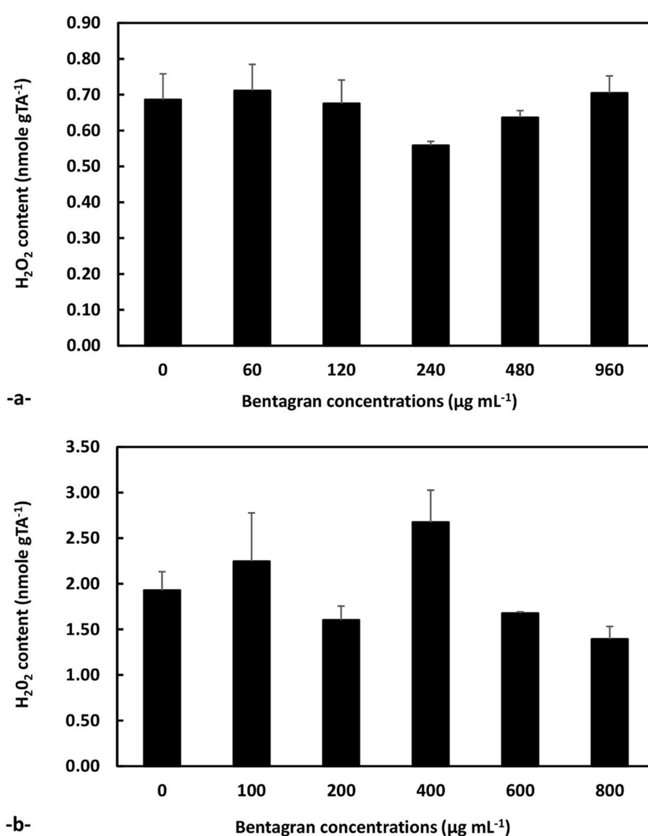


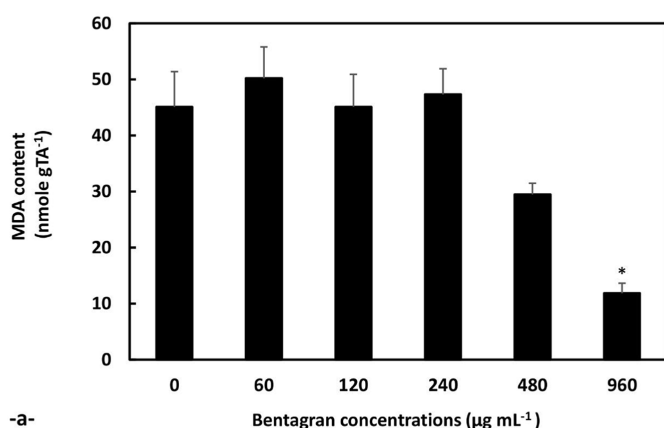
Fig. 6. Changes in amounts of H_2O_2 of (a) *C. vulgaris* and (b) *A. platensis* by adding different concentrations of Bentagran herbicide. Data are the means ± SD of three replicates. Mean values in columns with asterisk are significantly different at the 5% level according to least significant differences (Tukey) Test.

biosynthesis and/or accelerated degradation of photosynthetic pigments, or disruption of thylakoid integrity. Due to the effects on pigment degradation and photosynthetic inhibition, Bentagran may have suppressed the growth of *C. vulgaris* and *A. platensis.*

The intervention of the Bentagran in such photosynthetic processes is thought to be due to the formation of ROS in redox reactions. This situation causes oxidative stress in cells and creates an imbalance between membrane structure and antioxidant systems (Kortekamp, 2011). The activity of SOD increased only at 480 µg mL^{-1} in *C. vulgaris* when exposed to different concentrations of Bentagran. Also, 200 µg mL^{-1} concentrations showed a significant increase compared to the control in *A. platensis.* Galhano *et al.* (2010) studied the effect of Bentagran on *Anabaena cylindrica* antioxidant defense system. In their study, they observed that SOD activity increased due to increasing concentrations of Bentagran. Moreover, Galhano *et al.* (2011) showed that SOD activity increases when applying Bentagran on *Nostoc muscorum*. Moreover, in the strain of *Synechococcus elongatus* PCC7942 which is exposed to Bentagran, the activity of SOD increased 2–3-fold (Bagchi *et al.*, 2012). The SOD is an important endogenous antioxidant enzyme that functions as the first detoxification enzyme in cells and part of the primary defense system against ROS (Ighodaro and Akinloye, 2018). Kong *et al.* (1999) showed as the rate of contamination in algal cells increases the cellular detoxification system and SOD synthesis begins, thus it acts as the key enzyme that destroys active oxygen. Therefore, the SOD enzyme can be used as a sensitive biomarker and an early stimulus of pollution (Li *et al.*, 2005).

Ascorbate peroxidase (APX) and glutathione reductase (GR) are enzymes produced mainly in chloroplasts and other cell organelles that remove H_2O_2 and protect the redox state of the cell. Increased activity of these enzymes indicates cellular stress. Ascorbate is the most important reducing substrate in the detoxification of H_2O_2 in plant cells and it is broken down by APX into H_2O (Koç and Üstün, 2008). In our study with *C. vulgaris*, although APX activity increased compared to control, this increase was not statistically significant. The unchanged APX activity and unchanged H_2O_2 content similarity arise from the enzyme-substrate relationship in *C. vulgaris* application.

The ascorbate-glutathione cycle is a defense mechanism that provides detoxification of H_2O_2 to water and oxygen without generating ROS and is also present in peroxisome, chloroplast, cytosol, and mitochondria (Doğru, 2019). GR activity of *C. vulgaris* cultures exposed to Bentagran increased only at 960 µg mL^{-1} concentration indicating that this dose is the critical concentration for cellular damage. Although GR and APX enzymes are key enzymes related to each other, some studies have revealed that increases in glutathione pool and GR activity do not cause any increase in ascorbate levels under stress conditions. As the increase in ascorbate level is related to



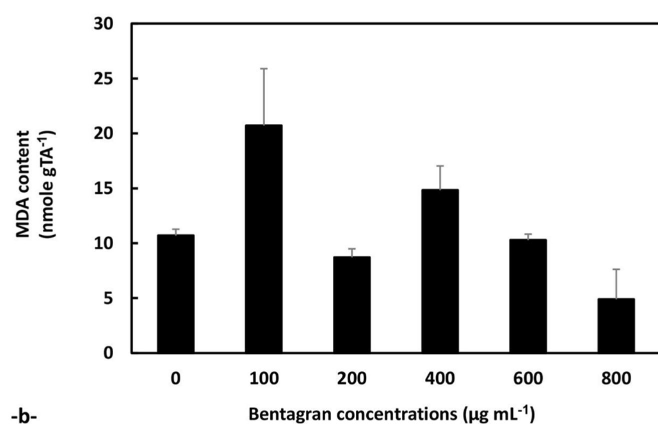
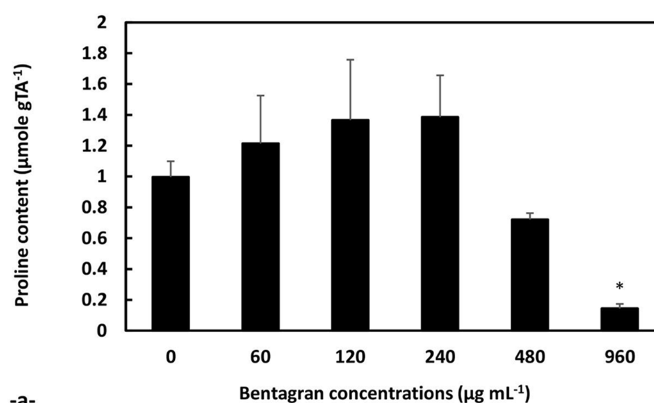


Fig. 7. Changes in amounts of MDA of (a) *C. vulgaris* and (b) *A. platensis* by adding different concentrations of Bentagran herbicide. Data are the means ± SD of three replicates. Mean values in columns with asterisk are significantly different at the 5% level according to least significant differences (Tukey) Test.



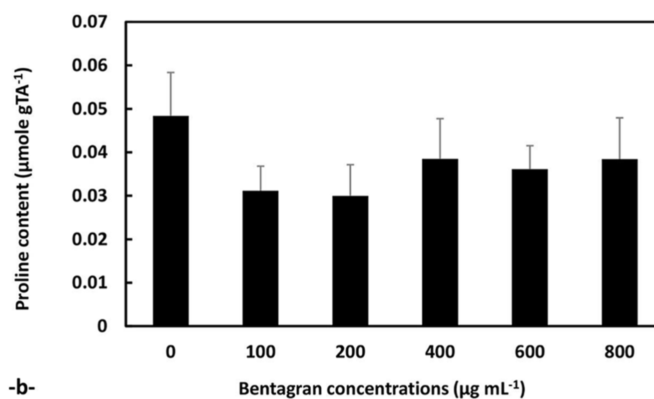


Fig. 8. Changes in amounts of Proline of (a) *C. vulgaris* and (b) *A. platensis* by adding different concentrations of Bentagran herbicide. Data are the means ± SD of three replicates. Mean values in columns with asterisk are significantly different at the 5% level according to least significant differences (Tukey) Test.

APX activity, this situation is also seen in our study (Karpuz and Çakır, 2021).

In *A. platensis* cultures, the activity of APX decreased at 400 and 600 $\mu g\,mL^{-1}$ concentrations. GR is required for the regeneration of ascorbate (Günsel *et al.,* 2018), and the activity of APX is related to the ascorbate and H_2O_2 contents present in the medium.

The GR activity increased compared to the control in *A. platensis* cultures exposed to Bentagran at 100, 200, and 400 $\mu g\,mL^{-1}$ concentration. Das and Bagchi (2012) reported that GPX-GR activity increase by 60% when applying Bentagran on mutant *S. elongatus* PCC7942 strain. It has been observed that GR activity increases as a defense strategy. It is thought that the decrease of GR activity in increasing concentrations affects the GSH pool which will form the substrate for GR activity thus suppressing the conversion of GSH to GSSG (Galhano *et al.*, 2010).

Both GR activity and APX activity decreased at 600 $\mu g\,mL^{-1}$ concentrations but they did not change at 800 $\mu g\,mL^{-1}$ concentrations in Bentagran application. The most important question is, why the activities increased at this concentration, at this point. Probably, the herbicide consumed the enzyme up to 600 $\mu g\,mL^{-1}$ concentrations in the cell but the gene expression of these enzymes was triggered at the 800 $\mu g\,mL^{-1}$ concentrations. Molecular analyzes are recommended to understand this issue.

In our study, the alterations of GR activity support the changes of APX activity at similar concentrations in these pesticide applications because the ascorbate pool is stabilized by the GR enzyme. It has been reported in previous studies that GR enzyme is associated with APX activity (Mallick and Rai, 1999; Teisseire and Vernet, 2001).

MDA is one of the major products of lipid peroxidation, which is associated with oxidative stress in cells. Total MDA amounts of *C. vulgaris* cultures decreased only at 960 $\mu g\,mL^{-1}$ concentration compared to the control. Increased GR activity has supported this information and also proline content has decreased at this concentration.

H_2O_2 is a strong oxidant that rapidly oxidizes thiol groups. Since photosynthesis is associated with thiol-regulating enzymes, H_2O_2 should not be allowed to accumulate in chloroplasts (Koç and Üstün, 2008). Total H_2O_2 content did not show significant change compared to control in *C. vulgaris* cultures exposed to increasing concentrations of Bentagran. The unchanged H_2O_2 content was supported by an increase in GR activity. Das and Bagchi (2012) observed that cell growth on the mutant *S. elongatus* PCC7942 strain was five times faster than that of wild type in Bentagran studies. They reported

they achieved this with the H_2O_2 detoxification mechanism and thus limited lipid peroxidation was observed. Galhano *et al.* (2010) reported H_2O_2 was removed by GR-GSH, CAT, and APX enzymes in *A. cylindrica* which they applied Bentazone. In our study, this situation can be explained by SOD and APX activities. Because if the SOD and APX activities decrease or increase, this situation reflects the H_2O_2 content. APX activity determines the H_2O_2 accumulation in cells and plays an important role (Asada, 1992). In this context, H_2O_2 accumulation was prevented in the medium because of the increase in the SOD and APX activity of *A. platensis* at 200 and 800 $\mu g\,mL^{-1}$ concentrations.

On the other hand, the total MDA content did not show significant change compared to control in *A. platensis* cultures exposed to all applied Bentagran concentrations. These changes are related to the changes in the amount of H_2O_2. When Lin and Kao (2000) applied the salt stress on *Oryza sativa* plant, they reported the unchanged content of both H_2O_2 and MDA due to the activity of other antioxidant enzymes.

While the free proline content decreased to the highest concentration of 960 $\mu g\,mL^{-1}$ in *C. vulgaris* cultures, the content did not change compared to the control in any concentrations of *A. platensis* treatment. Although there are studies in the literature that proline content increases under stress conditions, reverse studies are also available. *Arthrospira platensis* has decreased its proline content with increasing concentration of deltamethrin (Tunca *et al.*, 2021). In this study, they have suggested that sulfite inhibited the proline dehydrogenase enzyme in proline synthesis. Similarly, in our study, proline depletion may be an indication that toxic substances accumulate in the medium, proline synthesis may be inhibited and proline structure may be impaired. Furthermore, the decreased proline content suggests the possibility that free proline was used by free radicals. It is known that pesticide-acting proline production is almost absent in cyanobacteria (Galhano *et al.*, 2010). Moreover, when the changes in the proline content and the changes in H_2O_2 and MDA amounts are examined, the results suggest proline may prevent cell membrane damage and increase MDA amount. It has been shown that cadmium application does not change the MDA content and free radical damage has been prevented in the cell because proline has acted as an antioxidant in transgenic *Chlamydomonas reinhardtii* (Siripornadulsil *et al.*, 2002). The similarity between SOD and GR-APX enzyme activities can be supported by the fact that they perform good teamwork against lipid peroxidation in cells, and that there is no increase in the amount of MDA and proline, which are the indicators of lipid peroxidation in *C. vulgaris* cultures.

As a result, Bentagran herbicide reduces growth rates and causes oxidative damage by creating similar effects in both algae, despite the different cell structures. When EC 50 values are compared, the concentration in the treatment of *Chlorella vulgaris* has a higher value than in the *Arthrospira platensis* application, but the values are close to each other. This situation arises from the similarity of accumulation, detoxification, and degradation processes of Bentagran herbicide, in both algae. However, when the results are examined in detail, the antioxidant responses are different from each other. It is predicted that the most important reason of the case is the different free radicals amount produced by chloroplasts and mitochondria being important sources of ROS, in *C. vulgaris*. For this reason, different rates were observed in both algae especially in SOD and GR activities and in MDA amounts. Bentagran creates oxidative stress in phototrophs of aquatic ecosystems. The use of these small organisms, compared to large plants, in the detection of herbicidal contamination of the environment will shed light on further studies.

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