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ANTIOXIDATIVE DEFENSE SYSTEMS IN TWO SCENEDESMUS SPECIES EXPOSED TO COPPER AND LEAD

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Abstract

Heavy metals, including copper (Cu) and lead (Pb) depending on species, differentially affect algal growth and metabolism. *Scenedesmus obliquus* (*S. obl*), and *Scenedesmus regularis (S. reg*) are known to be affected by these environmental changes. Changes in the growth rates, level of oxidative stress markers, and antioxidants were analyzed following exposure to various concentrations of Cu (32, 44, 272, and 476 µM) and Pb (6, 8.4, 12.1, and 18.1 µM) for 25 days. Compared to the control groups, chlorophyll *a* amounts were low in the studied species. In addition, a consistent decrease in chlorophyll *a* level was detected depending on the applied stress doses, but it was not found to be statistically significant. Total antioxidant levels increased significantly in the dosage and concentration-dependent manners in both species when exposed only to Pb. The molecular antioxidant ratios in both species were higher, which was significant for total flavonoids. While the increase in SOD activity of the species against both metal stresses was observed at 18.1 µM Pb concentration (9.5%) in *S. regularis*, it was detected at 476 µM Cu concentration (13.2%) in *S. obliquus*. A similar situation was observed in GR activity at a Pb concentration of 12.1 µM (40.5%) in *S. regularis*, while it was at 476 µM Cu concentration (18.5%) in *S. obliquus*. Thus, the data indicate that the studied algae species exhibited similar strategies to alleviate long-term metal toxicity. This study also provided new data for Cu and Pb removal efficiency abilities of two microalgae species, where metals uptake and efficient antioxidant defense system protected species against oxidative stress induced by metals stress.

Key words: Microalgae, *Scenedesmus obliquus*, *Scenedesmus regularis*, Antioxidant, Copper metal, Lead metal.

Introduction

Metals according to their biochemical functions can be divided into two groups essential Cu, Fe, Ni, and Zn or non-essential heavy metals such as Pb, Cd, and Hg (Park *et al*., 2007; Collin *et al*., 2008). Although Cu is a vital trace element for the development of photosynthetic species, high levels of Cu heavy metal show biochemically toxic properties such as lead (Pb) heavy metal (Sabatini *et al*., 2009).

As a result of anthropogenic activities (industry and mining), the amount of heavy metals known to be toxic in ecosystems increases and there is a serious threat to living organisms (Nagajyoti *et al*., 2010; Walker, 2009). Pinto *et al*., (2003) in a study, showed that the discharge of Cu metal to the oceans was $9*10^6$ t / year. ILZSG reported that for the year, the Pb metal released as a result of human activities (leaded-gasoline, lead-acid batteries) was 11 million metric tons (Anon., 2019).

It is known that Cu stress slows down the growth of microalgae and increases oxidative stress (Machado & Soares, 2012). Non-redox active metals such as Pb may destroy electron transfer chains and depletion of cellular antioxidants (Pinto *et al*., 2003; Yadav, 2010). It is known that photosynthetic organisms cause the formation of reactive oxygen species of reactive electrons (ROS) due to electron transport activities occurring in the chloroplast, mitochondria and plasma membranes (Foyer & Noctor, 2005; Lepetit *et al*., 2013). Exposure to contaminating metals such as Cu and Pb has been shown to stimulate and increase ROS production (Szivák *et al*., 2009; Stoiber *et al*., 2013). Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GP), and glutathione reductases (GR) etc., of organisms exposed to a toxic compound enzymatic antioxidants, can serve as well biomarkers that reflect pollutant-induced oxidative stress (Geret *et al*., 2003; Torres *et al*., 2008).

Algal species found in surface waters are exposed to many heavy metals in varying concentrations over a long period. More research is needed to discover the potential of different algae species that have adapted to diverse natural conditions to survive the long-term toxic effects of heavy metals and to cope with oxidative stress. Following this purpose, the response of two Scenedesmus species obtained from Kırıkkale province (Turkey) on the algal growth process under long-term Cu and Pb metal stress, heavy metal removal potential, also long-term metal exposure affects antioxidant defense systems were investigated.

Material and Methods

In our study, two microalgae species, namely freshwater green microalgae, *Scenedesmus regularis*, and *Scenedesmus obliquus*, were isolated from the water samples of Kapulukaya Reservoir in Kırıkkale Province (Turkey). Morphological and molecular characterization was used to identify species and the diagnosis of Scenedesmus species was based on two gene regions (18S rRNA and ITS).

Algal cells were cultured and maintained in Basal Bold medium (Bischoff & Bold, 1963) at 25°C with a 16:8 hours light-dark cycle and in 4000 lux light-cool fluorescent. Cultures were grown in 200 ml sterile, and optimized culture medium, respectively, in laboratory conditions with 32, 44, 272, 476 µM Cu and 6, 8.4, 12.1, 18.1 µM Pb stock solutions from $Pb(NO_3)_2$ and $CuSO_4.5H_2O$ added. The initial cell density of $1.2*10^5$ cells was used for all experiments. The culture medium was kept under stable conditions by shaking at 115 rpm. During the growth stages of cell cultures, chlorophyll a, total carotene, total phenolic, total flavonoid, and total antioxidant changes were observed. SOD, CAT, GP, GR, etc. Immediately before the tests of the analysis of

enzymatic antioxidants, algal cells in the exponential growth phase were collected by centrifugation at 5000 rpm for 5 min, washed and resuspended in test buffers.

Algal growth rate: After 25 days of incubation, the algal cell density was diluted to the appropriate medium with the media and counted with the Invitrogen Countess Automated Cell Counter (Carlsbad, California, USA). The specific growth rate was determined by the method based on Guillard (1973) using the equation μ=ln (N(t)/N0)/(t–t0). In the equation, t and Nt define incubation time and cell density (cells ml^{-1}).

Antioxidant enzymes assays: The samples (100 mg) were placed in CAT and GR enzyme experiments in ice-cold buffer containing EDTA (1 mM) and potassium phosphate buffer (50 mM) pH 7.0. For SOD and GP enzyme experiments, EDTA (1 mM), and mannitol (210 mM) were mixed and centrifuged at 15000 rpm for 15 minutes in ice-cold HEPES buffer (20 mM) containing sucrose (70 mM) pH 7.2. Enzymatic antioxidant experiments; CAT, SOD, GR and GP (respectively, catalogue no. 707002, 706002, 703202 and 703102) produced by Cayman Chemical Company (Ann Arbor, MI, USA) were evaluated by repeated reading using the colourimetric assay kits according to the instructions.

Chlorophyll *a* and total carotene content: For chlorophyll a and total carotene measurements, 100 μl of algae cells was centrifuged at 15000 rpm. The precipitate was resuspended in cold acetone for 90% and sonicated for 5 minutes in the dark. After repeated centrifugation (15000 rpm), the supernatant was transported to a 96-well microplate. The optical density was determined with The BioTek PowerWave XS2 microplate reader for the wavelengths of A^{470} nm, A^{630} nm, A^{647} nm, A^{664} nm and A^{750} nm (Eqn-1) following the method proposed by Jeffrey and Humphrey, (1975).

$$\begin{aligned}
&\text{Chlorophyll } a && = 11.85 \times A^{664} - 1.54 \times A^{647} - 0.08 \times A^{630} \\
&\text{Chlorophyll } b && = -5.43 \times A^{664} - 2.66 \times A^{630} + 21.03 \times A^{647} \\
&\text{Total carotenoids (Tc)} && = (1000 \times A^{470} - 2.77 \times \text{chlorophyll } a) / 213 \qquad (1)
\end{aligned}$$

The metal concentrations measurement: Algae samples were digested in a microwave oven (CemMars6) and were determined by optical emission Spectrometry (ICP-OES, SpectroBlue, Germany) for Cu and Pb metals (Aluc & Ekici, 2019). Each experiment was repeated three times, and the average was taken. The analyzed heavy metals were quantified using calibration curves plotted from analytical standards (Merck, Germany). For analyses of heavy metals in samples, 0.1 mg of algae with 10 mL of 65% HNO_3 was mixed and digested. The instrumental parameters (Plant materials method) were set as power: 290–1800, ramp time: 20:00, hold time: 15:00, and temperature: 200°C.

Statistical analyses

Measurement results were statistically analyzed using One-way ANOVA (SPSS 20 software, IBM Corporation, Armonk, New York, USA), and the averages were simulated using Duncan's multiple range tests at 5%.

Result

Chlorophyll *a* content reached the maximum concentration after 25 days of incubation of the microalgal species. Decreases in the amount of chlorophyll *a* were detected at all doses of the applied metals in both microalgal species compared to the controls (Fig. 1A, B).

When the effect of Cu metal on the specific growth rate of *S. obliquus* was examined, insignificant change was observed in the first three concentrations tested, while a significant decrease was observed in 476 μM Cu concentration by 16.7%. Specific growth rates on *S. regularis* showed significant increases of 28.5% and 14.9% at 32 μM Cu and 44 μM Cu concentrations as a result of exposure to applied Cu stress, while as a result of exposure to high Cu stresses such as 272 μM Cu and 476 μM. Significant decreases of 26.5% and 28.5% were detected (Fig. 2A). On the algal growth rate were observed reductions for the two target species to different Pb concentrations. There were significant decreases in the 6 μM Pb concentration, in the specific growth rates of *S. regularis* and *S. obliquus* by 41.8% and 33.9%, respectively (Fig. 2B).

The removal efficiency of Cu and Pb was observed in all metal concentrations in both the algal species. When the removal efficiency of the two species was compared, it was found that *S. obliquus* for Cu metal was more effective. However, the difference was insignificant for Pb metal removal efficiency rates in either species (Fig. 3A, B). In both species, an increase in the removal efficiency was observed at 32 μM Cu concentration, while this effect was decreased at higher concentrations. The highest Cu metal removal efficiency rates for *S. obliquus* and *S. regularis* were obtained at 32 μM Cu concentrations at 91.6% and 76.9%, respectively. In both species, the Pb metal removal efficiency rate showed an average of 40% for all concentrations.

Catalase, superoxide dismutase activities, and reduced glutathione content were analyzed as antioxidant response parameters. Additionally, total antioxidant levels were monitored at different initial metal concentrations over 25 days for the responses of both species to both metal exposures. Concentration-dependent increases in total antioxidant levels were linear at all concentrations of Pb metal (Fig. 4A). *S. regularis* and *S. obliquus*, the highest increases in total antioxidant content were observed in 18.1 μM Pb concentrations compared to the control group, and it was measured as 32.3% and 17.3%, respectively. It was determined that there was a significant increase in the *S. obliquus* compared to the *S. regularis.* While significant increases were observed in all concentrations (11.9%, 64.8%, and 85.4%) after the 44 μM Cu concentration at the *S. regularis*. This species for a significant increase was observed only at the 476 μM Cu concentration (14.1%) (Fig. 4B).

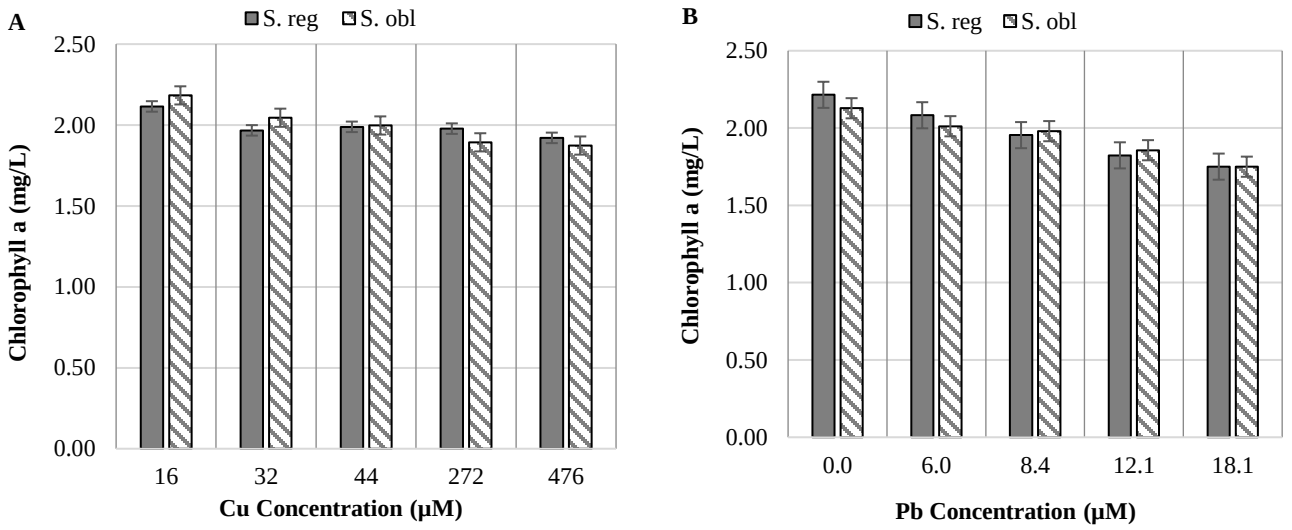

Fig. 1. (A, B). Chlorophyll *a* amounts according to all doses of applied metals concentrations in both species. Data are expressed as means ±S.D. Significant differences between control and treatments are indicated by asterisks: **p*<0.05 (A) Cu levels, (B) Pb levels.

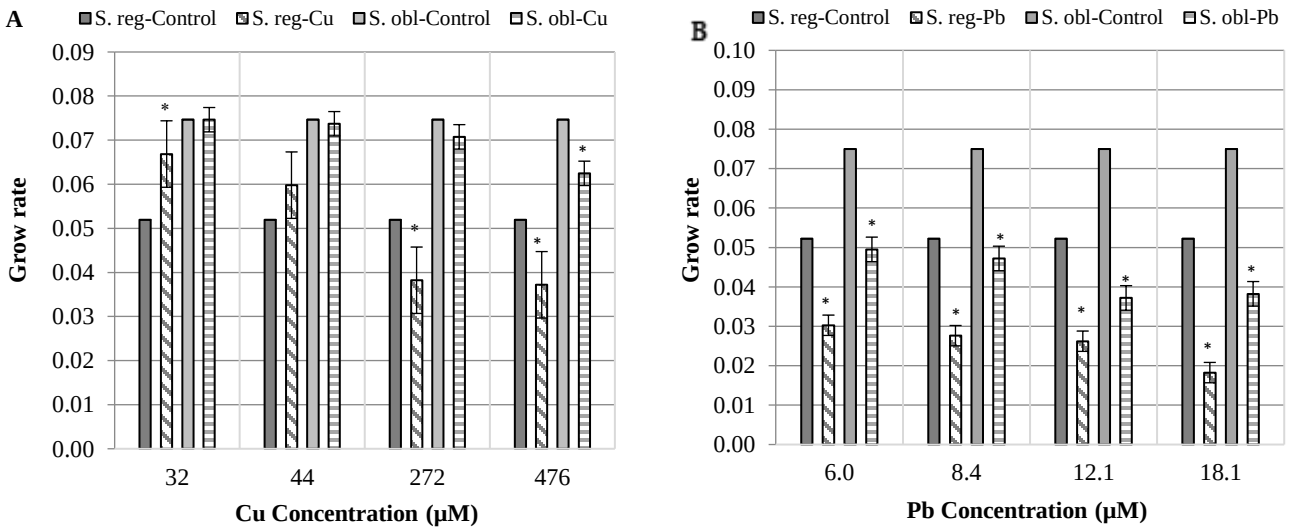

Fig. 2(A, B). Grow rates according to all doses of applied metals concentrations in both species. Data are expressed as means ±S.D. Significant differences betwen control and treatments are indicated by asterisks: **p*<0.05 (A) Cu levels, (B) Pb levels.

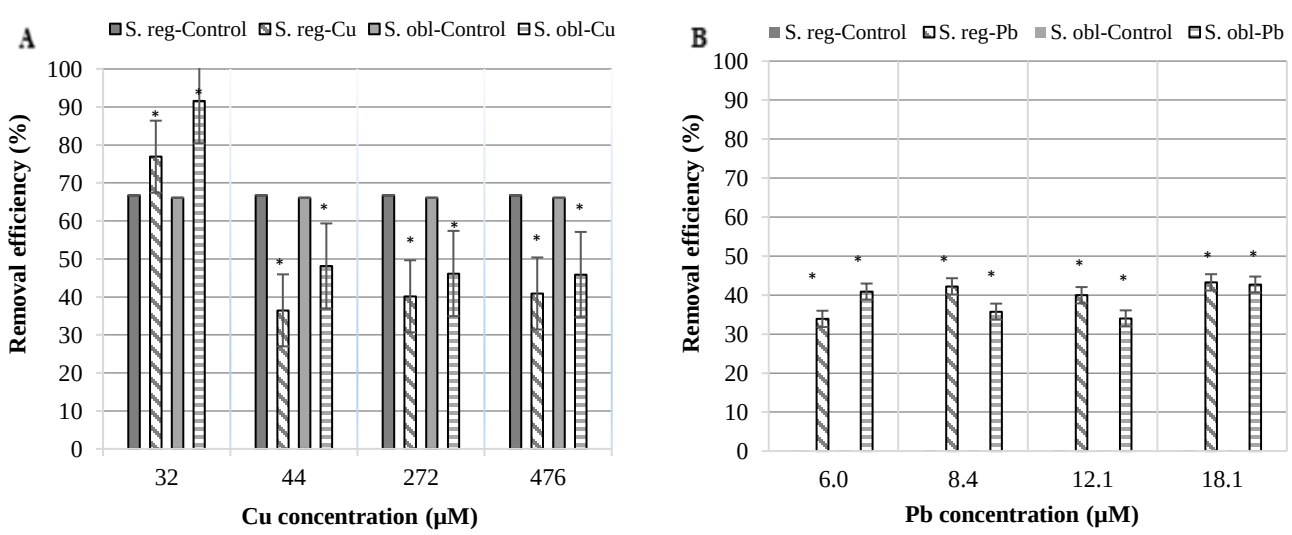

Fig. 3(A, B). % removal efficiency according to all doses of applied metals concentrations in both species. Data are expressed as means ±S.D. Significant differences betwen control and treatments are indicated by asterisks: **p*<0.05 (A) Cu levels, (B) Pb levels.

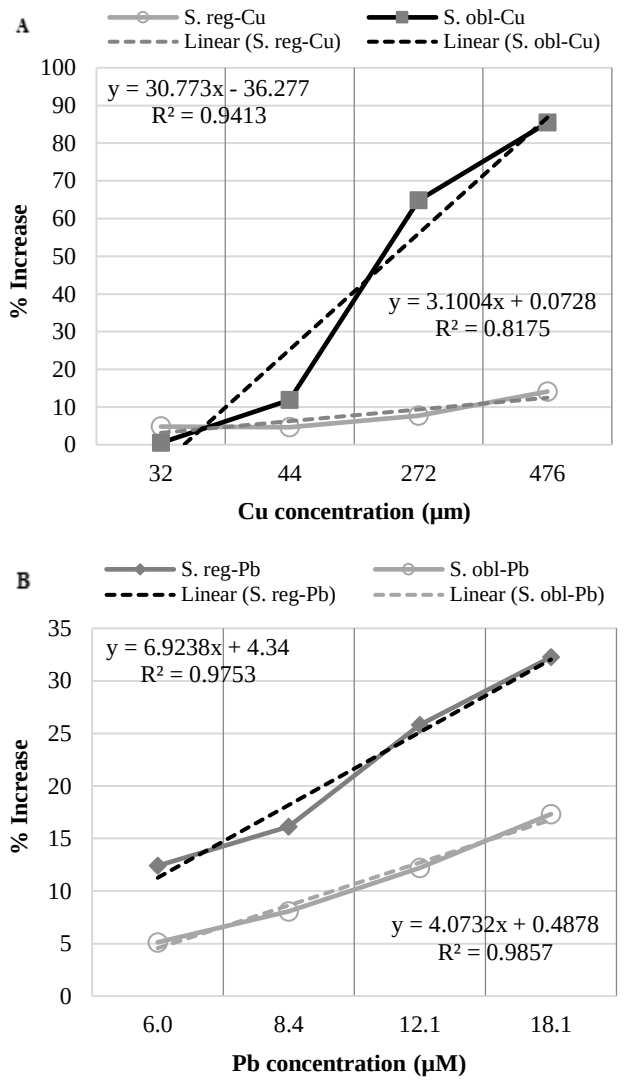

Fig. 4(A, B). Concentration-dependent increases in total antioxidant levels according to all doses of applied metals concentrations in both species. (A) Cu concentrations, (B) Pb concentrations.

Significant changes were observed in the ROS-scavenging enzymes of algae species exposed to Cu and Pb stresses. While remarkable increases were observed in SOD and GR activities against both metal stresses in the two algae species examined, significant decreases had detected in CAT and GP enzymes. The increase in SOD activity was observed in 18.1 µM Pb concentration (9.5%) in *S. regularis*, while had detected in 476 µM Cu concentration (13.2%) in *S. obliquus*. A similar situation was observed in the 12.1 µM Pb concentration (40.5%) in the *S. regularis*, while had observed in the 476 µM Cu concentration (18.5%) in the *S. obliquus* (Figs. 5, 6A-D).

The total phenol, total flavonoid and total carotene content, important secondary metabolites, were monitored for 25 days at different metal concentrations for the responses of both species to metal exposures. Increases in Molecular antioxidants were observed from day 15 on all tested metal concentrations in both species. The increases in the molecular antioxidant ratios of both species are listed as Total Carotene<Total Phenol<Total flavonoid (Figs. 7, 8A-C).

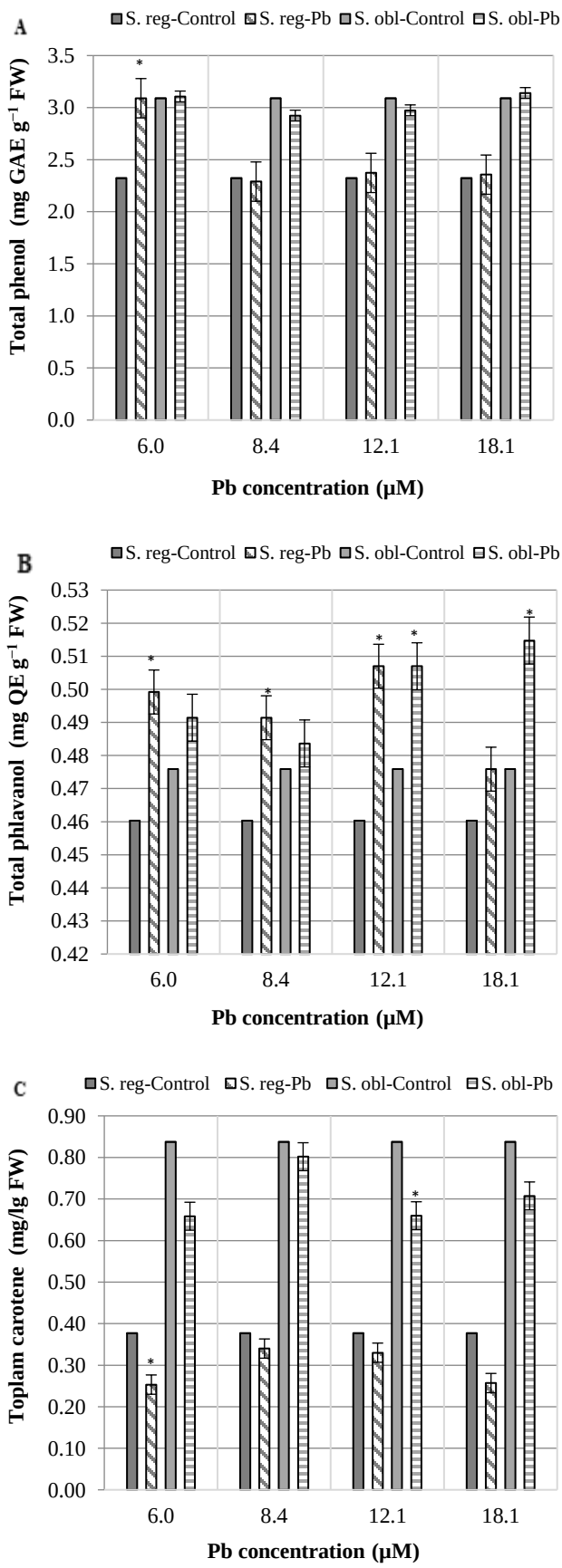

Fig. 5(A, C). Concentration-dependent secondary metabolites according to all doses of applied metals concentrations. Data are expressed as means ±S.D. Significant differences betwen control and treatments are indicated by asterisks: *p<0.05 (A) Total phenol, (B) Total phlavanol (C) Total Carotene.

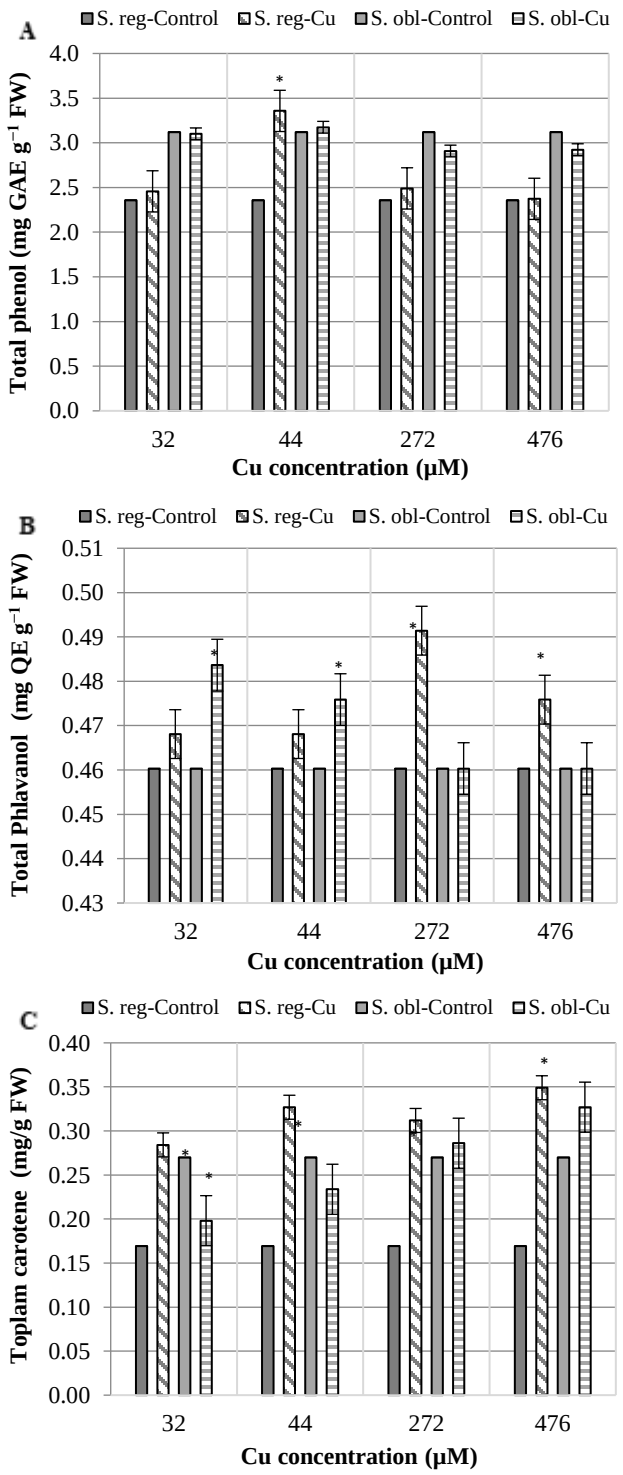

Fig. 6(A, C). Concentration-dependent secondary metabolites according to all doses of applied metals concentrations. Data are expressed as means ±S.D. Significant differences betwen control and treatments are indicated by asterisks: *p<0.05 (A) Total phenol, (B) Total phlavanol (C) Total Carotene.

Discussion

It is well known that algae are exposed to photosynthetic system damage by being affected by pollutants, and as a result, their growth is adversely affected (Bernal *et al*., 2006; Huang *et al*., 2013). Studies have shown that metal tolerance may have different responses among different microalgae species (Prasad *et al*., 1998; Baos *et al*., 2002; Yan & Pan, 2002; Schiariti *et al*., 2004; Sabatini *et al*., 2009; Kalinowska & Pawlik-Skowrońska, 2010). During the 25-day experiments, although reductions were detected in the chlorophyll *a* amount at all doses of chronically administered metals in both species compared to the control groups; these decreases were not found significant. It has been reported that when algae are exposed to metal, their volume increases (Wilde *et al*., 2006; Machado & Soares, 2014). The changes in the amount of chlorophyll *a* were not statistically significant in this study and the decreased cell density under increasing stress conditions was tolerated by the increase in cell volumes, as explained by Echeveste *et al*., (2017). It has also been reported that enlarged algal cells prevent the loss of pigment content caused by reduced cell density, resulting in increased pigment synthesis (Chen *et al*., 2016; Dong *et al*., 2020).

While high Cu concentrations applied in the study caused a significant decrease in the specific growth level in both species, it was determined that cell growth decreased at all doses of Pb. The inhibitory effects of Pb on growth and biomass production are known to suppress photosynthesis, especially by inhibiting the activity of carboxylation enzymes (Stiborová *et al*., 1987). Since Cu is an essential trace element in plants, it was observed that Cu at low concentrations had an encouraging effect on specific growth rates in both species, and this is in agreement with our study (Chen *et al*., 2016; Pham, 2019).

Algae can remove heavy metals from aqueous solutions (Abdel-Raouf *et al*., 2012). In the literature, several results have been obtained from metal removal efficiency in different Scenedesmus species (Monteiro *et al*., 2009; Abdel-Raouf *et al*., 2012; Sacristán *et al*., 2013; Wong *et al*., 2015). In this study, results similar to the high Cu removal rates of 89% reported by Pham (2019) were obtained as 91.6% and 76.9%, respectively, in the two species used.

It is known that cell surface/volume ratios have a positive effect on metal deposition (Sabatini *et al*., 2009). Due to the rule amount of algae in a unit volume, they have a higher Cell surface/volume ratio as they have a relatively larger surface area. It was reported that low-dimensional algae species generally had more metal accumulated than larger-cell algae (Khoshmanesh *et al*., 1997; Quigg *et al*., 2006). This study, which supported the knowledge of the literature, determined that the growth rate and the removal efficiency of *S. obliquus* were higher in Cu metal exposures compared to the type of *S. regularis* with a smaller size.

Heavy metals such as lead (Pb) cause environmental pollution, have no vital function and are toxic even at low concentrations (Wang & Chen, 2009). Studies on Scenedesmus species in the literature have shown that it had a removal efficiency of up to 89% of Pb from aqueous solutions. In this study, it was found to be an average of 43% for both species. However, it has been reported that algal surfaces (mucilage, cell walls, and membranes) having different metal affinities may be responsible for differences in Pb exposure sensitivity (Suresh Kumar *et al*., 2015). It exhibited similar strategies to alleviate Pb metal stress in the studied algae species. While total phenol and carotene levels did not change significantly in both species, they increased total flavonoid levels. A similar strategy was observed in the *S. regularis* for Cu stress. While there was no significant change in the Total phenol level of *S. obliquus*, significant increases had observed in total flavonoid and carotene levels. It is reported that flavonoids

remove metal ions by binding them to hydroxyl groups (Pinto *et al*., 2003). Consistency with our findings with Okamoto *et al.* (2001); Sabatini *et al*., (2009); Suman *et al*., (2015); Hamed *et al*., (2017) who reported a similar strategy in algae which prevented oxidative stress caused by metals using flavonoids.

Microalgae have several mechanisms to defend against the toxicity of heavy metals; the exclusion of metal ions by the cell wall, enhancing antioxidant enzyme activities, producing resistance proteins, increasing pyrenoids, and producing intracellular and extracellular metal ligands (Spain *et al*., 2021; Qiu *et al*., 2022). Organisms have developed various protective mechanisms to eliminate ROS before it can damage their cellular systems. ROS, catalase (CAT), ascorbate peroxidases (AP), glutathione peroxidases (GP), superoxide dismutase (SOD) and glutathione reductases (GR)) also, GSH are removed by compounds such as phenolics, ascorbate, flavonoid, tocopherols, and carotenoids (Nagajyoti *et al*., 2010).

In this study, the SOD levels of both species have been increased. Additionally, a positive correlation was found between Cu concentrations and SOD levels in *S. obliquus*. Results obtained in the present study were associated with the use of copper as a cofactor of enzymes such as SOD, which were involved in the elimination of superoxide radicals, as it can well gain and lose an electron, as stated in the literature (Nagajyoti *et al*., 2010). Nevertheless, enzymatic defense systems such as SOD and CAT can alleviate or eliminate the attack on membrane lipids by taking an active role in converting O_2 and H_2O_2 into less active substances. Pb inhibits the activity at the cellular level by reacting with the sulfhydryl groups of enzymes. High Pb concentration also induces oxidative stress by increasing the ROS production of plants (Reddy *et al*., 2005). While the increases in SOD values for *S. regularis* under Cu stress were observed at insignificant levels, on the contrary, significant increases had been detected under Pb stress. It has been reported in the literature that these cellular responses may differ depending on the algae (Pinto *et al*., 2003).

The absence of a significant correlation between CAT and Pb and Cu concentrations in both species suggested complexity in CAT activity in response to metals. However, CAT activity; has been reported, which can be affected by various factors such as heavy metal concentration, different algae species, exposure time and environmental conditions. In fact, in many studies, it has been reported in the literature that CAT enzyme activities show differences in the direction of decrease or increase due to heavy metals (Zutshi *et al*., 2008; Elbaz *et al*., 2010; Soto *et al*., 2011; Qiu *et al*., 2022).

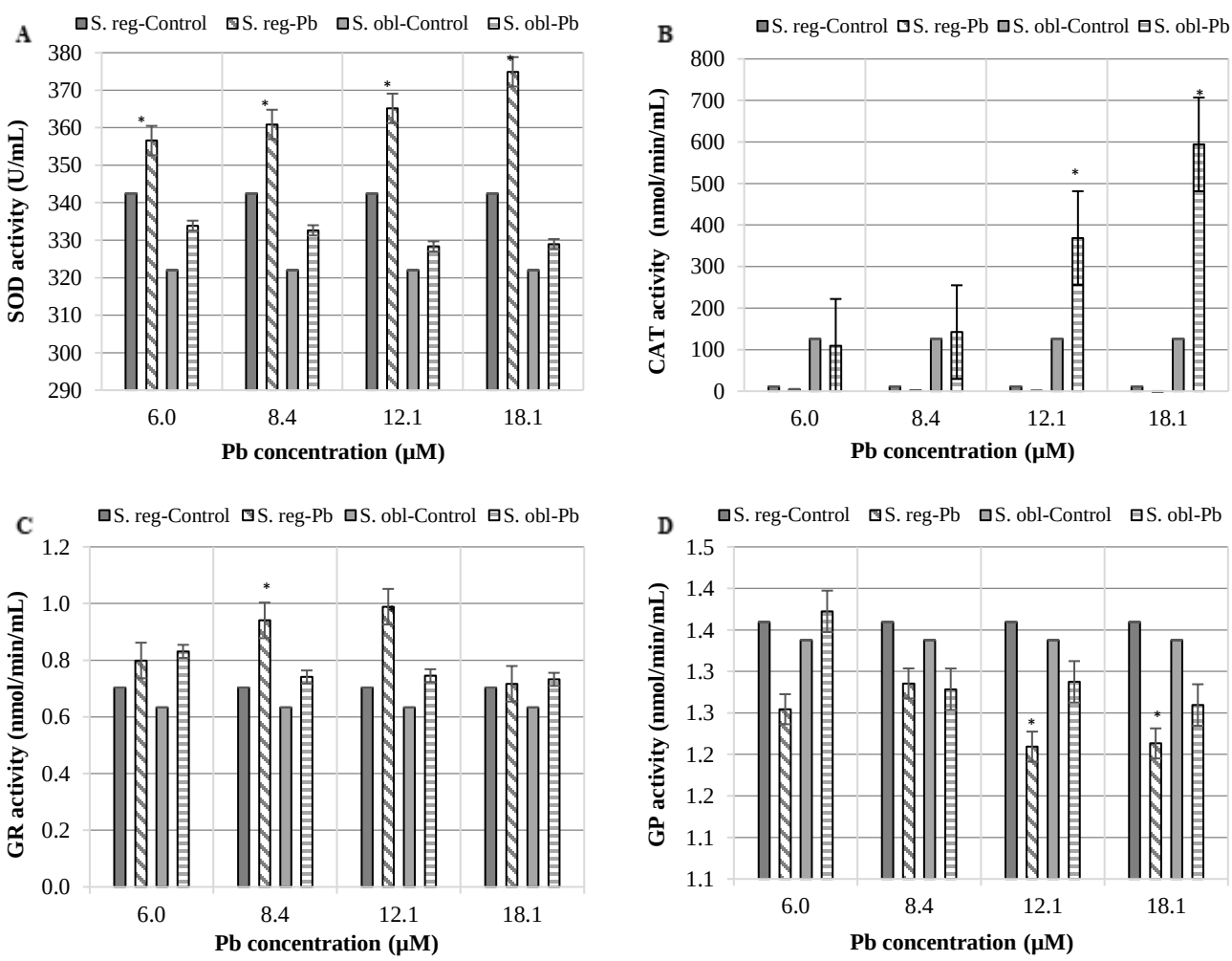

Fig. 7(A, D). Concentration-dependent ROS levels according to all doses of applied metals concentrations. Data are expressed as means ±S.D. Significant differences betwen control and treatments are indicated by asterisks: *p<0.05 (A) Superoxide dismutase, (B) Catalase activity (C) Glutathione reductase (D) Glutathione Peroxidase.

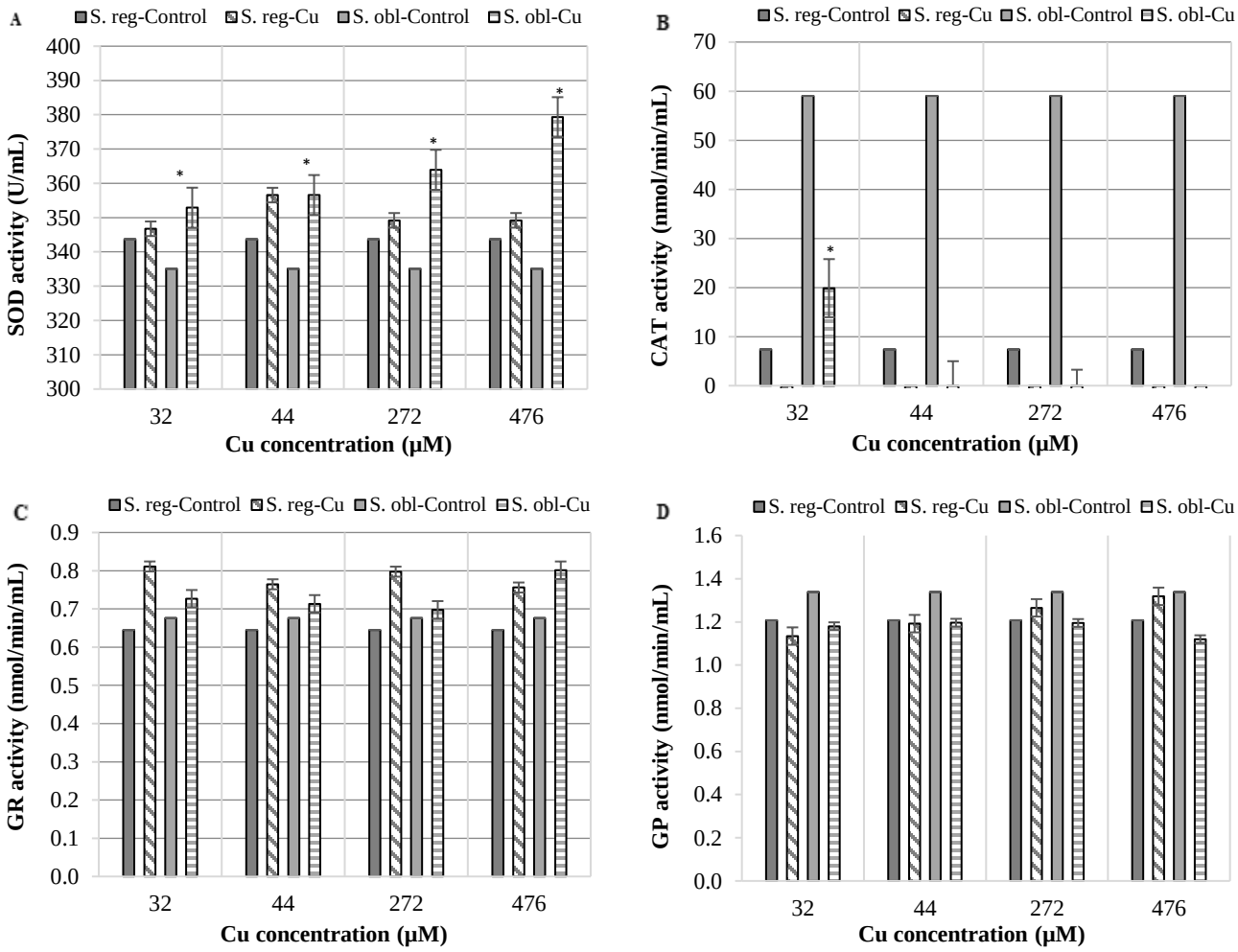

Fig. 8(A, D). Concentration-dependent ROS levels according to all doses of applied metals concentrations. Data are expressed as means ±S.D. Significant differences betwen control and treatments are indicated by asterisks: *p<0.05 (A) Superoxide dismutase, (B) Catalase activity (C) Glutathione reductase (D) Glutathione Peroxidase.

Conclusıon

Our study shows that microalgae cause different stress responses by affecting the biochemical composition of algal cells at different metal concentrations. In addition to sensitivity to diverse metal stress, different defenses were detected in antioxidant protection mechanisms. Moreover, *S. obliquus* exposed to chronic Cu stress for 25 days experienced more Cu metal oxidative stress with lower Cu accumulation and higher antioxidant levels than *S. regularis*. Although there were similar responses to chronic Pb stress, the most robust response was the increase in total flavonoid. *S. obliquus* may be a beneficial device to determine and predict copper contamination in SOD activity and Total antioxidant levels.

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