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Determination of the Effect of Nanoparticle Copper on *Navicula cryptocephala var. veneta* by Biomarkers and Bioaccumulation Amounts

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Abstract

Pollutants such as copper nanoparticles (CuNP), which form residues in the environment, especially in water environments, cause serious damage to the environment and the organisms living in the environment. In this study, *Navicula cryptocephala var. veneta* was selected as a model organism. The EC50 values of CuNP on *Navicula cryptocephala var. veneta* were determined as 4.94 mg\L and 3 different application concentrations were determined considering this ratio. The experiments were carried out in 3 replicates and samples were taken at 24 and 96 hours. At the end of 120 hours, samples were taken to determine the elimination values. CuNP accumulation amounts in the samples were measured by electrothermal atomic absorption spectrophotometer (ETAAS). For biomarker analysis, thiobarbituric acid (TBARS) and glutathione (GSH) levels and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were performed using CAYMAN ELISA kit. According to the study data, as CuNP concentration and application time increased, the amount of accumulation in *Navicula cryptocephala* also increased. Increasing-decreasing changes were observed in elimination. CuNP caused oxidative stress in N. cryptocephala and caused changes in TBARS and GSH levels. In addition, it caused increases in SOD activities and statistically significant decreases ($P<0.05$) in CAT and GPx activities.

Introduction

In recent years, copper and copper-based nanoparticles (CuNPs) have been used for industrial purposes, electrical equipment, construction materials, antimicrobial agents, and alloy formation with other metals. CuNPs are increasingly used in various sectors, including as catalysts in organic synthesis, in sensors for drug delivery (Albrecht et al., 2016), in agriculture and food preservation, and in paint and water purification (Ben-Sasson et al., 2016). Copper is abundant in the Earth's crust (Förstner and Wittmann 2012). It is a ductile and malleable heavy metal with a density greater than 5 g/cm^{-3} and low chemical reactivity. Copper is also an essential enzyme that plays an important role as a co-factor in critical enzyme reactions related to body processes necessary for survival in both humans and animals. In addition to its diverse uses, copper is also involved in enzymatic activities, including lysyl oxidase, tyrosinase, and dopamine hydroxylase, and is associated with the formation of copper chelates and complexes of Cu proteins (Watanabe et al., 1997). It plays an important biological role in oxygen transport. Hemocyanin is a counterpart of hemoglobin for oxygen transport found in molluscs and crustaceans (Malhotra et al., 2020). Therefore, to understand the mechanism of copper toxicity to organisms, it is essential to first understand its dominance as a chemical and its behavior in the environment.

Recently, nanomaterials have attracted great interest due to their versatile properties such as large specific surface area and high reaction activity (Chang et al., 2012). NP-containing wastes discharged from domestic, industrial and medical products are considered as a new biohazard to the environment (Marslin et al., 2017). CuO-NPs are among the most frequently used NPs, mainly used in anti-fouling agents, and used in superconducting material, sensing materials, glass and ceramics (Nations et al., 2011; Chang et al., 2012; Bao Sh et al., 2015). Several studies have shown that the in vitro toxicity of CuO-NPs is much higher compared to other metal oxide NPs and nanotubes (Chang et al., 2012).

As essential primary producers at the lowest trophic level in the food chain, algal populations often serve as model organisms and bioindicators for assessing the potential toxicity of hazardous substances in aquatic ecosystems (Wang et al., 2011). Algae are one of the main components of all aquatic ecosystems and have the capacity to accumulate various heavy metals and NPs (Marslin et al., 2017). The first step of NP accumulation primarily involves the adsorption of NPs onto cell wall functional groups (Nguyen et al., 2020). Positively or negatively charged NPs in the external environment adhere to the negatively charged cell surface through electrostatic interaction and associate with the cell wall (Shankar et al., 2016). Various algae and cyanobacteria hide extracellular substances such as exopolysaccharides and glycoproteins that can mediate the adsorption of uncharged NPs on the negatively charged surfaces of cells. Following adsorption, the cell surface and other extracellular matrices, NPs passively diffuse into the space between the cell wall and the cell membrane through nano-sized pores formed after the cell wall is broken (Nguyen et al., 2020). Some NPs, upon contact with the cell membrane, interact with groups of lipid molecules, resulting in pore formation in the cell membrane where the NPs enter the interior of the cell (Wang et al., 2016).

Oxidative stress represents the general response of organisms to a wide range of abiotic and biotic stresses. The intracellular level and rate of production of ROS typically increase several-fold after exposure of organisms to stimulating environments. Cellular concentrations of different ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen (O_2) and hydroxyl radical (OH^-) are transiently increased in aerobic organisms. Heavy metal-induced oxidative stress in algae and cyanobacteria has been widely recognized in the literature (Mehta and Gaur, 1999; Szivák et al., 2009; Tripathi and Gaur, 2004). Algae and cyanobacteria possess ROS scavenging mechanisms, including antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) (Mahana et al., 2021).

Microalgae are essential for aquatic ecosystems as they are the primary producers of oxygen for the survival of other organisms (Ribeiro et al., 2015; Wang et al., 2016; de Abreu et al., 2022). Microalgae are at the bottom of the food chain in the aquatic environment and their damage can jeopardize the entire biological system due to their role in cleaning the aquatic environment (Abdel-Raouf et al., 2012; Sathasivam et al., 2019). From the growth and physiological responses of algae, the cytotoxicity or functionality of a wide range of heavy metals or nanoparticles can also be screened and tested from an environmental or biotechnological perspective (Hassanpour et al., 2020).

While microalgae are considered suitable for monitoring pollution in the aquatic environment (van der Oost et al., 2003), they are also important because of their sensitive response to pollutant-induced biochemical and physiological changes (Lavado et al., 2006; Aydın and Serdar, 2023). The widespread use of nanoparticles for different applications has popularized their presence in the environment, especially in water. Many studies have been conducted to assess their effects on aquatic organisms. Microalgae are at the base of aquatic trophic chains. These organisms, which can be benthic or pelagic, i.e. interact with all types of particulate material regardless of their density, constitute an interesting model study (Déniel et al., 2019).

The aim of this study was to determine the accumulation and elimination of Cu Np in the microalgae *N. cryptocephala*, as well as to determine the levels of TBARS, GSH and SOD, CAT, GPx activities, which are oxidative stress responses.

Material Method

Cu Np Supply

Cu Np material, CAS-No: 7440-50-8 (25nm particle size) Sigma-Aldrich Co. It was purchased from the company.

Determination of Acute Toxicity (EC50) Value

Pure *N. cryptocephala* (Figure 1) (3.5-4×10^6 cells/ml) purchased from the University of Texas Algae Culture Collection (UTEX) were grown in 250 ml flasks on proteose nutrient medium (sterilized in autoclave) in a growth chamber at 24±1°C with a 12:12 h light:dark cycle (3200 lux) (Ashraf et al., 2011). Commercial fertilizer (Gübretaş 20:20:20:20 (N:P:K)+ Trace Element) was added to the starter culture (generation 0) at a rate of 40 mg/L (Ammar, 2016). *N. cryptocephala* cultures were harvested at logarithmic growth stage (average 1-2×10^6 cells/ml) (Regaldo et al., 2013).

EC50 values The inhibition of microalgae was based on the counting of viable cells. The Microalgae Inhibition Test was performed for 24, 48 and 72 hours as recommended by OECD (2011). At the end of the time, the samples were counted under a light microscope using a hemocytometer (Neubauer). Counts were repeated three times for each sample and average

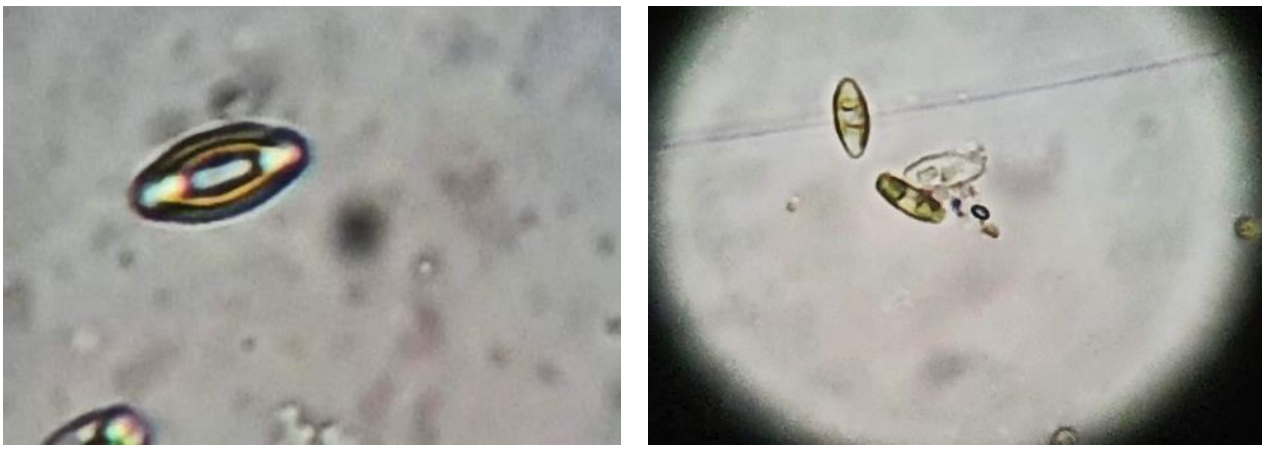

Figure 1. *N. cryptocephala*.

values were calculated (Yoon et al., 2007; Erdem et al., 2014; Özkaleli and Erdem 2017; OECD 2011).

Trial Design

As a result of the acute (EC50) tests, EC50 values of Cu Np pollutants were determined for microalgae. Compared to the EC50 values of Cu Np pollutants,

Group A; Not exposed to any pollutants (control)

Group B; pollutant 1/8 of the pollutant EC50 value,

Group C; pollutant 1/4 of the EC50 value of the pollutant,

Group D; pollutant at 1/2 the pollutant EC50 value,

Cu Np concentrations were determined and microalgae were directly exposed to these pollutants (Figure 2).

Experimental Application

N. cryptocephala var. veneta was applied separately in 3 replicates. A 1 liter sample was taken from the treatment groups every 24 hours for 4 days and precipitated by centrifugation. Ambient water was removed for bioaccumulation, microalgae samples were taken, passed through pure water, labeled for pre-treatment and stored at -18°C. For the detection of dead/living microalgae cells, 1 ml of microalgae Cu Np samples taken from each test vessel at 24, 48, 72 and 96 hours were stained with 0.1 ml of trypan blue stain and incubated in the dark for 10 min. The samples taken for biochemical analyses were dried on blotting paper, then taken into eppendörf tubes with the help of a spatula, labeled and stored in -80°C ultra-freezer for biochemical analyses. After the elimination samples were taken at the 96th hour, the microalgae in the application medium were serially precipitated by centrifugation and the water was removed from the medium. After the microalgae samples were washed with pure water, UV sterilized water was added to them and kept for 24 hours (120^{th} hour) and the elimination samples were preserved in the same way.

Determining the Amount of Accumulation

Samples were taken to determine the amount of accumulation in microalgae exposed to Cu Np pollutants in microalgae and pre-treatments were carried out in accordance with the following protocols. Protocol

Figure 2. Reproduction of *N. Cryptocephala*.

followed to determine the amount of Cu Np heavy metal accumulation in microalgae;

After Cu Nps were applied at three different sublethal concentrations (24, 48, 72, 96 h and elimination (120 h) to *N. cryptocephala* at logarithmic growth stage, 15 ml of each microalgae culture for each replicate was centrifuged at 6000 g for 10 min (Şişman-Aydın et al., 2013) and the algal biomass was filtered using a MF-Millipore filter (0.45 µ). They were then washed three times with 10 ml of 0.001 M EDTA (Ethylene diamine tetraacetic acid disodium salt dihydrate, Sigma Ultra grade, Sigma-Aldrich) solution to remove metals adsorbed on the algal cell walls. After filtering the microalgae, the filters were oven dried at 103°C for 2 hours. In the digestion process, 1 ml of pure nitric acid (70% HNO_3) and 125 µl of hydrogen peroxide (H_2O_2) were added to the samples in the tubes using an automatic pipette (Esmaeili, 2015). The samples dissolved in Ependorf tubes were transferred to high temperature resistant Teflon tubes and the dissolution process was carried out under fume hood. The amount of acid in the Teflon tubes was completed to 2 ml and after the mouths of the tubes were carefully closed, the samples were incinerated in a microwave oven (180-150ºC, 5-15 min). After dissolution, the samples were allowed to cool and the Teflon tubes were washed with ultrapure water and made up to 15 ml. Two ml of the obtained samples were transferred to ependorf tubes. CuNP accumulation amounts were measured by electrothermal atomic absorption spectrophotometer (ETAAS) (PerkinElmer, Inc., Shelton, CT, USA).

Obtaining Homogenates and Supernatants

For the determination of biochemical responses in the samples taken and preserved at 24, 48, 72, 96 and 120 hours in the bioassays conducted within the scope of the study, 0.5 g of sample was weighed and homogenized using a homogenizer with ice by adding 1/10 w/v PBS buffer (phosphate-buffered saline solution) (pH 7.4). These homogenized samples were centrifuged in a refrigerated centrifuge at 17000 rpm for 15 minutes and the supernatants obtained were stored in a deep freezer at -86°C until the measurement procedures were completed. Lipid peroxidation (TBARS) and reduced glutathione (GSH) levels, superoxide dismutase (SOD) enzyme activity, glutathione peroxidase (GSH-Px) enzyme activity, catalase (CAT) enzyme activity were determined by ELISA microplate reader (Figure 3).

TBARS level; Lipid peroxidation is a well-known mechanism of cellular damage in plants and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides derived from polyunsaturated fatty acids are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds such as malondialdehyde (MDA). MDA can be quantified by a controlled reaction with thiobarbituric acid and produces Thiobarbituric Acid Reactive Substances (TBARS). TBARS kit is based on the principle of colorimetric measurement of MDA-TBA adduct formed as a result of the reaction of MDA and TBA under high temperature (90-100°C) and acidic conditions at 530-540 nm. In the study, TBARS levels were measured with Elisa Kits purchased from a commercial company. By creating an absorbance curve with the standards in the kit, TBARS level was calculated by putting the absorbance value in the curve formula (Aydın and Serdar, 2024).

GSH level; GSH sulfhydryl group reacts with DTNB (5,5'-dithio-bis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow colored TNB. The disulfide mixture reduces GSH to recycle and GSTNB (between GSH and TNB), glutathione reductase to produce more TNB. The rate of TNB production is directly proportional to this recycle reaction, which is directly proportional to the GSH concentration in the sample. Measuring the absorbance of TNB at 405 or 412 nm provides an accurate estimate of GSH in the sample. GSH is easily oxidized to the disulfide dimer GSSG. Due to the use of

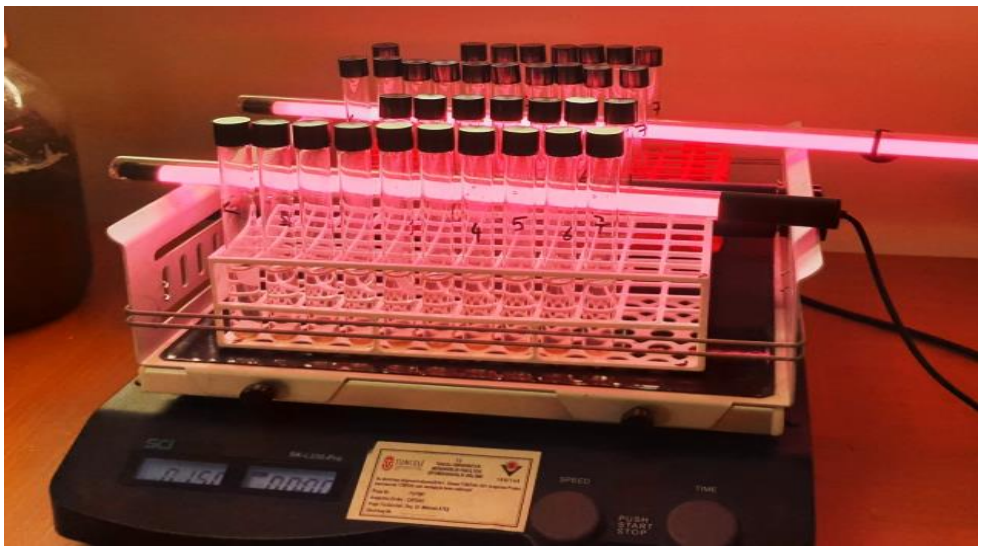

Figure 3. Obtaining samples from *N. Cryptocephala*.

glutathione reductase in the GSH test kit, both GSH and GSSG are measured and the test reflects total glutathione. GSH levels will be measured with Elisa Kits purchased from a commercial company in the study. GSH levels were calculated by creating an absorbance curve with the standards in the kit and downloading the company's calculation formulas from the relevant website (Serdar et al., 2024).

SOD enzyme activity was measured at 450 nm in a microplate reader with a commercial kit. The basis of the method is the elimination of superoxide formed in the medium by the superoxide dismutase (SOD) enzyme and the formation of O_2^- by xanthine with xanthine oxidase (XO), which is based on the remaining amount being colored by staining, and this color intensity is measured spectrophotometrically by forming a colored compound with NBT. In the study, SOD enzyme activities were measured with Elisa Kits purchased from a commercial company. Absorbance curves were created with the standards in the kit, the company's calculation formulas were downloaded from the relevant web page, and SOD activities were calculated.

CAT enzyme activity was measured at 540 nm in a microplate reader with a commercial kit. As a result of the measurements, a standard graph was drawn according to the protocol provided in the kit content and an absorbance curve was created, the company's calculation formulas were downloaded from the relevant web page and CAT enzyme activity values were calculated. The kit we will use uses the peroxidatic function of CAT to determine enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal H_2O_2 concentration. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as a chromogen. 1,2 Purpald forms a bicyclic heterocycle, especially with aldehydes; a change from colorless to purple is observed depending on the oxidation change.

GPx enzyme has a molecular weight of 84000 daltons and contains 4 atoms of selenium per molecule. GPx catalyzes hydrogen peroxide to water in the presence of glutathione. GPx enzyme catalyzes the chain breaking effect in H_2O_2 and lipid peroxidation. GPx enzyme uses reduced GSH as an electron acceptor during the reaction and the formed oxidized glutathione (GSSG) is regenerated by the NADPH dependent GSH-Rd enzyme). GPx enzyme activity in the obtained supernatant samples was measured in a microplate reader at 340 nm using a commercial kit. The kit used indirectly measures GPx activity by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced as a result of the reduction of an organic hydroperoxide by GPx, is converted back to its reduced state by GR and NADPH. The oxidation of NADPH to NADP+ triggers a decrease in absorbance at 340 nm. The decrease rate in A340 (6 minutes at 1-minute intervals) is calculated as directly proportional to the GPx activity in the sample. The calculation formulas of the company from which the kit was purchased were downloaded from the relevant web page and GPx enzyme activity values were calculated.

Statistical Analysis

SPSS 24.0 package program one-way ANOVA (Duncan 0.05) was used to evaluate biochemical analyses.

Results

EC50 Values

According to the results of probit analysis, the acute toxicity of Cu Np heavy metal on *N. cryptocephala* (Table 1) was calculated by probit analysis.

Cu Np Heavy Metal Bioaccumulation in *N. cryptocephala*

Within the scope of the study, bioaccumulation amounts were determined in *N. cryptocephala* organisms exposed to Cu Np heavy metal pollutant for 24, 48, 72, 96 and 120 hours. As the concentration and duration of treatment increased, the amount of Cu Np heavy metal bioaccumulation in microalgae increased (Figure 4). Decreases in bioaccumulation amounts were recorded in the elimination group.

Biomarker Responses

Within the scope of the study, in order to determine the TBARS level values of CuNP heavy metal on *N. cryptocephala* microalgae, TBARS level (Figure 5a), GSH level (Figure 5b), SOD activity (Figure 5c), CAT activity (Figure 5d) and GPX activity (Figure 5e) were measured by taking samples from three different concentration groups and one control group every 24 hours.

TBARS level increased in the groups exposed to Cu Np compared to the control and a statistically significant difference was found at 24, 72 and 96 hours ($P>0.05$). In different time periods of the same groups, the groups at 72 hours were statistically different from the other groups (24, 48, 96 and 120 hours) ($P<0.05$).

A statistically significant difference was found between the Cu Np concentration group samples and the control group samples in all groups of GSH level values (72, 96 and 120 hours) ($P<0.05$). The difference between the changes between the other groups was not statistically significant ($P>0.05$). In different time periods of the same groups, the groups at 72 hours were statistically different from the other groups (24, 48, 96 and 120 hours) ($P<0.05$).

SOD enzyme activity was statistically significantly different in different concentration application groups compared to the control ($P<0.05$). In different time periods of the same groups, the groups at 72 hours were

Table 1. EC50 values of CuNP heavy metal on *N. cryptocephala* calculated by probit analysis

N. cryptocephala	EC50mg/l
Repetition 1	4,87
Repetition 2	5,01
Repetition 3	4,87
Average value	4,94
Standard deviation	0,10

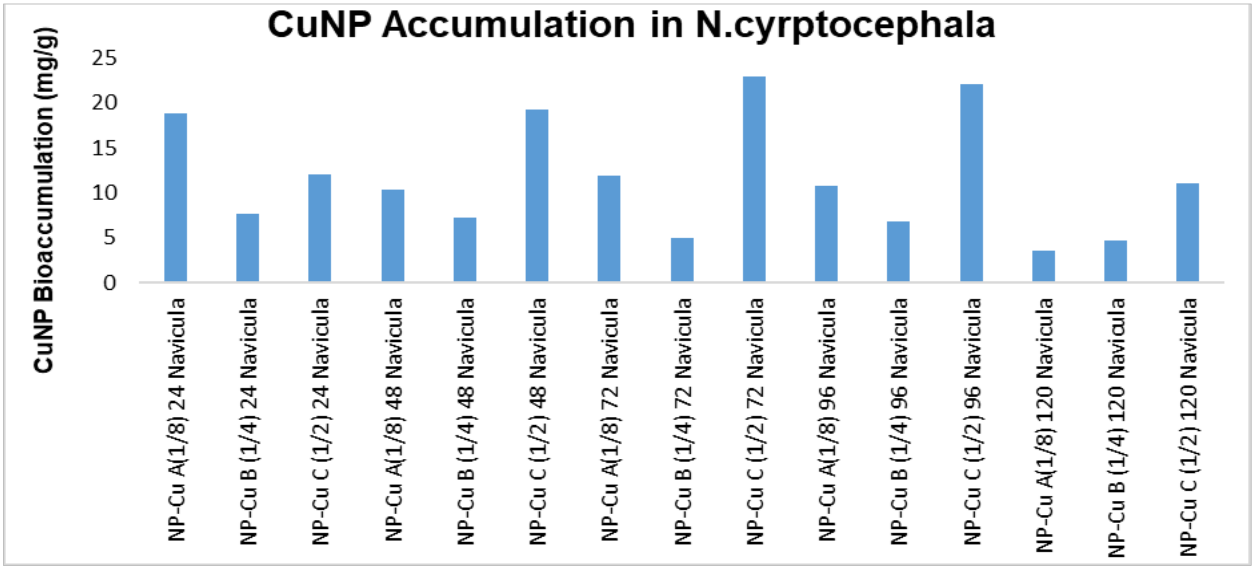

Figure 4. Cu Np heavy metal bioaccumulation amounts in *N. cryptocephala* (mg/g).

statistically different from the other groups (24, 48, 96 and 120 hours) ($P<0.05$).

CAT enzyme activity was found to be statistically significantly different ($P<0.05$) between the Cu Np heavy metal exposure samples in all treatment groups except the elimination (120th hour) group and the control group samples. In different time periods of the same groups, the groups at 120th hour were statistically different from the other groups (24, 48, 72 and 96th hours) ($P<0.05$).

The changes in GPx enzyme activity between all Cu Np heavy metal exposure samples and control group samples were statistically significant ($P<0.05$).

Discussion

A comprehensive study of the behavior of NPs in the aquatic environment is essential to assess their potential toxic effects on aquatic organisms. There are many studies examining various aspects of Cu Np impact on various aquatic organisms. Janova et al., 2021, in their study, examined Cu Np toxicity in *Chlamydomonas reinhardtii* species and confirmed inhibition of growth, reduction of chlorophyll levels in cells, cell penetration and increased ROS production. Fazelian et al., 2019 examined the toxic effects of CuO-Np on *Nannochloropsis oculata* and reported that CuO-Np significantly reduced cell growth in *N. oculata* cells. Perreault et al., 2012, examined the effect of polymer coating on CuO NP toxicity in the green alga *C.reinhardtii* by comparing bare and polymer coated CuO NPs prepared from the same CuO nanoparticle and reported that CuO NPs were toxic in *C. reinhardtii* in all cases. Solomonova et al., 2023 observed a 30% decrease in cell numbers of Cu Np in *Prorocentrum cordatum* and *Dunaliella salina* species in both species. Wan et al. 2018, reported that copper sulfate ($CuSO_4$) and copper oxide nanoparticles (CuO Np) caused toxic effects and inhibited cell growth in *Chlorella* sp. Xia et al. (2015) reported that NP TiO_2 significantly inhibited the growth rate of microalgae *Nitzschia closterium*. Solomonova et al. (2023) examined the changes of CuO-Np in different algal species (*D. salina, Isochrysis galbana, Thalassiosira weissflogii* and *Prorocentrum cordatum*) and reported that Cu-NP has an effect on almost every aspect of algal function. Hurtado-Gallego et al. (2020), in their study, stated that superparamagnetic iron oxide nanoparticles had a toxic effect on *C. reinhardtii* and changed ROS values. Johari et al. (2018), in their study, stated that citrate-coated silver nanoparticles had a toxic effect on *D. salina* and inhibited growth. Bibi et al. (2021), in their study, stated that while lipid content increased, fatty acid content decreased and algal growth was inhibited in the model organism *C. vulgaris* with the effect of α-Fe_2O_3 NP. Tayemeh et al. (2020), reported that silver nanoparticles (AgNPs) and silver nitrate ($AgNO_3$) produced toxic effects in *C. vulgaris*. Wan et al. (2021), examined the combined toxicity of nanoplastics (NPs)

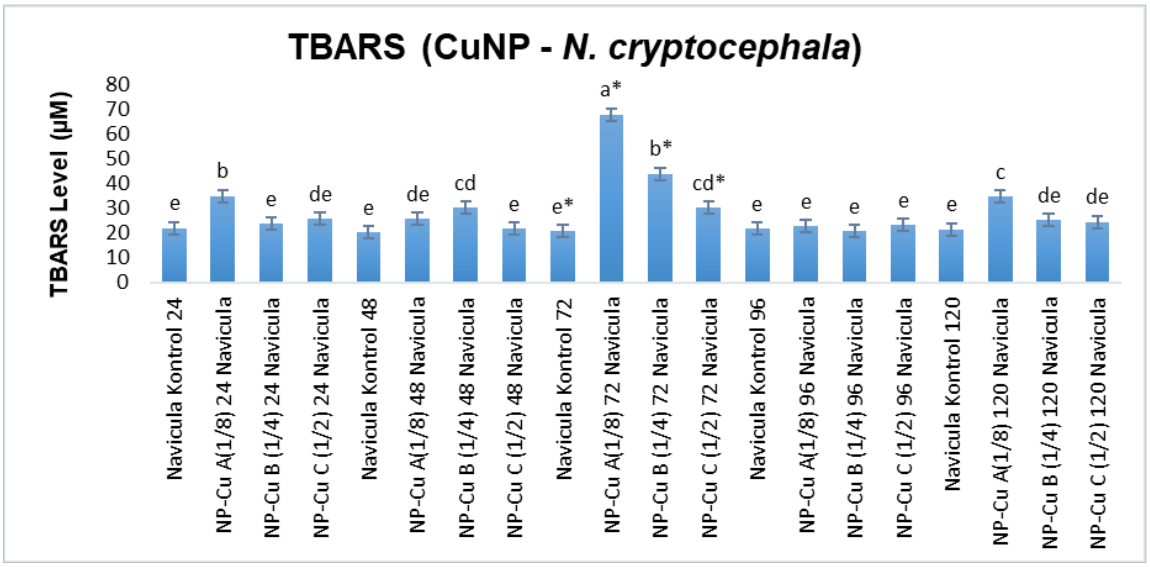

Figure 5a. TBARS level values of Cu Np heavy metal on *N. Cryptocephala*. There are differences at P<0.05 level between the data shown with different letters on the column within the same time group.*The statistical difference in different time periods of the same groups is indicated with "*" (asterisk).

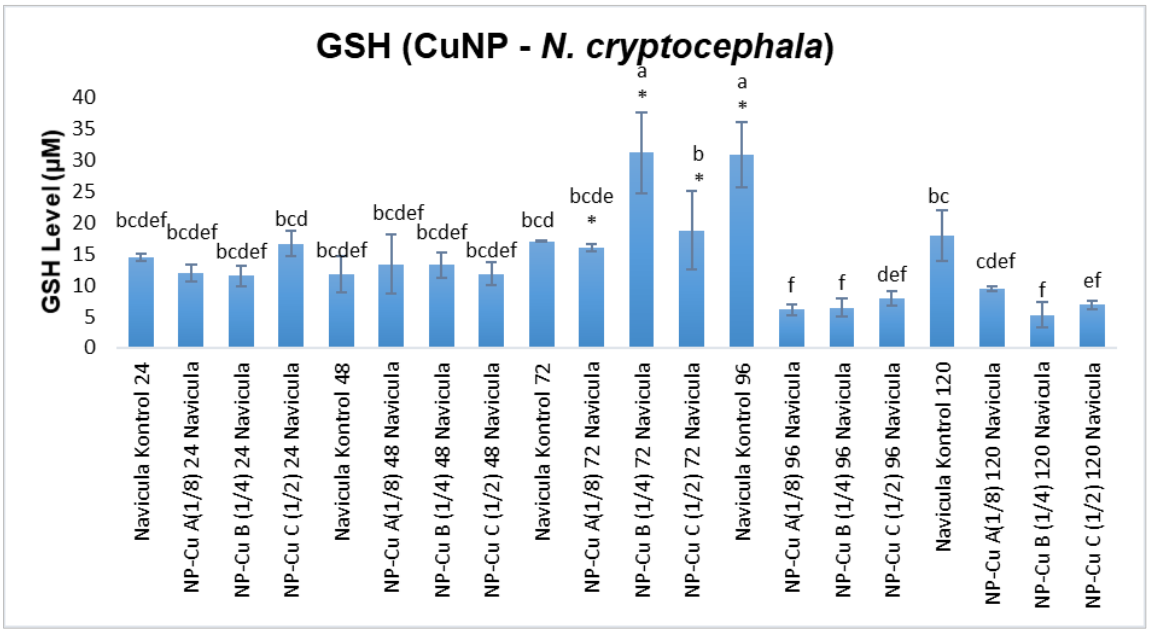

Figure 5b. GSH level values of Cu Np heavy metal on *N. Cryptocephala*. There are differences at P<0.05 level between the data shown with different letters on the column within the same time group. *The statistical difference of the same groups in different time periods is indicated with "*" (asterisk).

and Cu in *Chlorella* sp and *Pseudokirchneriella subcapitata* and reported that they caused high oxidative stress and morphological and structural changes in both microalgae. Fathi et al. (2020), examined the effect of copper oxide nanoparticles on Chlorella algae in the presence of humic acid and reported that high concentrations of these compounds increased the amount of intracellular ROS and reduced the growth rate of Chlorella algae.

When CuNPs penetrate the organism, they accumulate in the cells. It is thought that the amount of accumulation in *N. cryptocephala* is due to the cell structure of the organism, exposure time and concentration of the pollutant and this idea is supported by the literature review. Li et al. (2016), in their study, stated that copper accumulation increased as the copper concentration increased as a result of Cu Np exposure in *Scenedesmus obliquus*. Regier et al. (2015), in their study, stated that accumulation occurred in *Elodea nuttallii* species as a result of Cu NP exposure. Yang et al. (2022), in their study, stated that Cu accumulation occurred in *Procambarus clarkii* with Cu Np effect.

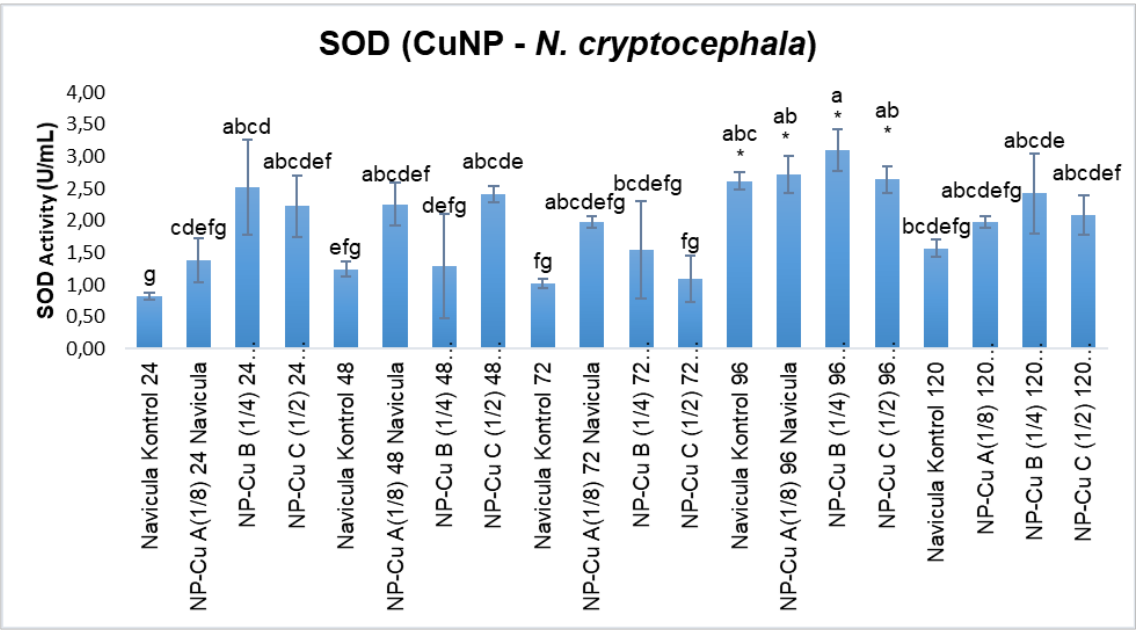

Figure 5c. SOD enzyme activity values of Cu Np heavy metal on *N. Cryptocephala*. There are differences at P<0.05 level between the data shown with different letters on the column within the same time group. *The statistical difference of the same groups in different time periods is indicated with "*" (asterisk).

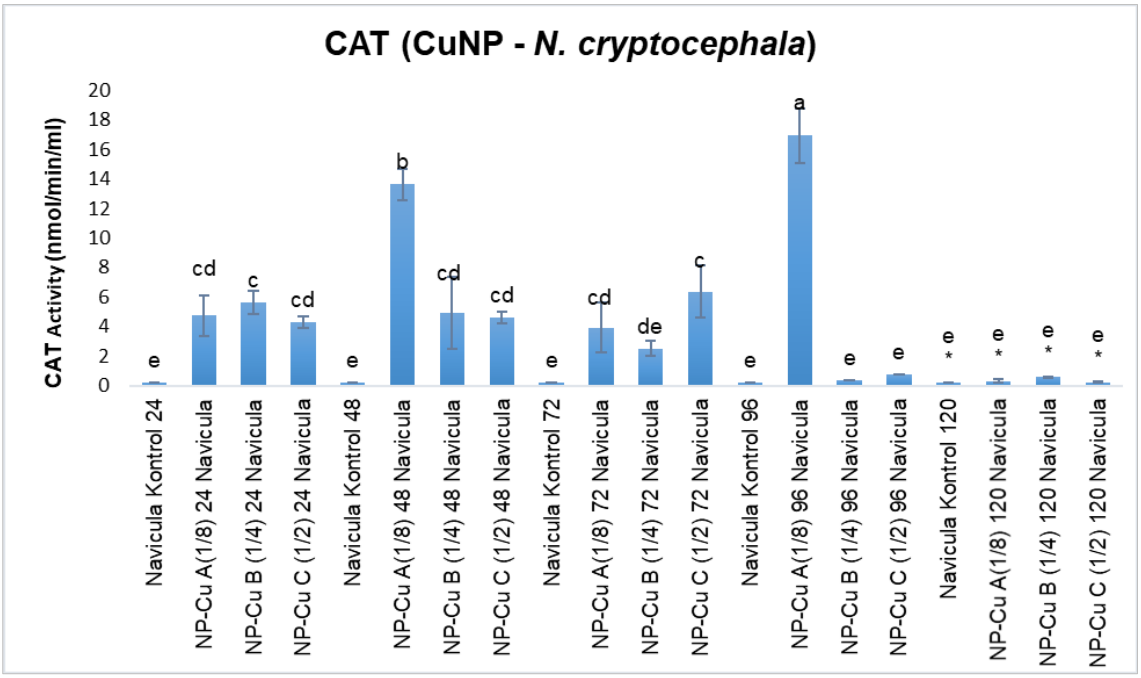

Figure 5d. CAT enzyme activity values of Cu Np heavy metal on *N. Cryptocephala*. There are differences at P<0.05 level between the data shown with different letters on the column within the same time group. *The statistical difference in different time periods of the same groups is indicated with "*" (asterisk).

A common consequence of the harmful effects of ROS is damage to membrane lipids. Since TBARS is the end product of lipid peroxidation, measurement of TBARS is a powerful index of oxidative stress leading to cell death (Ates et al., 2015; Marslin et al., 2017). Increased TBARS content and induction of oxidative stress have been reported in Cu Nps. Melegari et al. (2013), they stated that there were changes in TBARS level in *Chlamydomonas reinhardtii* with the effect of CuO Np. Suman et al. (2015), they examined the effect of ZnO NPs on the marine microalga *Chlorella vulgaris* and stated that there were increases in TBARS levels due to ZnO NPs. Yao et al. (2012), they stated that changes occurred in TBARS levels in *C. vulgaris* with the effect of nano-Fe_3O_4. Xia et al. (2015), in their study examining the effect of TiO2 particles on *Nitzschia closterium*, they

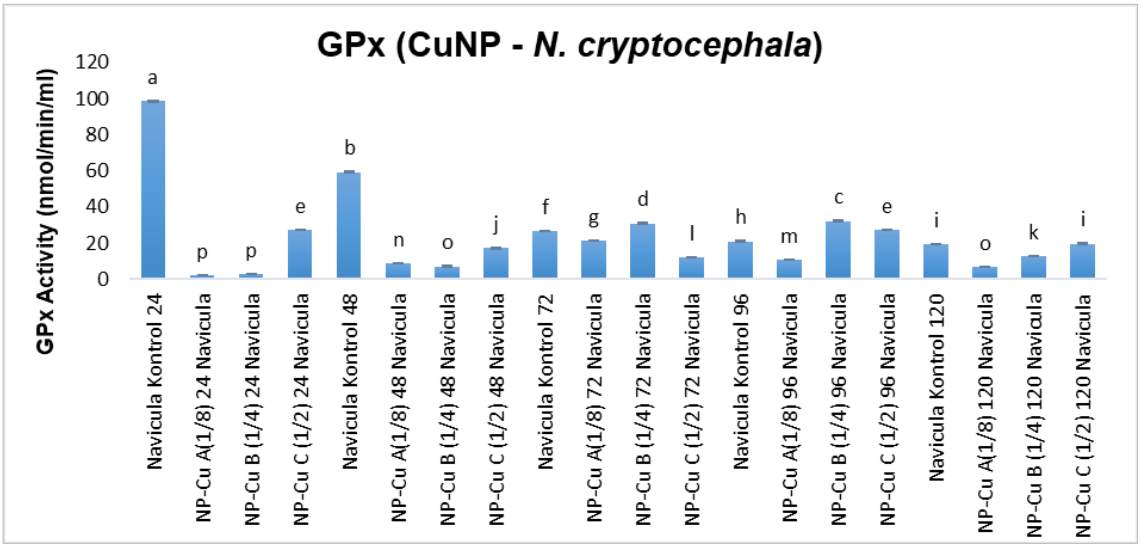

Figure 5e. GPx enzyme activity values of Cu Np heavy metal on *N. Cryptocephala*. There are differences at P<0.05 level between the data shown with different letters on the column within the same time group. *The statistical difference in different time periods of the same groups is indicated with "*" (asterisk).

stated that there were increases in MDA levels. Zhao et al. 2021, they stated that silver nanoparticles (Ag-NPs) caused increases in MDA levels in *C. reinhardtii*. Komazec et al. 2023, they observed that there were increases in MDA levels in *C. vulgaris* with the effect of AgNP. Liu et al. 2018 study, investigated the toxicity of titanium dioxide (TiO_2), silicon dioxide (SiO_2), (ZrO_2) NPs to *Scenedesmus obliquus* separately and in double and triple combinations, and observed increases in MDA levels. Dinç et al. 2022, stated that elenium nanoparticles (SeNPs) caused changes in MDA levels in *C. vulgaris*. Bahador et al. 2019, stated that MDA contents increased in *Dunaliella saline* (AgNP) and with the effect of salicylic acid (SA). Lei et al., in their 2016 study, stated that iron-based nanoparticles caused increases in MDA levels in *Chlorella pyrenoidosa*. Oukarroum et al. 2012 study, observed increases in TBARS levels in *C. vulgaris* and *Dunaliella tertiolecta* species under the influence of AgNP. Li et al. 2015, reported increases in MDA levels as a result of nano-TiO_2 application in *Karenia brevis* and *Skeletonema costatum* species. Xin et al. 2020, changes in TBARS levels were observed with the effect of P25, nano-ZnO and triclosan on *Asterococcus superbus*. Fazelian et al. 2019, in their research, stated that there were increases in MDA levels in *N. oculata* with the effect of CuO-NP. Liv et al. 2016, stated that the MDA content of *S. obliquus* increased with increasing copper concentration. Fu et al. 2019, examined the effect of microplastic polyvinyl chloride and Cu individually and in combination and observed increases in TBARS levels in single applications. Xia et al. 2015, reported increases in microalgae *N. closterium* TBARS levels in NP TiO_2 exposure. Franzitta et al. 2020, reported an increase in TBARS levels with increasing concentration as a result of CuO Np exposure in the model organism *Pheodactylum tricornutum.* Zhu et al. 2017, reported that CuNps and $CuSO_4$ caused increases in MDA levels in *P. tricornutum* species. Shi et al. 2021, reported that Cu and humic acids (HA) increased MDA levels in the microalgae *C. vulgaris*. The increases in TBARS levels of CuNP heavy metal in *N. cryptocephala* were in parallel with the literature studies and the results were supported by the literature. It was determined that the changes in TBARS levels in the elimination amounts occurring at 120th hour in *N. cryptocephala* were statistically insignificant (P>0.05).

GSH is an antioxidant and important free radical scavenger. Algae can respond to heavy metal stress by increasing GSH concentration. *N. cryptocephala* is thought to increase GSH levels to counter Cu Np stress. GSH acts as a proton donor and reduces the disulfide form and oxidized glutathione (GSSG) (Ma et al., 2013). Suman et al. 2015, they examined the effect of ZnO NPs on the marine microalga *C. vulgaris* and stated that there were increases in GSH levels due to ZnO NPs. Yao et al. 2012, they stated that changes occurred in GSH levels in *C. vulgaris* with the effect of nano-Fe_3O_4. Komazec et al. 2023, they observed that there were decreases in GSH levels in *C. vulgaris* with the effect of AgNP. Thakkar et al. 2016, reported that there were decreases in GSH levels in *D. tertiolecta* with the effect of carbon nanotubes. Janani et al. 2020, reported that there were decreases in GSH levels in *Pseudomonas aeruginosa* and *Staphylococcus aureus*, *Daphnia magna* species with the effect of CuO NPs. Li et al. 2016, reported that GSH concentrations gradually increased in both copper and copper/nano-Al_2O_3 systems with increasing copper concentration in *S. obliquus*. Çimen et al. 2020, stated that *A. salina* exposed to Cu and CuO NP exposure showed increases in GSH levels. In *N. cryptocephala*, statistically significant (P<0.05) decreases in GSH level occurred during 120 hours (elimination). While significant increases occurred in the elimination of SOD activities, CAT activities were

determined to be close to the control in the elimination amounts. Significant decreases were observed in the elimination amounts of GPx activities at 120th hour.

SOD is an enzymatic antioxidant that can scavenge reactive oxygen species (ROS) produced by heavy metal stress (Li et al., 2016). Li et al. 2016, reported that exposure of *S. obliquus* to 1.0 mg/L nano-Al_2O_3 did not cause any change in SOD activity, but with increasing copper concentration, SOD activity initially increased at a low concentration and then decreased with or without nano-Al_2O_3. Suman et al. 2015, they examined the effect of ZnO NPs on the marine microalga *C. vulgaris* and stated that there were increases in SOD aktivity due to ZnO NPs. Yao et al. 2012, they stated that changes occurred in SOD aktivity in *C. vulgaris* with the effect of nano-Fe_3O_4. Xia et al. 2015, in their study examining the effect of TiO_2 particles on *Nitzschia closterium*, they stated that there were changes in SOD activities. Zhao et al. 2021, they stated that silver nanoparticles (Ag-NPs) caused increases in SOD aktivity in *C. reinhardtii*. Komazec et al. 2023, they observed that there were increases in SOD aktivity in *C. vulgaris* with the effect of AgNP. Liu et al. 2018, study investigated the toxicity of TiO_2, SiO_2, ZrO_2 NPs to *S. obliquus* separately and in double and triple combinations, and observed significant increases in SOD activities. Wang et al. 2016, stated that the effect of nTiO_2 caused increases in SOD activities on *Phaeodactylum tricornutum*. Li et al. 2015, reported changes in SOD activity as a result of nano-TiO_2 application in Karenia brevis and *Skeletonema costatum* species. Xin et al., 2020 observed the effects of P25, nano-ZnO and triclosan on SOD activities on *Asterococcus superbus*. Morelli et al. 2018, investigated the effect of TiO_2 NPs on *Dunaliella tertiolecta* and observed increases in SOD activities. Das et al. 2022, reported increases in SOD activities in *Scenedesmus obliquus* with the effect of nTiO_2. Regier et al. 2015, in their study, stated that decreasing and increasing fluctuations in SOD activities were observed in *Elodea nuttallii* species with Cu Np effect. Che et al. 2018, *Chlorella* sp. and *Scenedesmus* sp. stated that there were increases in SOD activity in both species with CuO Np effect. Fu et al. 2019, examined the effect of microplastic polyvinyl chloride and Cu individually and in combination and observed increases in SOD activities in both applications. Xia et al. 2015, observed that SOD activities in the microalgae *N. closterium* were first induced and then inhibited following exposure to TiO_2 Np. Franzitta et al. 2020, reported that SOD activities increased with increasing concentration as a result of CuO Np exposure in the model organism *P. tricornutum*. Zhu et al. 2017, reported that Cu Nps and $CuSO_4$ caused increases in SOD activities in *P. tricornutum* species. Shi et al. 2021, reported that Cu and HA increased the SOD activities of the microalgae *C. vulgaris*. Pradhan et al. 2015, in their study, stated that increases in SOD activities occurred in 5 water mushroom isolates with CuO Np effect. Braz-Mota et al. 2018, in their study, stated that decreasing and increasing fluctuations in SOD activities occurred in *A. agassizii* and *P. axelrodi* species exposed to CuO Np exposure. Kumar et al. 2023, reported that Cu and Cu Nps increased SOD activities in *P. hypophthalmus*. Fan et al. 2012, reported increases in SOD activities in *D. magna* with Cu exposure. Serdar et al. 2024, observed changes in SOD activities in *G. pulex* with the effect of NP γ-Al_2O_3 and α-Al_2O_3 in their study. It is thought that the increases in SOD activity of *N. cryptocephala* as a result of Cu Np exposure are related to the concentration and application time and that the cell increases SOD activity to protect itself. Statistically significant ($P<0.05$) increases in SOD activity were observed in *N. cryptocephala* at 120 hours (elimination).

CAT are hematin-containing enzymes that facilitate the removal of hydrogen peroxide (H_2O_2) metabolized to molecular oxygen (O_2) and water (Unfried et al., 2007). Since the increase in enzyme activities is probably due to the increase in ROS, it is thought that the increase in CAT activity in *N. cryptocephala* by Cu Np effect is due to the increase in ROS. Fazelian et al. 2019, reported increases in CAT activities in *N. oculata* with CuO-NP effect in their research. Xia et al. 2015, observed that NP TiO_2 exposure first induced and then inhibited CAT activities in the microalgae *N. Closterium* after exposure. Franzitta et al. 2020, stated that CAT activities increased with increasing concentration as a result of CuO Np exposure in the model organism *P. tricornutum*. Melegari et al. 2013, they stated that there were changes in CAT activities in *Chlamydomonas reinhardtii* with the effect of CuO Np. Xia et al. 2015, in their study examining the effect of TiO_2 particles on *Nitzschia closterium*, they stated that there were changes in CAT activities. Komazec et al. 2023, they observed that there were increases in CAT aktivity in *C. vulgaris* with the effect of AgNP., Liu et al. 2018 study investigated the toxicity of TiO_2, SiO_2, ZrO_2 NPs to *S. obliquus* separately and in double and triple combinations, and observed significant increases in CAT activities. Bahador et al. 2019, stated that CAT activities increased in *D. saline* (AgNP) and with the effect of salicylic acid (SA). Li et al. 2015, reported changes in CAT activity as a result of nano-TiO_2 application in *Karenia brevis* and *Skeletonema costatum* species. Xin et al. 2020, observed the effects of P25, nano-ZnO and triclosan on CAT activities on *Asterococcus superbus* Morelli et al. 2018, investigated the effect of TiO_2 NPs on *D. tertiolecta* and observed increases in CAT activities. Das et al. 2022, reported increases in CAT activities in *Scenedesmus obliquus* with the effect of nTiO_2. Braz-Mota et al. 2018, stated in their study that increases in CAT activities occurred in *A. agassizii* and *P. axelrodi* species, which were exposed to CuO Nps. Fan et al. 2012, reported that there were changes in CAT activities with Cu exposure in *D. magna*. It was determined that CAT activity levels were close to control in *N. cryptocephala* during the 120-hour (elimination) period. Statistically significant decreases ($P<0.05$) were observed in the elimination amounts of GPx activities at the 120th hour.

GPx catalyzes the metabolism of HO to water, which involves the simultaneous oxidation of reduced GSH to its oxidized form (GSSG) (Lesser 2006). Abdel-Latif et al. 2021, stated in their study that there were significant increases in GPx activities in *O. niloticus* species with the effect of CuONP. Pradhan et al. 2015, they stated that increases in GPx activities occurred in 5 aquatic fungus isolates with the effect of CuO NPs. Gopi et al. 2019 stated that there were changes in GPx activities in *O. niloticus* due to the effect of Cu. Dawood et al. 2020, stated in their study that there were increases in GPx activities in carp fish with the effect of Cu Np. Srikanth et al. 2016, stated that there were increases in GPx activities in *Chinook salmon* with the effect of CuNP. Tunçsoy and Erdem 2018 study, observed that there were increases in the GPx activities of *O. niloticus* with the effect of $CuSO_4$ and Cu Np. Srikanth and Nutalapati, 2022 study, stated that $CuFe_2O_4$ NPs caused significant increases in GPx activities by causing oxidative stress in *Channel catfish*. Tunçsoy et al. 2017, reported in their study that there were increases in GPx activities in *O. niloticus* with the effect of CuNP. It was observed that the decreases in GPx activity in *N. cryptocephala* during the 120 hour (elimination) period were statistically significant ($P<0.05$). It is thought that the decreases in GPx activity in *N. cryptocephala* are due to the cell structure of the organism, duration of exposure to the pollutant and concentration.

Conclusion

The production and application of large amounts of NPs results in the unintentional discharge of NPs into the environment, and most environmental NPs enter the aquatic environment through winds, precipitation, and surface runoff, posing a potential threat to organisms and ecosystems (Fan et al., 2018; Hanna et al., 2013; 2018; Zha et al., 2022). Cu and other NPs mixed into the water environment *N. cryptocephala*. It is thought that affecting microalgae will negatively affect the oxygen level of the water environment, and considering that algae constitute the first step of the food chain, it will affect higher level organisms. It was also determined in our study that Cu Nps accumulate in *N. cryptocephala* and affect the vital activities of the organism, causing oxidative stress in the organism and causing changes in TBARS, GSH levels and SOD, CAT and GPx activities, and the cell tries to protect itself.

Ethical Statement

All authors declare that there is no ethical violation in this manuscript. Also, this manuscript does not contain data belonging to others. The authors declare that they have no conflict of interest. The authors alone are responsible for the content and authoring of the present paper.

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Author Contribution

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Conflict of Interest

There is no conflict of interest between the authors.

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