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RESEARCH ARTICLE

Single and combined toxic effects of nCu and $nSiO_2$ on *Dunaliella salina*

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Abstract

There are many studies on the toxic effects of single nanoparticles on microalgae; however, many types of nanoparticles are present in the ocean, and more studies on the combined toxic effects of multiple nanoparticles on microalgae are needed. The single and combined toxic effects of nCu and $nSiO_2$ on *Dunaliella salina* were investigated through changes in instantaneous fluorescence rate (Ft) and antioxidant parameters during 96-h growth inhibition tests. It was found that the toxic effect of nCu on *D. salina* was greater than that of $nSiO_2$, and both showed time and were dose-dependent with the greatest growth inhibition at 96 h. A total of 0.5 mg/L nCu somewhat promoted the growth of microalgae, but 4.5 and 5.5 mg/L nCu showed negative growth effects on microalgae. The Ft of *D. salina* was also inhibited by increasing concentrations of nanoparticles and exposure time. nCu suppressed the synthesis of TP and elevated the MDA content of *D. salina*, which indicated the lipid peroxidation of algal cells. The activities of SOD and CAT showed a trend of increasing and then decreasing with the increase of nCu concentration, suggesting that the enzyme activity first increased and then decreased. The toxic effect of a high concentration of nCu was reduced after the addition of $nSiO_2$. SEM and EDS images showed that $nSiO_2$ could adsorb nCu in seawater. $nSiO_2$ also adsorbed Cu^{2+} in the cultures, thus reducing the toxic effect of nCu on *D. salina* to a certain extent. TEM image was used to observe the morphology of algal cells exposed to nCu.

Keywords *Dunaliella salina* · Cu nanoparticles · SiO_2 nanoparticles · Toxic effect · Antioxidant · Marine pollution

Introduction

Nanoparticles are particles with a size between 1 and 100 nm and are used in daily life, food safety, agriculture, and medicine due to their superior physical, chemical, and mechanical properties (Niknam et al. 2022; Saraswat et al. 2023). While nanotechnology brings us great benefits, it also leads to some environmental problems because of its universality and non-degradability (Bhuyar et al. 2020). Nanomaterials are discharged into the ocean through sewage discharge, causing harm to some marine organisms (Pourebrahimi and Pirooz 2023).

Microalgae are primary producers and the essential link in the food chain of marine organisms (Ning et al. 2022). Because of its short growth cycle, easy culture, and sensitivity to poisons, microalgae are widely used to explore the toxicity of nanoparticles and conduct ecological assessments (Chen et al. 2012). *D. salina* is a salt-tolerant unicellular eukaryote that can adapt to NaCl concentrations from 0.05 to 5.5 M (Einspahr et al. 1988). Without a cell wall, *D. salina* possesses a resilient cell membrane that changes in size and morphology depending on the salinity of the environment (Monte et al. 2020). Nanoparticles can be adsorbed on the surface of algal cells, reduce photosynthesis, and cause physical damage to the microalgae (Ghazaei and Shariati 2020). Some metal nanoparticles release toxic metal ions in seawater, which also inhibit the growth of microalgae (Sun et al. 2017). The attack of nanoparticles can lead to the increase of oxygen free radicals and ROS content in microalgae cells and trigger oxidative stress (Wang et al. 2022a).

nCu, as a superplastic and ductile pure material with more chemical activity than ordinary copper, has a wide range of applications in catalysts and alloy engineering

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(Eid et al. 2019). The concentration of ocean-engineered nanoparticles was expected to be between 100 pg/L and 10 ng/L (Sun et al. 2017; Zhao et al. 2021) and even reached 1–10 mg/L (Gottschalk et al. 2013). Zhang et al. (2022) found that low concentration of Cu^{2+} could improve the antioxidant reaction and immune response of *Nile tilapia*, but high concentration of Cu^{2+} reduces the antioxidant reaction. In order to reduce the environmental damage caused by nCu due to the release of Cu^{2+}, Z. Wang et al. (2019) prepared $nCuAl_2O_4$, which greatly reduced the leaching rate of Cu^{2+}; however, the number of *Escherichia coli* in the medium is greatly reduced by adding such nanoparticles, demonstrating that nCu also has antibacterial activity. Janova et al. (2021) found that nCu showed extreme toxicity on *Chlamydomonas reinhardtii* by decreasing chlorophyll content and increasing the ROS level of the algae as the concentration of nCu was higher than 25 mg/L. $nSiO_2$, widely used in anti-aging and anti-UV fields, is more easily transferred to the ocean because it is an important component of quartz sand (Wang et al. 2022a). The global $nSiO_2$ market size is projected to reach approximately US$ 5.14B by 2025 (Ahamed et al. 2021). Sousa et al. (2019) found that the toxicity of $nSiO_2$ on freshwater algae was caused by the nanoparticles themselves through reducing photosynthetic efficiency and leading to the accumulation of ROS to inhibit the algae growth. Fujiwara et al. (2008) found that $nSiO_2$ with a small particle size had a large toxic effect on *Chlorella kessleri*.

The combined toxicity may be higher or lower than that of a single nanoparticle when microalgae are exposed to two or more types of nanoparticles, which is called the synergistic or antagonistic effect. However, most of the previous studies on the toxic effects of nanoparticles on marine organisms were confined to single nanoparticles. The amount of nCu in the ocean is increasing with the widespread use of nCu in the field of sterilization, so the toxic effect of nCu on microalgae cannot be underestimated. To our knowledge, there are limited studies on the toxic effects of $nSiO_2$ on microalgae, the investigation of combined toxic effects of $nSiO_2$ and nCu on marine algae is limited, especially for *D. salina*. Therefore, the combined toxic effects of $nSiO_2$ and nCu on *D. salina* should be explored. These results will provide the foundation for the complex toxic effects of multiple nanomaterials on marine microalgae.

In this study, nCu and $nSiO_2$ were selected as two types of nanoparticles to explore their toxic effects on *D. salina*. It was hypothesized that the combined toxic effects of two types of nanoparticles are not a simple superposition of single toxic effects. The presence of one nanoparticle may affect the toxic effect of another nanoparticle on microalgae. Acute toxicity tests, including growth inhibition, instantaneous fluorescence rate, and antioxidant changes, were investigated and verified the hypothesis. In addition, this study also demonstrated the interaction of $nSiO_2$ and nCu in seawater through the combination of SEM, EDS, and Cu^{2+} exposure experiments. TEM image was used to observe the morphology of algal cells exposed to nCu. The toxic effect of nCu on microalgae was composed of physical contact and Cu^{2+} toxicity by comparing the growth inhibition rates of Cu^{2+} and nCu on *D. salina*.

Materials and methods

Microalgae culture

D. salina was obtained from the Algal Center of Key Laboratory of Marine Chemistry Theory and Technology, Ocean University of China. Microalgae were cultured in an f/2 medium made of sterile seawater filtered through a 0.45-μm membrane. *D. salina* was cultivated at 20 ± 1 °C and irradiated with cold continuous white fluorescence at 55 μmol photons/m^2/s and 12/12 h of light/dark cycle. The algae were shaken three times a day to promote the dissolution of CO_2 in the algal solution and to prevent the algae from adhering to the walls of the flasks. All flasks used for the experiments were soaked in 10% HCl for 24 h. The seawater was collected from the coast of Qingdao with a salinity of 32.

Preparation of nanoparticle suspensions

nCu (99.9% purity, 30 nm particle size distribution, black powder) was purchased from Aladdin Industries. $nSiO_2$ (99.9% purity, 30 nm particle size distribution, white powder) was purchased from Sigma-Aldrich. The nanoparticle suspensions were configured to a concentration of 1000 mg/L, respectively. A total of 0.1 g nanoparticles were weighed, and Milli-Q water was added to a volumetric bottle with a constant volume of 100 mL and then treated with ultrasound for 30 min.

Growth inhibition experiments

Growth inhibition of nCu on *Dunaliella salina*

The algal cultures were divided into 300-mL flasks, and each flask contained 150 mL of algal cultures. nCu suspension was added to the algal cultures in corresponding doses so that the concentration gradient of nCu was 0, 0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 mg/L. No addition of nanoparticles was set as the control group. Three parallel groups were performed for each concentration. The flasks

were placed in an incubator with the conditions the same as 2.1. One milliliter of the algal solution was removed at 0, 24, 48, 72, and 96 h, respectively, and the algal cells were fixed with Ruger's reagent. The density of algal cells was counted by hemocytometry through the microscope.

Growth inhibition of $nSiO_2$ on *Dunaliella salina*

The concentration gradients of $nSiO_2$ were set to 0, 5, and 10 mg/L. The rates of growth inhibition were calculated as the following equation.

$$IR = \frac{\mu_c - \mu_0}{\mu_c} \times 100$$

where μ_c and μ_0 were the specific growth rates of the test and control groups on the same day, respectively. $IR > 0$ indicates growth inhibition, and $IR < 0$ indicates growth promotion.

Measurement of photosynthetic parameters

The instantaneous fluorescence rate (Ft) of the samples was measured by a portable plant efficiency analyzer—AquaPen (Ecotech, Beijing, China) to analyze the effects of different concentrations of nCu and $nSiO_2$ on the photosynthetic system of algal cells. Three milliliters of algal cultures treated by nanoparticle at 0, 48, and 96 h was transferred into a quartz colorimetric dish and dark adaption for 10 min, and then, Ft was recorded.

Measurement of antioxidant parameters

Samples were collected at 96 h, and the kits were used to determine total protein (TP) content, malondialdehyde (MDA), superoxide dismutase (SOD) activity, and catalase (CAT) activity of the algal exposed to nCu and $nSiO_2$. The kits were purchased from Nanjing Jiancheng Institute of Biological Engineering.

Total protein (TP) Determination of total protein content was done using the Protein Quantification Kit. The principle of measurement is that the anion in the Caulmers Brilliant blue colorant binds to $-NH_3^+$ in the protein, and the absorbance is measured at 595 nm.

Malondialdehyde (MDA) Determination of malondialdehyde content was done using the malondialdehyde test kit. The principle of the measurement is that the algal cell metabolite malondialdehyde condenses with thiobarbituric acid (TBA) to form a red product with a maximum absorption peak at 532 nm.

Superoxide dismutase (SOD) and catalase (CAT) Determination of superoxide dismutase activity and catalase activity was done using the superoxide dismutase test kit and catalase test kit. SOD can decompose O_2^- into O_2 and H_2O_2. CAT can decompose H_2O_2. The activity of SOD and CAT can indirectly indicate that microalgae are subjected to oxidative stress.

Combined toxicity of nCu and $nSiO_2$ on *Dunaliella salina*

To investigate the effects of $nSiO_2$ on the toxicity of low and high concentrations of nCu, the combination of nCu and $nSiO_2$ with concentrations of (0.5 + 5), (5.5 + 5), (0.5 + 10), and (5.5 + 10) mg/L was chosen, and the control group was the algal solution without the addition of nanoparticles. Experimental procedures and calculation equations were the same as in the "Growth inhibition experiments" section to the "Measurement of antioxidant parameters" section.

SEM and EDS images were used to analyze the interaction between $nSiO_2$ and nCu in seawater after 96 h exposure. TEM image was used to observe the morphology of algal cells exposed to nCu.

Exposure of Cu^{2+} to *Dunaliella salina*

Fifty milliliters of samples was collected from all incubation groups at 96 h that had been treated with nCu alone and nCu in combination with $nSiO_2$. The samples were centrifuged at a speed of 5000 rmp at 4 °C for 10 min. The concentration of Cu^{2+} in supernatant was measured to explore Cu^{2+} released from nCu in the presence and absence of $nSiO_2$. After the supernatant was removed, the centrifuged algal cell bullets were collected and washed several times with normal saline, and Cu^{2+} content in the washed solution was measured to calculate the adsorption amounts of Cu^{2+} by algal cells. The washed algal cells were crushed by ultrasound, and Cu^{2+} content in the crushed algal cell solution was measured in order to explore the internalization amounts of Cu^{2+} by algal cells.

The sum of Cu^{2+} contents obtained above was regarded as the amount of Cu^{2+} released by nCu. The same concentration of Cu^{2+} was performed for 96 h exposure experiments to investigate the effect of Cu^{2+} on *D. salina*.

Statistics analysis

All data were analyzed by one-way analysis of variance (ANOVA) and LSD test ($P < 0.05$). Significant differences between the treatment groups were tested by IBM SPSS Statistics 20.0. All data are mean ± relative standard deviation. The values of the nCu concentration gradient and growth inhibition rates of microalgae were entered into SPSS 20.0 software with a confidence interval of 95% to calculate the EC50.

Results and discussion

Inhibition of algal growth

Growth inhibition of *Dunaliella salina* by different concentrations of nCu

nCu toxicity to *D. salina* increases with the duration of exposure. The algal density of the control group (0 mg/L) and the experimental groups with concentrations of 0.5, 1.5, 2.5, and 3.5 mg/L nCu showed an increasing trend with the time as shown in Fig. 1A; however, the experimental groups exposed to 4.5 and 5.5 mg/L nCu showed negative growth, indicating that the algal growth was greatly inhibited at such nCu concentrations. Fifty percent effective concentration (EC50) of nCu on *D. salina* was calculated as shown in Table 1. Li et al. (2015a) investigated the growth inhibition of nCu on *Skeletonema costatum* and found that the algal growth was negative under a higher concentration of nCu, which was consistent with the result of this experiment. Compared with the control group, the inhibition effect of nCu on *D. salina* was more obvious with the increase of nCu concentration. The growth inhibition rate of 5.5 mg/L nCu on *D. salina* could reach 94.7% at 96 h as shown in Fig. 1B. Compared with the control group, 0.5 mg/L nCu seems to have a slight promotion on the growth of *D. salina*, but there was no statistical difference between the two groups. As a transition metal, a small amount of Cu^{2+} released by low

Table 1 EC50 of nCu on *Dunaliella salina* at 24, 48, 72, and 96 h exposure

Kinds of materials	50% effective concentration EC50 (mg/L)			
	24 h	48 h	72 h	96 h
nCu	5.1 (4.5–6.2)	2.7 (1.4–3.8)	1.8 (1.6–2.3)	0.6 (0.5–1.1)

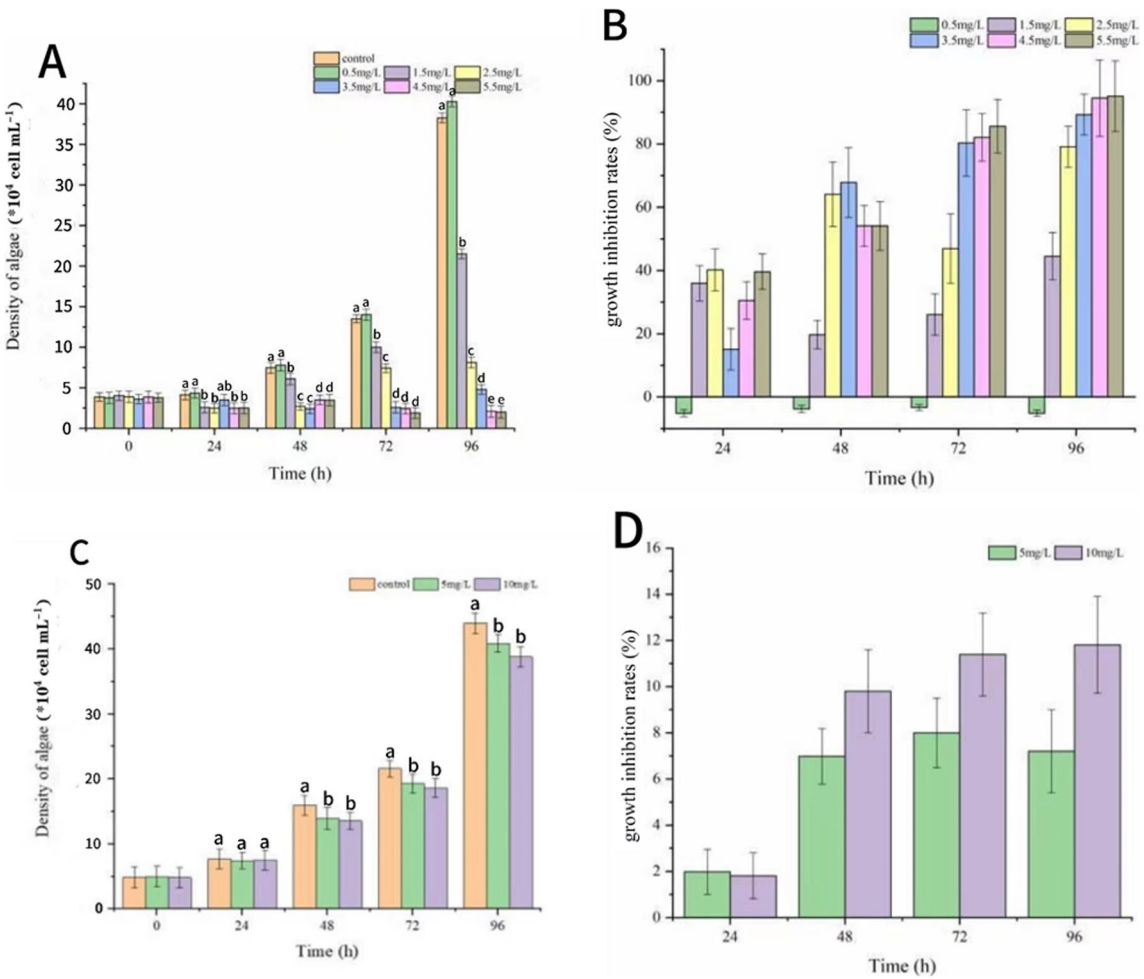

Fig. 1 Algal density (**A**) and growth inhibition rates (**B**) exposed to different concentrations of nCu. Algal density (**C**) and growth inhibition rates (**D**) exposed to different concentrations of $nSiO_2$. The control group received no nanoparticles. Values were reported as mean of 3 replicates ± standard deviation. Different letters indicate significant differences between groups with different treatment conditions ($P < 0.05$)

concentration nCu can be used as a cofactor of enzymes related to photosynthesis of algae to promote the growth of algae (Li et al. 2015a). Janova et al. (2021) found that nCu could promote the growth of *Chlamydomonas Rhine* when its concentration does not exceed 5 mg/L; however, when its concentration exceeded 10 mg/L, nCu would significantly inhibit the growth of *C. Rhine*. Low concentration of nCu may promote algal growth, while a high concentration inhibits algal growth. Fang et al. (2022) found that a high concentration of nCuO would form nCuO clusters in seawater, and algal cells would be severely deformed after contact with nCuO clusters.

Growth inhibition of *Dunaliella salina* by different concentrations of $nSiO_2$

The algal density increased with increasing exposure time in the $nSiO_2$ treatment groups as shown in Fig. 1C. Compared with the control group, the decrease of algal density exposed to 5 and 10 mg/L $nSiO_2$ was significant over 48 h exposure. The toxicity of $nSiO_2$ gradually increased with exposure time. Wang et al. (2022a) found that the density of *Heterosigma akashiwo* was significantly reduced when it was exposed to $nSiO_2$ with a concentration higher than 5 mg/L. *H. akashiwo* showed a time-dependent and dose-dependent adverse response as it was exposed to $nSiO_2$, which is consistent with this study. The growth inhibition rate increased gradually with the exposure time as shown in Fig. 1D. The greatest growth inhibition rate occurred at 96 h as algae were exposed to 10 mg/L $nSiO_2$. The effects of silicate released by $nSiO_2$ on *D. salina* can be excluded due to the low solubility of $nSiO_2$ in seawater and not a diatom of *D. salina*, which does not need silicate for growth. Previous studies have shown that the toxicity of $nSiO_2$ on *Pseudomonas subpitiata* was mainly caused by the nanoparticles themselves, as the supernatant has no effect on cell viability (Sousa et al. 2019).

The hydrophilic surface of $nSiO_2$ makes it easier to react with hydrophilic groups such as carboxyl and hydroxyl groups on the algae surface, which increases the likelihood of contact between $nSiO_2$ and microalgae (Pikula et al. 2020). It is speculated that the toxic effect of $nSiO_2$ is due to the heterogeneous aggregation with algae. $nSiO_2$ also can be adsorbed on the surface of algal cells, which may destroy cell integrity and cause physical damage to algal cells. In addition, nanoparticles may reduce the algal cell area which received light, thus affecting photosynthesis. Certainly, nCu has a similar effluence on the algae in addition to releasing ions.

Effects on photosynthesis

Photosynthetic parameters can reflect damage to the photosynthetic system of algal cells. The instantaneous fluorescence rate (Ft) reflects the content of chlorophyll in algal cells and the growth of algal cells. Ft of the control group and the experimental group with nCu concentration lower than 4.5 mg/L showed an increasing trend with the increase of exposure time as shown in Fig. 2A. With the increase of nCu concentration, Ft decreased slowly, indicating that the damage to the algal photosynthetic system was dose-dependent. Ft of the 5.5 mg/L experimental group was the lowest among all groups. Al-Khazali and Alghanmi (2019) also found that the content of *Coelastrella terrestris* chlorophyll was inhibited as treated with nCu.

Ft of the $nSiO_2$ experimental groups (5 and 10 mg/L) increased with the increase of time as shown in Fig. 2B. Ft in the 10 mg/L $nSiO_2$ experimental group decreased significantly

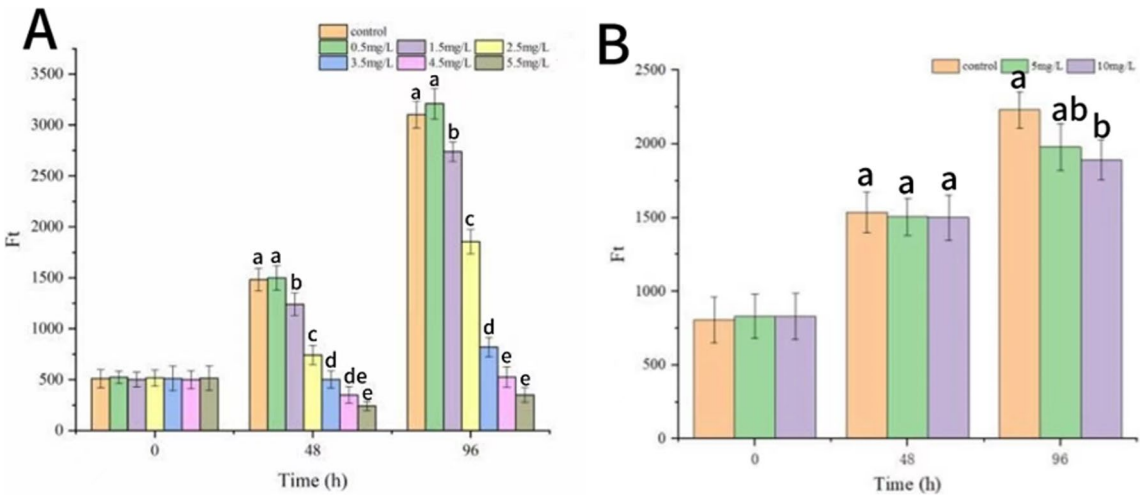

Fig. 2 The instantaneous fluorescence rate of algae exposed to different concentrations of nCu (**A**) and $nSiO_2$ (**B**). The control group received no nanoparticles. Values were reported as mean of 3 replicates ± standard deviation. Different letters indicate significant differences between groups with different treatment conditions ($P < 0.05$)

compared with the control group at 96 h. Wang et al. (2022a) found that a concentration of 30 mg/L of $nSiO_2$ reduced the Ft of *H. akashiwo* by 27.7%. Ft value of 5.5 mg/L nCu experimental group decreased by 90.3%; in addition, the 5 mg/L $nSiO_2$ experimental group decreased by 12.7%, and the 10 mg/L $nSiO_2$ group decreased by 20.7% compared with the control at 96 h. Moreover, the toxicity of $nSiO_2$ on *Heterosigma akashiwo* is concentration-dependent (Wang et al. 2022a), and it could be judged that the Ft value decreased between 12.7 and 20.7% when 5.5 mg/L $nSiO_2$. The toxicity of $nSiO_2$ is less than that of nCu from photosynthesis.

Measurement of antioxidant parameters

Total protein (TP) content

Protein is the basis for synthesizing enzymes and carrying out all physiological activities for algal cells (Sueda 2020). When algal cells are subjected to different degrees of stress, protein content will change (Wang et al. 2022b; Ni et al. 2023). Compared with the control group, TP content increased by 10.5% as exposed to 0.5 mg/L nCu as shown in Fig. 3. When nCu concentration increased continuously, TP content significantly decreased by 50.7%, 53.9%, 55.3%, and 57.2% as exposed to 2.5, 3.5, 4.5, and 5.5 mg/L nCu compared with the control group. Low concentration of nCu improved TP content, but a high concentration of nCu improved the toxicity and inhibited algal growth and TP content. Due to the adsorption of nCu on algal cells at high concentrations, it caused cell rupture and cytoplasm outflow, resulting in protein loss and TP content decrease. Zhu et al. (2022) observed nZnO aggregation and adsorption on the surface of microalgae *Gymnodinium*, which resulted in changes in cell size and morphology and rupture of cell walls. This type of destruction would result in the loss of its protein. TP content showed an increasing trend under $nSiO_2$ stress as shown in Fig. 3. Wang et al. (2022a) investigated the toxic effect of $nSiO_2$ on *H. akashiwo* and found that the TP content of *H. akashiwo* showed an increasing trend under the condition of $nSiO_2$ at low concentration, which was consistent with this study.

MDA content

MDA is the main product of lipid peroxidation. Changes in MDA content can explain the oxidative damage and lipid peroxidation of algal cells produced by different concentrations of nanoparticles (Li et al. 2015b). When stressed by nanoparticles, algal cells accumulate more oxygen radicals and ROS, which attack the algal cell membrane. MDA is the final product of this reaction. Therefore, MDA can be used as an important indicator to evaluate lipid peroxidation (Ni et al. 2018). When the concentration of nCu was 4.5 and 5.5 mg/L, MDA content significantly increased by 65.6% and 84.1% compared with the control. As the concentrations of $nSiO_2$ were 5 and 10 mg/L, MDA content significantly increased by 59.7% and 86.1% compared to the control as shown in Fig. 3. Bahador et al. (2019) investigated the toxic effect of nAg on *Salvia dubliniensis* and found that the concentration of nAg was positively correlated with MDA content. Huang et al. (2021) found that the MDA content of *Gymnodinium aeruginosum* increased with the increasing concentration of polystyrene with 0.1 μm particle size.

Antioxidant enzyme SOD and CAT activity

The enzyme SOD can dismutate superoxide anion into H_2O_2. CAT is an enzyme that decomposes hydrogen peroxide (H_2O_2) and usually works together with SOD to remove excess oxygen free radicals and ROS in microalgae cells. Therefore, the extent of microalgae cells affected by oxidation stress can be evaluated by measuring the activities of SOD and CAT (Zhao et al. 2020). The activity of SOD showed a trend of first increasing and then decreasing with the increased concentration of nCu as shown in Fig. 3. When the concentration of nCu was 1.5, 2.5, and 3.5 mg/L, the activity of SOD showed an increasing trend with nCu concentration, and the activity significantly increased by 458.8%, 361.2%, and 667.1% compared with the control. The enhanced activity of SOD can dismutate more oxygen free radicals and ROS caused by the increasing nCu concentration. When the concentration of nCu exceeded 3.5 mg/L, much more oxygen free radicals and ROS were triggered by nCu and destroyed the SOD structure directly (Chen et al. 2019), showing a decreasing trend of SOD activity. Ni et al. (2023) found that the SOD activity of *S. costatum* decreased with the increasing concentration of polystyrene microplastics. The activity of SOD exposed to 5 and 10 mg/L $nSiO_2$ showed a significant increase compared with the control as shown in Fig. 3, indicating that the activity of SOD was positively correlated with the concentration of $nSiO_2$.

Similar to SOD, the activity of CAT showed a trend of first increasing and then decreasing as exposed to nCu. With the increase of nCu concentration from 2.5 to 5.5 mg/L, the activity of CAT significantly increased by 300%, 428.6%, 569.6%, and 365.2%, respectively. High concentration of nCu caused excessive oxidative stress, which led to a decrease in the activity of CAT. SOD and CAT work together to eliminate oxygen radicals, so the activity change of the two enzymes showed a similar trend. As the concentration of $nSiO_2$ increased, the activity of CAT was significantly increased by 38.8% and 57.5% exposed to 5 and 10 mg/L $nSiO_2$ compared to the control as shown in Fig. 3. Wang et al. (2022a) found a significant increase in CAT activity of *H. akashiwo* with the increased concentration of $nSiO_2$ exposed.

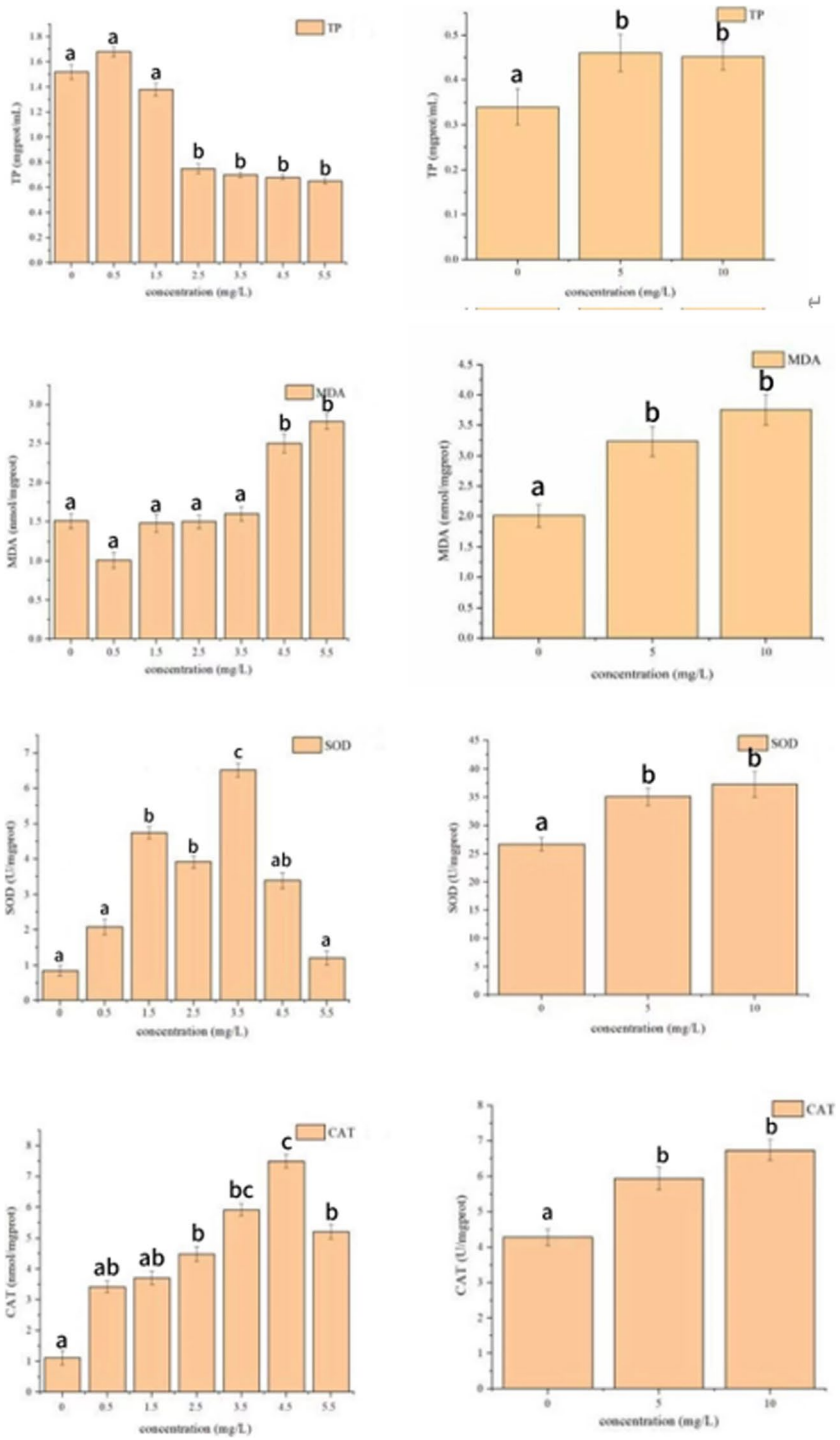

Fig. 3 TP, MDA, SOD, and CAT changes of algae cells after exposure to different concentrations of nCu and different concentrations of $nSiO_2$ at 96 h. The control group received no nanoparticles. Values were reported as mean of 3 replicates ± standard deviation. Different letters indicate significant differences between groups with different treatment conditions ($P < 0.05$)

Combined toxicity of nCu and $nSiO_2$ to *Dunaliella salina*

Growth inhibition

A total of 0.5 and 5.5 mg/L of nCu and 5 and 10 mg/L of $nSiO_2$ were selected to investigate whether $nSiO_2$ affects the toxic effect of nCu on *D. salina*. After adding both concentrations of $nSiO_2$ to 0.5 mg/L nCu, the algal density decreased after exposure 72 h as shown in Fig. 4A. Cu^{2+} can no longer be used as a trace element to promote the algal growth because the concentration of Cu^{2+} became very low after adsorption by $nSiO_2$, while $nSiO_2$ can attack algal cells to hinder their growth and showed toxicity to the algae. A total of 5.5 mg/L nCu significantly inhibited the algal growth. When $nSiO_2$ was added, the growth inhibition

of algal cells was greatly alleviated. The growth inhibition rate of the combination of (5.5 + 10) mg/L nanoparticles was lower than that of the combination of (5.5 + 5) mg/L as shown in Fig. 4B, because higher concentration $nSiO_2$ adsorbed more nCu and Cu^{2+}, which is consistent with the results of Zhu et al. (2022). Zhu et al. (2022) explored that the addition of nZnO reduced the toxicity of graphene quantum dots (GQD) to microalgae *Gymnodinium* due to the formation of heterogeneous aggregates of nZnO with GQDs. Ullah et al. (2019) prepared a cyclic peptide-conjugated silver nanoparticle, and it was used to absorb Hg^{2+} in human blood and water. These results postulated that $nSiO_2$ formed heterogeneous aggregates with nCu and adsorbed Cu^{2+}. The adsorption of nCu on $nSiO_2$ prevented the contact between nCu and algal cells, reducing its physical damage. $nSiO_2$ also adsorbed Cu^{2+} to reduce its concentration and toxicity. The concentration of Cu^{2+} release by nCu showed a relatively steady state over 96 h, and the experimental result (Fig. 9) was supplemented in the supplementary materials.

Photosynthetic parameters

Compared with 0.5 mg/L nCu, Ft of the (0.5 + 5) and (0.5 + 10) mg/L experimental groups decreased by 9.0% and 11.6% at 96 h as shown in Fig. 4C. $nSiO_2$ suppressed Ft, therefore inhibiting the growth of algal cells. Compared with 5.5 mg/L nCu, the decrease of Ft was alleviated to a certain extent after the addition of $nSiO_2$ at 96 h. $nSiO_2$ reduced the contact of nCu to algal cells by adsorption and alleviated the toxic effect by adsorption of Cu^{2+}.

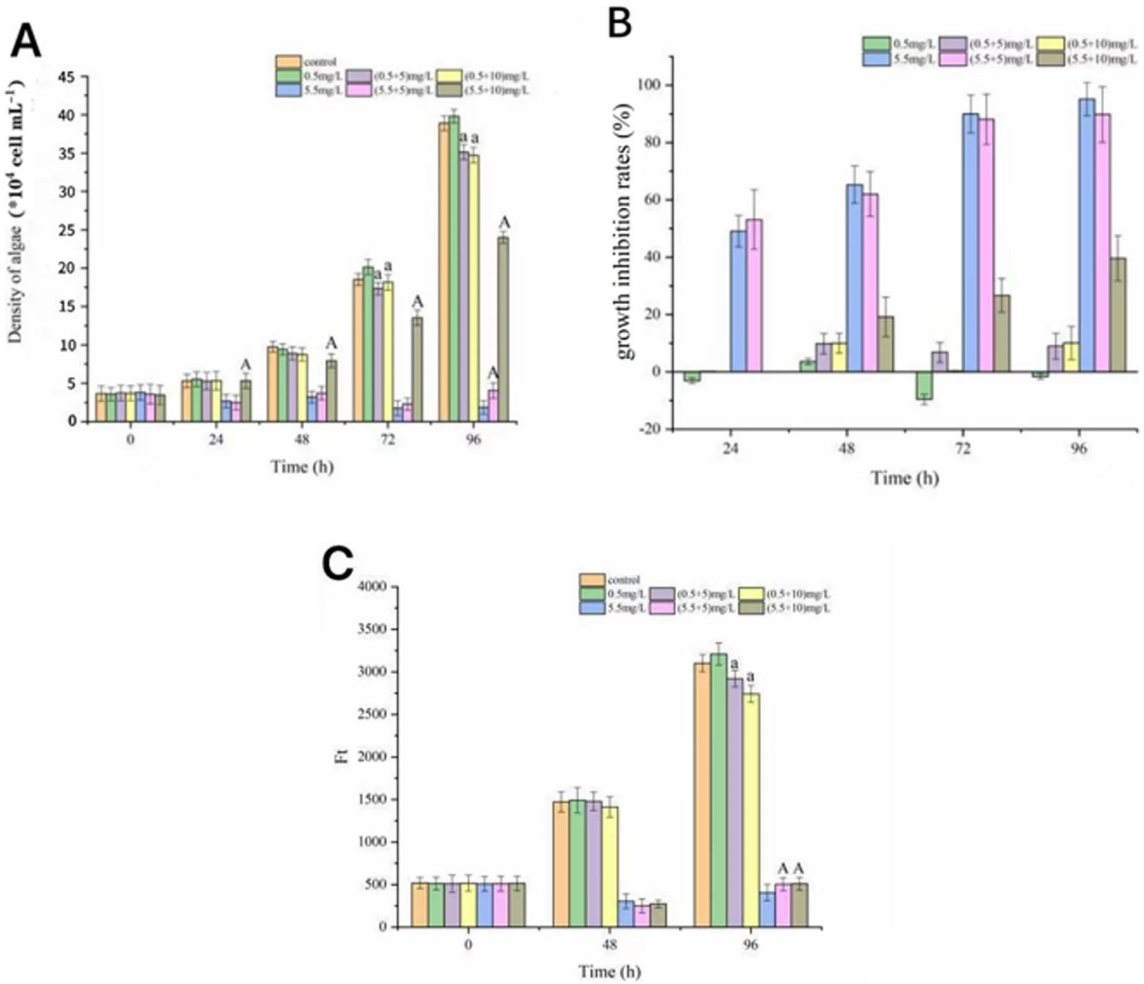

Fig. 4 Cell density (**A**) and growth inhibition rate (**B**) of algae exposed to the coexistence of nCu (0.5, 5.5 mg/L) and $nSiO_2$ (5, 10 mg/L). The instantaneous fluorescence rate (**C**) of algae exposed to the coexistence of nCu (0.5, 5.5 mg/L) and $nSiO_2$ (5, 10 mg/L). The control group received no nanoparticles. The value represents the mean ± standard deviation of 3 replicates. "a" means that there is a significant difference between the experimental groups of 0.5 mg/L nCu before and after adding $nSiO_2$. "A" means that there is a significant difference between the 5.5 mg/L nCu experimental groups before and after adding $nSiO_2$

Antioxidant system

TP content increased after the addition of $nSiO_2$ in the 5.5 mg/L nCu experimental groups as shown in Fig. 5A, which was due to the adsorption of nCu and Cu^{2+} by $nSiO_2$, thus reducing the toxic effect. When $nSiO_2$ was added, MDA content in the 0.5 mg/L nCu group increased; however, it decreased in the 5.5 mg/L group as shown in Fig. 5B. The adsorption of nCu on algal cells will damage the algal cell membrane, resulting in protein loss and MDA content increase. The changes in MDA contents in the 0.5 and 5.5 mg/L groups were reversed after adding $nSiO_2$. In the 0.5 mg/L nCu experimental groups, the toxic effect of nCu was negligible, and the toxicity was mainly caused by $nSiO_2$, resulting in the increase of MDA content with the increasing concentration of $nSiO_2$. In the 5.5 mg/L nCu experimental groups, $nSiO_2$ adsorbed nCu and Cu^{2+}, which reduced the toxicity of nCu to microalgae, so MDA content decreased.

nCu and $nSiO_2$ caused oxidative stress on cells, and the activities of SOD and CAT increased to eliminate ROS and oxygen free radicals produced. In the 0.5 mg/L nCu experimental groups, $nSiO_2$ triggered oxidative stress, and the activity of SOD increased. In the 5.5 mg/L nCu experimental groups, the activity of SOD increased first and then decreased after the addition of $nSiO_2$. A total of 5.5 mg/L of nCu directly caused the decrease in SOD activity. The excess toxicity of nCu decreased after the addition of $nSiO_2$, so the activity of SOD increased. With the increase of $nSiO_2$ concentration, the toxicity of nCu continued to diminish so the activity of SOD decreased. The activity of CAT increased after the addition of SiO_2 in the 0.5 mg/L nCu experimental groups as shown in Fig. 5D. The toxic effect is caused by

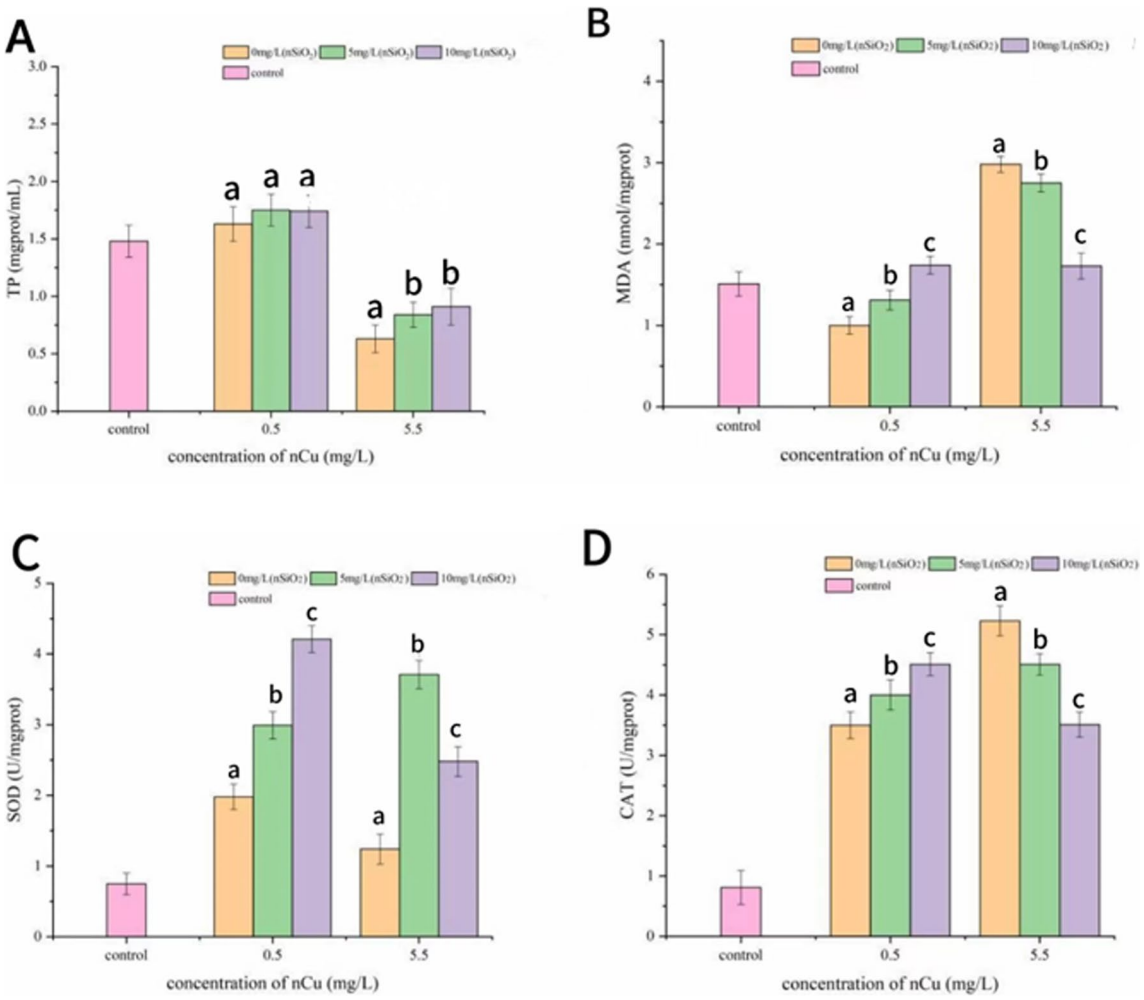

Fig. 5 TP (**A**), MDA (**B**), SOD (**C**), and CAT (**D**) Changes of algae cells under the coexistence of nCu (0.5, 5.5 mg/L) and $nSiO_2$ (5, 10 mg/L) at 96 h. The control group received no nanoparticles. This value represents the mean ± standard deviation of 3 replicates, and different letters indicate significant differences between groups with different treatment conditions ($P < 0.05$)

$nSiO_2$ in this nCu concentration, and the activity increased after the addition of $nSiO_2$. Five and ten milligrams per liter of $nSiO_2$ reduced the toxic effect of 5.5 mg/L nCu due to the adsorption of nCu and Cu^{2+} to $nSiO_2$; therefore, the activity of CAT decreased.

Toxic effect of Cu^{2+} on *Dunaliella salina*

The amount of Cu^{2+} in the supernatant was measured by centrifugation as shown in Fig. 6A. Cu^{2+} released by nCu increased with the increasing concentration of nCu. When $nSiO_2$ was added, Cu^{2+} concentration was less in the supernatant. The greater the concentration of $nSiO_2$ added, the less Cu^{2+} concentration was determined. The addition of 5 and 10 mg/L $nSiO_2$ reduced the concentration of Cu^{2+} by 30.4% and 55.4% respectively in the 5.5 mg/L nCu group. $nSiO_2$ adsorbed nCu and Cu^{2+}, thus affecting the concentration of Cu^{2+} in supernatant.

The amount of Cu^{2+} adsorbed on the cell surface and internalized in the cell was measured when the algae were exposed to 3.5 mg/L nCu, combined with 5 and 10 mg/L $nSiO_2$. Most of the Cu^{2+} released by nCu were internalized in the cell, and the least amount was adsorbed on the cell surface as shown in Fig. 6B. Fang et al. (2022) investigated the toxic effect of nCuO on *T. obliqua* and found that most of Cu^{2+} were internalized by the algal cells, which are consistent with this study. The addition of 10 mg/L of $nSiO_2$ resulted in a significant decrease of Cu^{2+} concentration in solution, on the cell surface, and within the cells compared to no addition of $nSiO_2$. The toxic effect of nCu on microalgae cells is greatly due to the physical damage of nCu and Cu^{2+} internalized into the cells.

The sum of Cu^{2+} released by 3.5 mg/L nCu in solution, on cells, and inside cells was calculated to be 1.25 mg/L. The algal density was observed after exposed to 1.25 mg/L Cu^{2+} in order to investigate the toxic effect of Cu^{2+}. An addition group was also set up by adding 10 mg/L $nSiO_2$ combined with 1.25 mg/L Cu^{2+}. The cell density exposed to 1.25 mg/L Cu^{2+} significantly decreased compared with the control after 24 h exposure as shown in Fig. 7A. After the addition of $nSiO_2$, the algal density showed an increasing trend from 48 h compared with no addition of $nSiO_2$. The added $nSiO_2$ adsorbed Cu^{2+} to reduce the toxic effect of Cu^{2+} on the algae. In addition, the toxic effects of 3.5 mg/L nCu and 1.25 mg/L Cu^{2+} on algal cell growth were compared as shown in Fig. 7B. A total of 3.5 mg/L nCu inhibited the growth of algal cells more than 1.25 mg/L Cu^{2+}. In addition to releasing a certain concentration of Cu^{2+} to damage algae, nCu also caused physical damage to algae, thus inhibiting the algal growth more.

Characterization of nCu and $nSiO_2$ mixed in seawater

A totol of 3.5 mg/L nCu and 10 mg/L $nSiO_2$ were mixed in seawater and characterized by SEM and EDS images as shown in Fig. 8B, C, compared with the TEM characterization of nCu in seawater in Fig. 8A (Zhang et al. 2018). It can be seen that nCu in transmission electron microscopy is clustered together due to agglomeration, but the particles are still visible. However, the morphology of the heterogeneous aggregates observed in Fig. 8B is complex. Wang et al. (2022b) found that nZnO and graphene quantum dot tended to form heterogeneous aggregations in

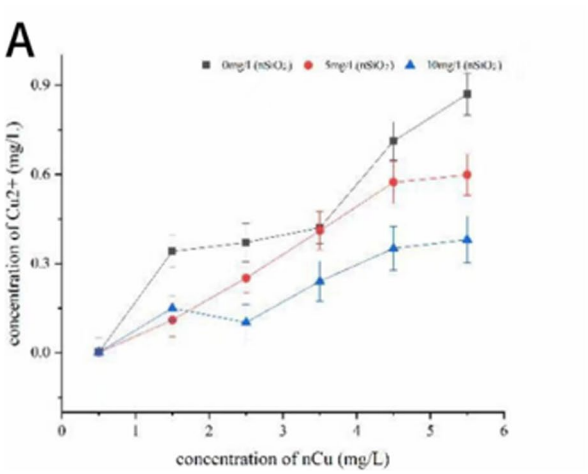

Fig. 6 Release of Cu^{2+} from nCu in the presence of $nSiO_2$ in the supernatant (**A**) and distribution of the amount of $Cu.^{2+}$ released by 3.5 mg/L nCu at different concentrations of $nSiO_2$ (**B**). This value represents the mean ± standard deviation of 3 replicates, and different letters indicate significant differences between groups with different treatment conditions ($P < 0.05$)

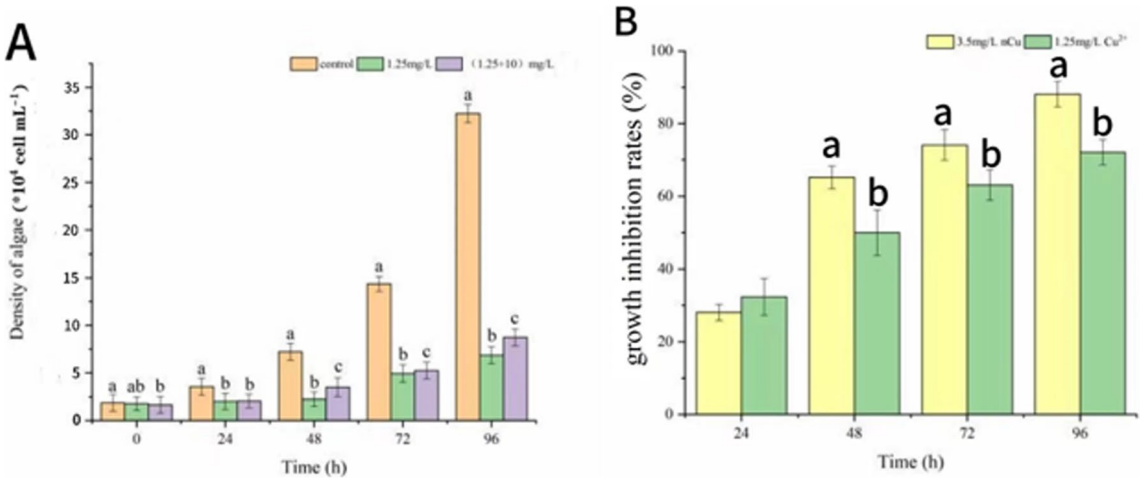

Fig. 7 Cell density (**A**) of the algae after exposure to Cu^{2+} (1.25 mg/L) and $nSiO_2$ (10 mg/L) and the comparison of growth inhibition rates (**B**) between exposure to 1.25 mg/L $Cu.^{2+}$ and 3.5 mg/L nCu. The control group received no nanoparticles. This value represents the mean ± standard deviation of 3 replicates. Different letters indicate significant differences between groups with different treatment conditions ($P < 0.05$)

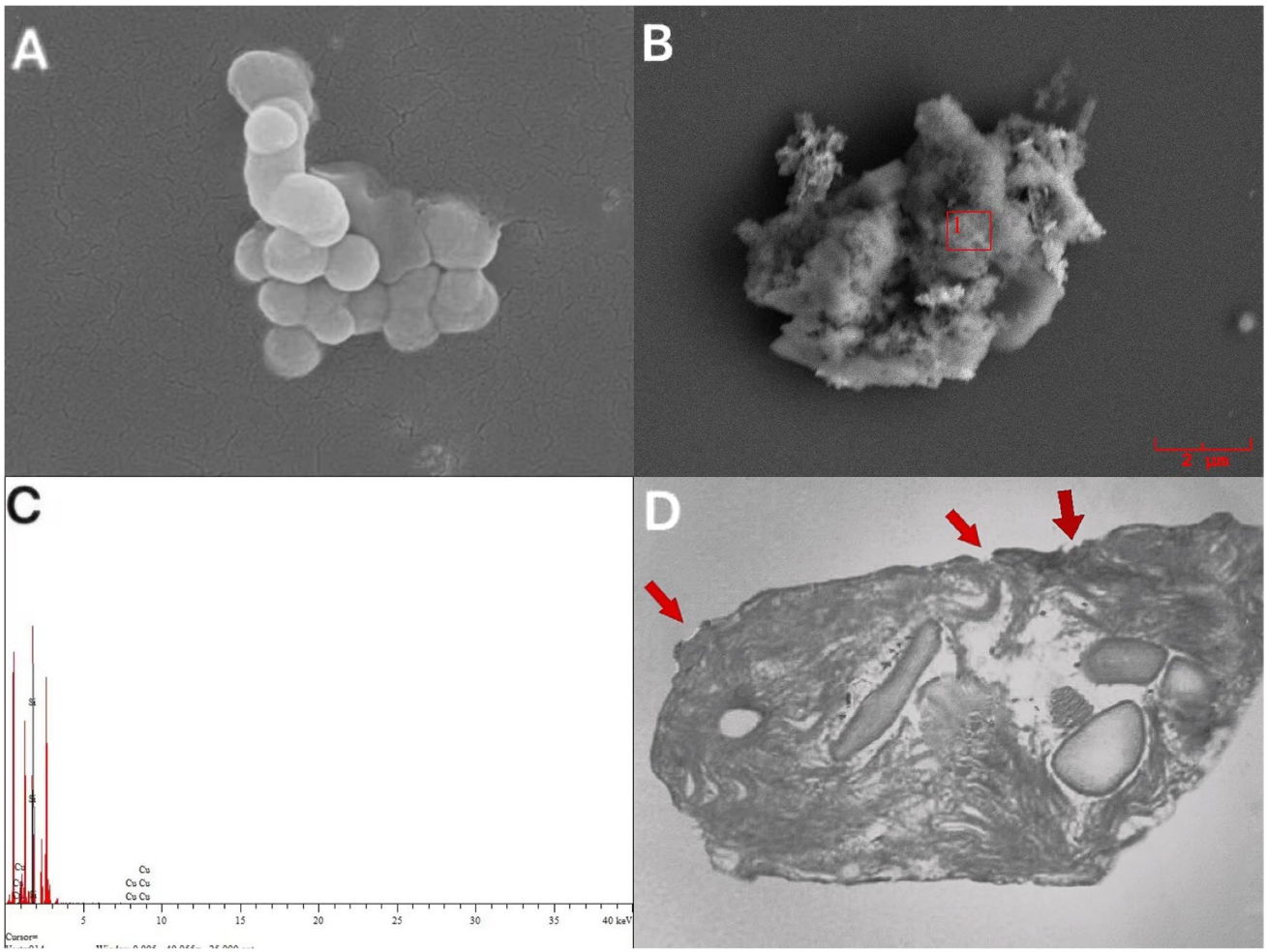

Fig. 8 TEM (**A**) characterization of nCu in seawater, SEM (**B**) and EDS (**C**) characterization of nCu (3.5 mg/L) and $nSiO_2$ (10 mg/L) mixed in seawater. TEM (**D**) characterization of *Dunaliella salina* exposed to 3.5 mg/L nCu (red arrows represent cell membrane rupture)

seawater through SEM images. The EDS image of $nSiO_2$ and nCu is shown in Fig. 8C. The appearance of Si and Cu peaks in the image proved that the heterogeneous aggregations are composed of nCu and $nSiO_2$. The two diagrams showed that $nSiO_2$ adsorbed nCu in seawater, thus reducing the toxicity of nCu on algae. The TEM image (Fig. 8D) showed that nCu disrupted the membrane of the algal cell.

Conclusion

In this study, a series of changes in growth, instantaneous fluorescence rate, and antioxidant system of *D. salina* were investigated under single and combined stress of nCu and $nSiO_2$. The toxic effects of the two nanoparticles on *D. salina* were both time- and dose-dependent. The two nanoparticles induced oxidative stress in algal cells and triggered the increased content of MDA, SOD, and CAT activities. $nSiO_2$ increased the toxic effect of nCu on *D. salina* at low concentrations, and the toxic effect was caused by $nSiO_2$; however, it reduced the toxic effect of nCu on *D. salina* at high concentration since $nSiO_2$ adsorbed nCu and Cu^{2+}. The two nanoparticles interacted with each other and produced complex toxic effects on the algae.

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Qi Li, Liju Tan, and Jiangtao Wang. The first draft of the manuscript was written by Qi Li, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article [and its supplementary material].

Declarations

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Consent to participate Not applicable.

Consent for publication All authors approved the final manuscript.

Competing interests The authors declare no competing interests.

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