RESEARCH ARTICLE

Combined toxicities of copper nanoparticles with carbon nanotubes on marine microalgae *Skeletonema costatum*

Cai Zhang[1,2] · Xiaohua Chen[1] · Liju Tan[1] · Jinagtao Wang[1]

Abstract

To investigate the combined toxicities of copper nanoparticles (nano-Cu) with carbon nanotubes (CNTs) on marine microalgae *Skeletonema costatum*, algal growth inhibition tests were carried out. Toxicities of nano-Cu with CNTs and without CNTs on microalgae were determined, respectively. Chlorophyll content and photosynthetic efficiency (ΦPSII) were determined to compare negative effects of nano-Cu with CNTs and without CNTs on photosynthesis. The concentration of Cu^{2+} released by nano-Cu into the medium was determined, and interactions between nano-Cu and CNTs were analyzed to study toxic mechanisms of combined toxicities of nano-Cu with CNTs. It was found that both nano-Cu and CNTs could inhibit the growth of the microalgae; however, the toxicity of CNTs on the microalgae was far lower than that of nano-Cu. The maximum growth inhibition ratio (IR) of nano-Cu on the microalgae was 86% appearing at 96 h under 1.0 mg/L nano-Cu treatment, while the maximum IR of CNTs on the microalgae was 58% at 96 h under 200 mg/L CNT treatment. CNTs could reduce the toxicity of nano-Cu on the microalgae in processes of growth and photosynthesis. Adsorption of Cu^{2+} on CNTs and aggregate between Cu and CNTs in the medium were main reasons for attenuation of toxicity of nano-Cu with adding CNTs.

Keywords Copper nanoparticles · Carbon nanotubes · Combined toxicities · *Skeletonema costatum*

Introduction

A variety of nanomaterials which have nano-dimensions and unique physicochemical properties play an important role in commodity production all over the world (Navarro et al. 2008). The heavy use of nanomaterials increases the potential for their release to the environment. Nanomaterials in aquatic environment pose a threat to aquatic organisms, which has already been proved by many studies (Bouldin et al. 2008; Li et al. 2015; Schwab et al. 2011). Oceans are the final destination for all waste nanomaterials (Moreno-Garrido et al., 2015), and coastal systems are likely the ultimate sink for

the nanomaterials, deliberately discharged into the environment (Klaine et al., 2008). Many kinds of nanomaterials usually exist in the same area. For simulating real conditions and evaluating potential environmental risks, the combined toxicities of different nanomaterials need to be investigated. Marine microalgae as model organisms are often used in marine ecotoxicological tests for their important implications to marine ecosystems. Toxic effects of nanomaterials on microalgae have been reported in many studies (Bouldin et al., 2008; Miller et al., 2010; Röhder et al., 2014).

Nanoparticles are not stable for their high specific surface energy and high reactivity in the environment. They aggregate and interact with other substance to keep themselves stable in the aquatic environment (Jiang et al. 2009). Nanoparticles interacting with other materials develop combined toxicity different from single toxicity (Glover et al. 2011). Schiavo et al. (2016) found that nano-Ag with coating could well disperse in the medium and release more Ag^+, which enhance the toxicity of nano-Ag on microalgae. Nano-TiO_2 could adsorb Cd^{2+} and reduce the bioavailability and toxicity of Cd on microalgae *Pseudokirchneriella subcapitata* (Hartmann et al. 2010). Carbon nanomaterials such as fullerene (C_{60}) could adsorb 85% of phenanthrene and 10% of pentachlorophenol (PCP) in the medium, which increased the

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toxicity of phenanthrene, but decreased the toxicity of PCP (Baun et al. 2008). The combined toxicities of different nanoparticles were not the same and combined toxicities of more nanoparticles should be noted (Chen et al. 2015, Wang et al. 2016).

Copper nanoparticle (Nano-Cu) as a kind of conductive materials has many applications such as catalysts and solid lubricants (Müller 2015). Compared with nano-Cu, it was clear that Cu^{2+} released by nano-CuO was the main toxic mechanism of nano-CuO on microalgae (Bondarenko et al. 2012, Heinlaan et al. 2008). Cu^{2+} could induce generation of reactive oxygen species (ROS), DNA damage, disturbance of protein function, and cell membrane injury (Saison et al. 2009, Wang et al. 2011). Studies about toxicity of nano-Cu were less than that of nano-CuO; however, Cu^{2+} released by nano-Cu was the dominant factor for the toxicity of nano-Cu on microalgae (Minocha & Mumper 2012, Reyes et al. 2015). Carbon nanotube (CNT) as an emerging carbon nanomaterial with lots of physical-chemical characteristics such as electrical conductivity, lipotropic ability, and large specific surface area also has toxic effects on microalgae (Hull et al. 2009, Jia et al. 2005). Generation of reactive oxygen species and lights shading caused by CNTs were potential toxic mechanisms to inhibit the growth of microalgae; however, the toxicity of CNTs was far below nano-Cu (Long et al. 2014, Navarro et al. 2008).

To investigate the combined toxicity of nano-Cu with CNTs on microalgae *Skeletonema costatum*, algal growth inhibition tests were carried out, and chlorophyll content and ΦPSII were determined to study effects of nano-Cu with CNTs on photosynthesis. Adsorption of Cu^{2+} on CNTs was also analyzed to study toxic mechanisms of nano-Cu with CNTs. This study provided results of the combined toxicity of two nanomaterials to understand interactions between microalgae and more than one nanomaterial.

Materials and methods

Chemicals

Nano-Cu (99.9% purity, particle size distribution 10–30 nm) was purchased from Shanghai ST-Nano Science & Technology Co.,

LTD, China. CNTs (95% purity, 10–30 μm long, 20–30 nm wide) were purchased from Beijing DK nano technology Co., LTD, China. Both nano-Cu and CNTs were black dry powders. Nano-Cu were spheroidal particles and CNTs were threadlike tubes in SEM images (Fig. 1). Nano-Cu was dispersed in the f/2 medium to the final concentration of 10 mg/L. A drop of the medium sample with nano-Cu was put on the conducting resin. The medium sample on the conducting resin was dried with nitrogen and observed by a scanning electron microscope (SEM, Hitachi S-4800 cold field-emission). The aggregates of nano-Cu were observed by SEM.

Organisms

The algal strain *Skeletonema costatum* (Bacillariophyta) was obtained from the Algal Center of Key Laboratory of Marine Chemistry Theory and Technology, Ocean University of China. The microalgae were cultivated in the f/2 medium (Guillard & Ryther 1962) made with sterile seawater (filtered by a 0.45-μm membrane) from Qingdao, China. The microalgae were cultivated in a 5-L Erlenmeyer flask at 20 ± 1 °C under cool continuous white fluorescent lights (4000 lx) with a 12-h light/dark cycle and manually shaken twice a day to prevent sedimentation of algae. The incubation was lasted 5–7 days until log phase growth prevailed. Cell density was measured by a hemacytometer under an inverted microscope (Leica, DM4000B) with 400 magnification times.

Preparation of nano-Cu and CNT suspensions

Stock suspensions of nano-Cu were prepared through dispersing nano-Cu powders into Milli-Q water to the final concentration of 10 mg/L. The same method was used for stock suspensions of CNTs and the final concentration was 1000 mg/L. The well-dispersed stock suspensions through shaking and sonicating (30 min at 50 W) were diluted with Milli-Q water and then ultrasound bathed 30 min at 50 W for dispersion before the toxicity assay.

Fig. 1 SEM images of nano-Cu and CNTs
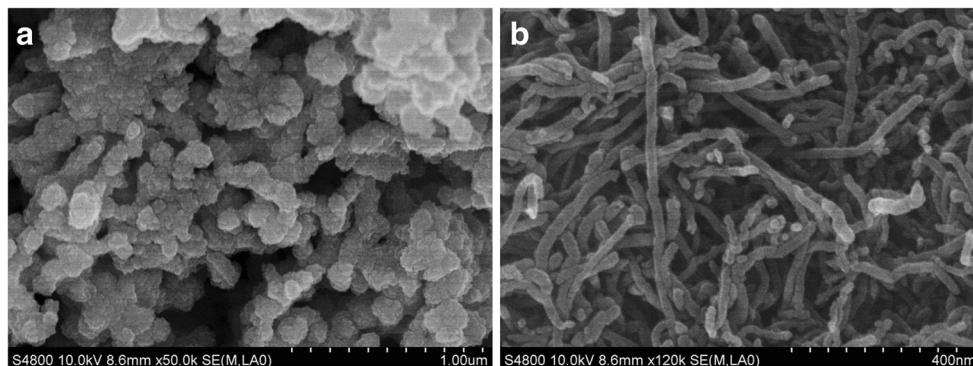
Algal growth inhibition test

All tests in the study complied with the OECD (Organization for Economic Cooperation and Development) Guidelines 201. Glass wares were acid-soaked, cleaned with Milli-Q water, and then autoclaved before the test. One hundred milliliters of pre-cultured algal cells in log phase growth was transferred into a 250-mL Erlenmeyer flask, then nano-Cu and CNT suspensions under different concentrations were added into the test flask in triplicate, respectively. In algal growth inhibition tests, microalgae were exposed to 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/L nano-Cu with 5 mg/L CNTs or without CNTs in the medium. In another test, the final concentrations of CNTs were 0, 5, 50, 100, and 200 mg/L for testing the toxicity of CNTs alone. The test flasks were randomly placed in the growth incubator for 96 h under the condition in accordance with pre-cultured condition. The cell density was counted every 24 h and the 96-h growth curve was created.

Determination of chlorophyll and photosynthetic efficiency (ΦPSII)

Chlorophyll content and photosynthetic efficiency (ΦPSII) were determined by Algae Lab Analyzer based on fluorescent technique (bbe Moldaenke GmbH, Germany). After 96 h exposure, every sample was prepared with dark treatment for 20 min. Twenty milliliters of the sample was transferred into the test container. Chlorophyll content and ΦPSII were calculated by parameters of fluorescence. All data from the instrument were converted to percentage of control (% control) for easy comparison. Chlorophyll content reflected the content of algal photosynthesis and ΦPSII represented the efficiency of algal photosynthesis.

Adsorption curve

Analysis of Cu^{2+} released by nano-Cu in the f/2 medium was performed using an inductively coupled plasma-atomic emission spectrometry (Thermo Fisher Scientific, ICAP-6300). Three replicates for each treatment in each set were analyzed. Nano-Cu was dispersed in the f/2 medium to the final concentrations of 0, 5, 10, 20, 50, and 100 mg/L. Nano-Cu suspensions were shaken for 24 h and centrifuged at 8000 rpm for 20 min. Five milliliters of the supernatant liquid was taken into a volumetric flask with 5% HNO_3 to a final volume of 10 mL for Cu^{2+} determination. For another set of experiments, 50 mg/L CNTs was dispersed to nano-Cu suspensions of the same concentrations and Cu^{2+} was determined by the same method. The concentration difference of Cu^{2+} determined in two groups was the adsorbing capacity of Cu^{2+} on CNTs.

Data analysis

One-way analysis of variance with Duncan comparisons (used software SPSS 19) was applied in order to test significant differences in effects among treatments (unless otherwise noted, significance level was set at $p < 0.05$). Data were expressed as average ± standard deviation in the study. Growth inhibition ratio (IR) was calculated as follows: IR (%) = $(1 - T/C) \times 100\%$, where T and C were cell density in the experimental group and control group, respectively. The difference of IR between separate toxicity tests (only nano-Cu) and combined toxicity tests (nano-Cu with CNTs) under the same concentrations of nano-Cu represented the combined effects on microalgae.

Results and discussion

Toxicities of nano-Cu and CNTs on microalgae

For comparing with combined toxicity of nano-Cu with CNTs on the *Skeletonema costatum*, separate toxicities of nano-Cu and CNTs were determined, respectively. The algae density decreased with nano-Cu concentration increasing as shown in Fig. 2a. All treatments had a significant difference ($p < 0.05$)
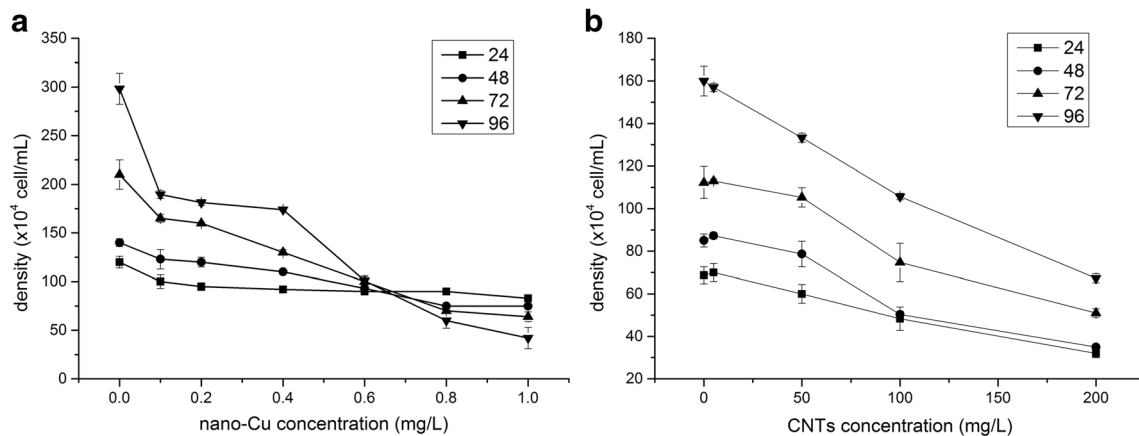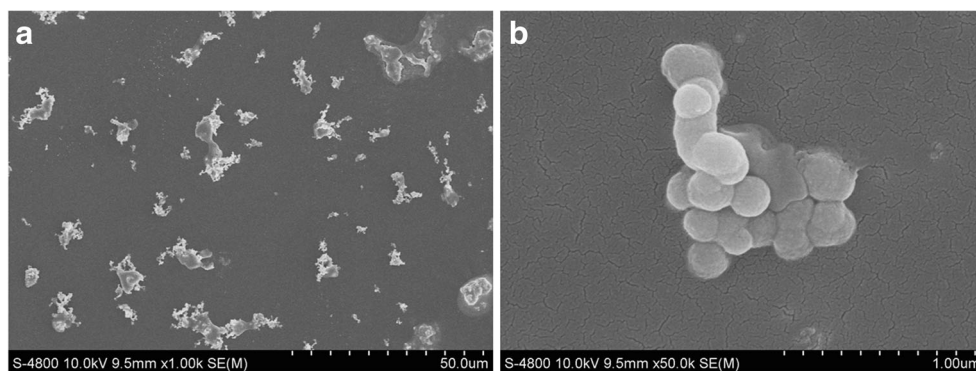

Fig. 2   The algae density under different concentrations of nano-Cu and CNTs at 24, 48, 72, and 96 h

Fig. 3 SEM images of aggregates of nano-Cu dispersed in the f/2 medium (**a** aggregates, **b** enlarged view of aggregates)

with corresponding control in every time. Under high concentrations (0.8 and 1.0 mg/L) of nano-Cu treatments, the algae density decreased with time. Toxic effects of nano-Cu on the microalgae were enhanced with increasing of nano-Cu concentration and time. The IR of nano-Cu on microalgae reached up to 86% under 1.0 mg/L concentration of nano-Cu at 96 h. Even under low concentration (0.1 mg/L) of nano-Cu treatments, the IR could reach up to 36% at 96 h. Nano-Cu could inhibit the growth of *Skeletonema costatum*.

Most studies considered that Cu^{2+} in a medium released by nano-Cu was the dominant factor of toxicity on microalgae. Müller (2015) found only 1–2% of Cu^{2+} was released to media by nano-Cu (6–7 nm); however, it resulted in a strong inhibition of micro algal photosynthesis. Minocha and Mumper, 2012 found the content of Cu^{2+} released by nano-Cu with carbon coating was four times less than that of Cu^{2+} released by nano-Cu without carbon coating, which caused lower toxicity of nano-Cu with carbon coating than without carbon coating. Nano-Cu was easy to aggregate in the f/2 medium to the average diameter of 10 μm (Fig. 3a). Big aggregates could precipitate on the bottom of Erlenmeyer flasks and reduce the probability of interactions between nano-Cu and microalgae, which had a potential to reduce the toxicity of

nano-Cu. The maximum concentration of nano-Cu in algal growth inhibition tests was 1 mg/L, which was much lower than the maximum concentration of other nanoparticles (nano-ZnO or nano-TiO$_2$) (Li et al. 2015, Manzo et al. 2013). Nano-Cu of such low concentrations could not wrap up cells of microalgae like nano-TiO$_2$ which could inhibit the growth of microalgae by wrapping up microalgae (Li et al. 2015). In addition, the dissolution of nano-Cu was also observed in the f/2 medium (Fig. 3b). The broken edge of the aggregate indicated that nano-Cu dissolved in the medium and released Cu^{2+} to the medium (Fig. 3b). In brief, the main toxicity of nano-Cu on *Skeletonema costatum* resulted from Cu^{2+} released by nano-Cu.

CNTs also had negative effects on the growth of *Skeletonema costatum*. Under low concentration (5 mg/L) of CNT treatment, the algae density had no significant difference ($p > 0.05$) with control (Fig. 2b). Then the algae density decreased with CNT concentration increasing. CNTs of high concentrations (≥ 50 mg/L) had obvious inhibition on the growth of the microalgae and the maximum IR was 58% under 200 mg/L CNT treatment at 96 h. Oxidative damage and physical damage caused by CNTs could inhibit growth and photosynthesis of microalgae (Schwab et al. 2011).
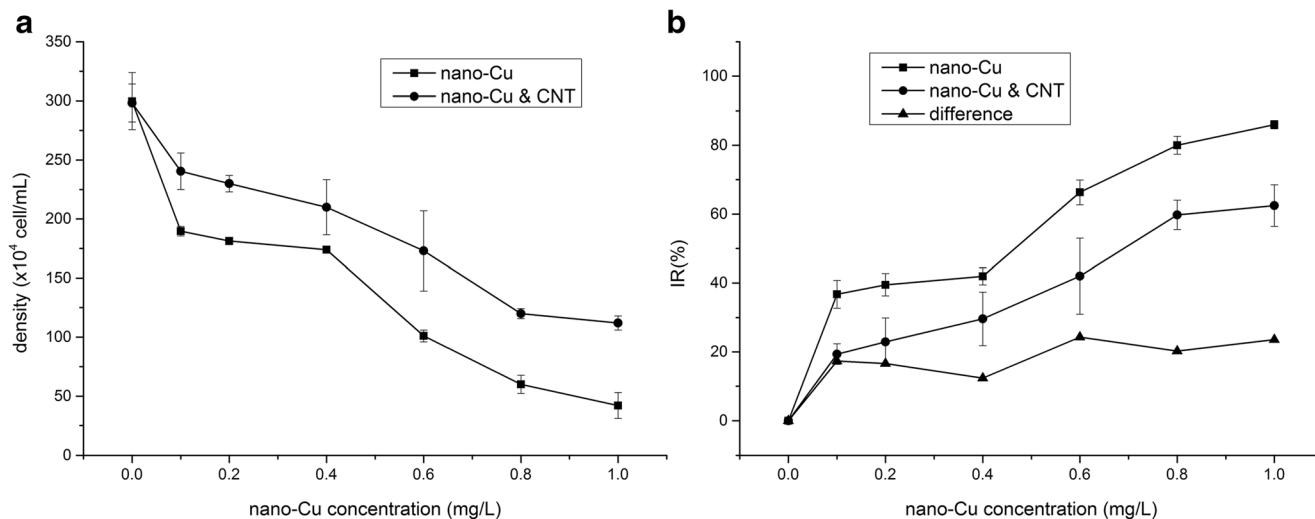

Fig. 4 Toxic effects of nano-Cu with and without CNTs on growth of *Skeletonema costatum* with 96 h exposure (**a** algal density, **b** IR)
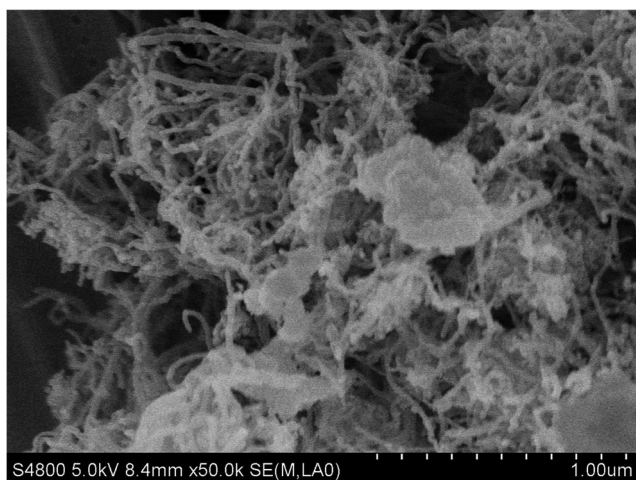
Fig. 5 SEM images of aggregates of nano-Cu and CNTs in the f/2 medium

Though CNTs of high concentrations (≥ 50 mg/L) inhibited the growth of microalgae, 5 mg/L CNTs had no effects on the growth of *Skeletonema costatum*.

Toxicities of nano-Cu with 5 mg/L CNTs on microalgae

Five milligrams per liter of CNTs had no effects on the growth of *Skeletonema costatum* in a separate experiment; however, CNTs had an influence on toxicity of nano-Cu when added in the f/2 medium. The algae density decreased with nano-Cu concentration increasing as shown in Fig. 4a. However, under the same concentration of nano-Cu treatment, the algae density with CNT treatment was significantly higher (independent sample t test, $p < 0.05$) than that without CNT treatment, which indicated toxicity of nano-Cu on the growth of the microalgae was reduced with the presence of CNTs. The IR

of microalgae decreased with adding CNTs and the difference reached up to about 20% under the same concentration of nano-Cu (Fig. 4b). The decrease of IR also indicated CNTs could reduce toxicity caused by nano-Cu. In previous study, the bioavailability of Cu and Cu^{2+} could be reduced by adsorption of them on nano-TiO_2, which decreased toxicity of Cu and Cu^{2+} on microalgae (Chen et al. 2015, Fan et al. 2011). The CNTs, hollow on the inside, had large superficial area like nano-TiO_2, which could adsorb much Cu and Cu^{2+}. Aggregation had a significant influence on transport and reactivity of nanoparticles in the environment (Hotze et al., 2010). Aggregation of nanoparticles could change their toxicities in different environment (Jiang et al., 2009). Aggregates of nano-Cu could also be combined with aggregates of CNTs to form the bigger aggregates (Fig. 5), reducing the probability of interactions between nano-Cu and microalgae. The size of aggregates between nano-Cu and CNTs could reach to a micron level (Fig. 5). Big aggregates were not easy to absorb on microalgae and could precipitate on the bottom of the medium. In contrast, nanoparticles were easy to adsorb on the surface of microalgae, increasing the probability of interactions between nanoparticles and microalgae (Ma et al., 2015). Adsorbed nanoparticles could dissolve more metal ions into cells of microalgae (Xia et al., 2008). Nanoparticles could also enter the cell as a whole, causing intracellular damages (Li et al., 2015; Zhang et al., 2016). For these two potential reasons, toxicity of nano-Cu on *Skeletonema costatum* was reduced with adding CNTs.

Results of chlorophyll content had the same trend as growth inhibition tests (Fig. 6a). Whether or not with 5 mg/L CNTs, the chlorophyll content of the microalgae decreased under nano-Cu treatments, but CNTs could reduce the negative effects of nano-Cu on chlorophyll content of the microalgae. No significant difference ($p > 0.05$) of ΦPSII
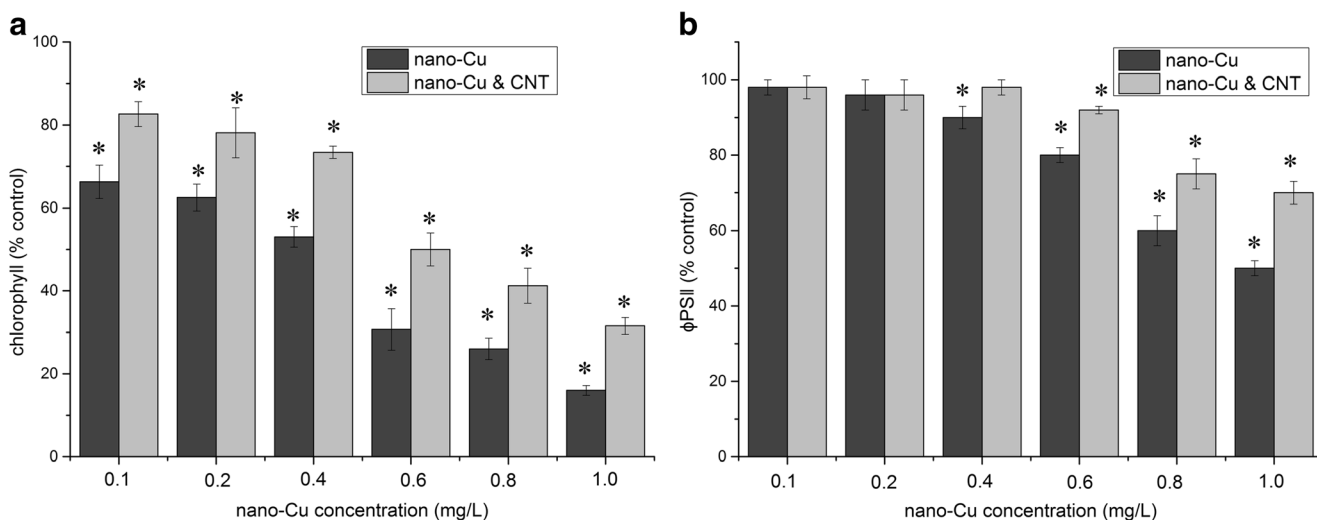

Fig. 6 Effects of nano-Cu and CNTs on photosynthesis of *Skeletonema costatum* with 96 h exposure. Asterisks represent significant difference ($p < 0.05$) with control (100%). **a** Chlorophyll. **b** ΦPS II

was found between treatments and control under 0.1 and 0.2 mg/L nano-Cu (Fig. 6b). The ΦPSII decreased under nano-Cu treatments of 0.4, 0.6, 0.8, and 1.0 mg/L. Under the same concentration of nano-Cu, the value of ΦPSII with 5 mg/L CNTs was higher than that of ΦPSII without 5 mg/L CNTs. CNTs could also reduce the negative effects of nano-Cu on ΦPSII of microalgae. Cu^{2+} could destroy the chlorophyll, inhibit cell division, and reduce the photosynthetic efficiency in algal cells (Stauber and Florence, 1987). Results obtained in photosynthesis tests inferred that the toxicity of nano-Cu on the microalgae caused by Cu^{2+} released into the medium was reduced by adding CNTs.

Adsorption of Cu^{2+} on CNTs

Adsorption tests were carried out to confirm the above inference. The concentration difference of Cu^{2+} in the f/2 medium under nano-Cu treatment with adding CNTs and without adding CNTs was determined to calculate adsorption capacity of CNTs to Cu^{2+}. The concentration of Cu^{2+} in the f/2 medium increased with nano-Cu concentration increasing, but the release rate was decreased as shown in Fig. 7. Three percent of Cu^{2+} was released to the medium under 5 mg/L nano-Cu treatment; however, only 1% of Cu^{2+} was released to the medium under 100 mg/L nano-Cu treatment. The concentration of Cu^{2+} in the f/2 medium reduced 20% with adding CNTs under 5 and 10 mg/L nano-Cu treatments. Under the high concentration of 100 mg/L nano-Cu treatment, the concentration of Cu^{2+} in the f/2 medium still reduced 15% with adding CNTs. The IR also reduced about 20% with adding CNTs in algal growth inhibition tests. Hartmann et al., 2010 found nano-TiO_2 adsorb much Cd^{2+} and reduce the bioavailability of Cd, which decreased the toxicity of Cd on *Pseudokirchneriella subcapitata*. Cerrillo et al. (2015) found humic acids could increase the stability of nano-TiO_2 and nano-CeO_2 in the medium and reduce the bioavailability of them, which reduced the toxicity of nanoparticles on
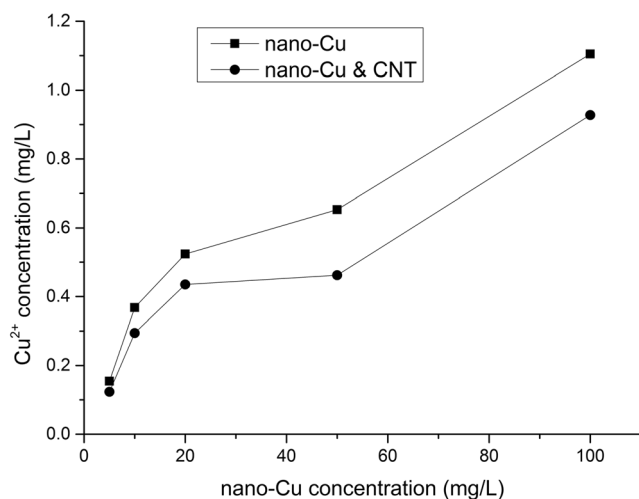

Fig. 7 The concentration of Cu^{2+} in the f/2 medium under nano-Cu and CNT treatment

microalgae. Adsorption of Cu^{2+} on CNTs decreased the concentration of Cu^{2+} in the medium. Aggregates of nano-Cu with CNTs reduce the probability of interactions between nano-Cu and microalgae. The bioavailability of Cu^{2+} and nano-Cu reduced by adsorption and aggregation results in attenuate toxicity of nano-Cu on microalgae.

Conclusion

To investigate toxicities of nano-Cu with CNTs on marine microalgae *Skeletonema costatum*, algal growth inhibition tests were carried out. Chlorophyll content and ΦPSII were determined and adsorption of Cu^{2+} on CNTs was analyzed to study toxic mechanisms of nano-Cu with CNTs. Both nano-Cu and CNTs could inhibit the growth of microalgae; however, the toxicity of CNTs on microalgae was far lower than that of nano-Cu. CNTs could reduce the toxicity of nano-Cu on growth and photosynthesis of *Skeletonema costatum*. Adsorption of Cu^{2+} on CNTs and aggregate between nano-Cu and CNTs in the medium were main reasons for attenuation of toxicity of nano-Cu with adding CNTs.

Compliance with ethical standards

All tests in the study complied with the OECD (Organization for Economic Cooperation and Development) Guidelines 201.

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