Effects of copper ions and copper nanomaterials on the output of amino acids from marine algae

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Abstract

In this study, the effects of different copper's forms, metal salt (Cu^{2+}), nano-metal (nano-Cu) and nano-metal oxide (nano-CuO), were tested on two marine algae named *Skeletonema costatum* and *Nitzschia closterium*. During a 96-hour exposure to nanoparticles (NPs) and salt, cell number, Cu^{2+} concentration in the culture medium, morphology and intracellular amino acids was measured to assess the toxicity of those copper materials and the toxicity mechanism of NPs. It was found that the toxicity of Cu^{2+}, nano-Cu and nano-CuO on marine phytoplankton decreased in order. The EC_{50} values of Cu^{2+} and nano-Cu for *S. costatum* and *N. closterium* ranged from 0.356 to 0.991 mg/L and 0.663 to 2.455 mg/L, respectively. Nano-Cu inhibited the growth of marine phytoplankton mainly by releasing Cu^{2+}, however, nano-CuO mainly produced toxic effects on microalgae through the effect of NPs. The secretion of extracellular polymeric substances by microalgae could be another possible reason for nano-Cu and nano-CuO to impose implications for microalgae. *S. costatum* was more sensitive to copper than *N. closterium*. Cu^{2+}, nano-Cu and nano-CuO all reduced the total output of algae-derived amino acids by affecting the growth of phytoplankton and per-cell amino acids. This manuscript is of important implications to fill the data gaps for nano-Cu and nano-CuO risk assessment on marine algae.

1. Introduction

Nanoparticles (NPs) refer to particles with a nanometer-scale structural characteristic, generally ranging from 1 ~ 100 nm in least two dimensions (Shi 2007). Nanomaterials have been widely used in aerospace, electronics, chemical, metallurgy, military, nuclear, medical and biological engineering industry since the 1960s because of their outstanding properties (Zhai et al. 1999). Copper is an essential metal element for the growth of marine microalgae (Marangoni et al. 2017). An excessive amount of copper leads to significant adverse effects on algae, such as pool growth, inhibition of photosynthesis, and even death of cells in severe cases (Wang et al. 2018; Fawaz et al. 2018; Guo et al. 2019).

Nano-Cu and nano-CuO are wildly used for catalysis, drug additives, superconducting materials, etc., and tend to accumulate in the ocean during laboratory research, industrial production, transportation, consumption and disposal (Cui et al. 2013). Thus, it is necessary to study their ecotoxicity to nature environment. Marine microalgae are regarded as the significant primary producer in the ocean and the important source of marine amino acids (Li et al. 2009; Zhang et al. 2015), which are the most widely used test organism (Bondarenko 2016; Wang et al. 2019; Nguyen et al. 2020). Previous studies have reported nano-Cu and nano-CuO toxicity on freshwater algae (Müller et al. 2016; Zhao et al. 2016).

This study aimed to investigate the toxic effects and mechanism of different copper NPs on marine algae *Nitzschia closterium*, a commonly used marine bait in aquaculture (Sun et al. 2016), and *Skeletonema costatum*, a red tide species commonly found near the offshore area of China (Li et al. 2017). Cu^{2+} concentrations in the culture media were analyzed to assess the impact of NPs on microalgae. The number of cells and the cell morphology of algae observed by scanning electron microscope (SEM) were

used to assess the toxicity mechanism and the interactions between NPs and microalgae. The production of algae-derived amino acids was determined to explore the effects of NPs on the ecosystem.

2. Materials And Methods

2.1. Chemicals

$CuSO_4 \cdot 5H_2O$ (purity > 99.9%) was purchased from Sinopharm Chemical Reagent Co., Ltd, China. Nano-copper powder (nano-Cu, purity > 99.9%, 10–30 nm) and nano-copper oxide powder (nano-CuO, purity > 99.5%, 40 nm) were purchased from Shanghai Aladdin Co., Ltd., China. NPs suspensions and $CuSO_4 \cdot 5H_2O$ solutions were prepared according to the procedure used by Zhang (2018). The SEM (Fig. S1) of nano-Cu, similar to nano-CuO, were shown in the previous study at our laboratory (Zhang et al. 2018). Hydrodynamic diameter (HDD) of nano-Cu and nano-CuO was measured by dynamic light scattering using a Malvern Zetasizer (Nano ZS, Malvern Panalytical Ltd, UK) every 24 hours up to 96 hours at the concentration of 10 mg/L to assess the aggregation of nano-Cu and nano-CuO in seawater (Fig. S2). The NPs tended to gather in the seawater, and were of stability in the experiment period with an average size of 194 nm for nano-Cu and 525 nm for nano-CuO. Zeta potential of nano-Cu and nano-CuO was also measured at the concentration of 10 mg/L at 24 h using Malvern Zetasizer. Zeta potential of nano-Cu and nano-CuO was − 13 mV and − 19 mV, respectively.

2.2. Algae cultures

The test species, *N. closterium* and *S. costatum*, were obtained from the Algal Center of Key Laboratory of Marine Chemistry Theory and Technology, Ocean University of China.

Algae were cultivated in the incubator under fluorescent lights (4000 lx) with a 12 h light-dark cycle at 20 ± 1°C in f/2 medium (Guillard and Ryther 1962) based on sterile filtered (0.45 μm) seawater collected from the coastal sea near Qingdao, China. Containers were shaken three times every day to avoid the sedimentation of microalgae. The toxicity test was performed as the exponential growth phase approached.

2.3. Test methods

2.3.1. Inhibition test of Cu^{2+}, nano-Cu and nano-CuO on microalgae

The inhibition test was performed in 96 h with the Guideline 201 of OECD (OECD Test No. 201 2011) adopted. *N. closterium* and *S. costatum* in the exponential growth phase were added into f/2 medium in several 500 mL Erlenmeyer flasks. Cu^{2+} and well-dispersed nano-Cu and nano-CuO were then added into the flasks, respectively. Flasks were shaken three times every day to avoid the sedimentation of microalgae and allow gas exchange with outside air, and placed randomly to remove the influences of light or temperature.

According to previous studies (Aruoja et al. 2009; Manusadžianas et al. 2012; Li et al. 2015; Zhang et al. 2018) and our pre-test, the concentrations of copper materials were set as follow. In the *N. closterium* treatment, copper was added at concentrations of 0.1, 0.3, 0.5, 0.7 and 1.0 mg/L for Cu^{2+}, 0.5, 1.0, 1.5, 2.5 and 5.0 mg/L for nano-Cu, and 5, 10, 30, 50 and 70 mg/L for nano-CuO. In the *S. costatum* treatment, Cu^{2+} was of 0.05, 0.15, 0.20, 0.50 and 1.00 mg/L, nano-Cu of 0.1, 0.5, 1.0, 2.0 and 5.0 mg/L and nano-CuO of 1, 5, 10, 20 and 50 mg/L, respectively. The treatment without any copper material was set as control (0 mg/L). Each of the treatments was run in triplicates. The cultures were sampled every 24 h for counting cell numbers using an inverted biological microscope with a hemocytometry. Samples after 48 h exposure were collected to access growth inhibition ratio (*IR*) (calculated according to the following Eq. (1)) and concentration for 50% of maximal effect (EC_{50}).

$$IR\% = (1 - T/C) \times 100\% \quad (1)$$

which *T* is the density of microalgae cells in the treatment group, and *C* is the density of microalgae cells in the control group (Šepič et al. 2003).

2.3.2. Measurement of Cu^{2+} concentration

The concentrations of Cu^{2+} in the cultures were measured using Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Thermo Fisher Scientific, USA) every 24 hours up to 96 hours at the concentration of 5 mg/L for nano-Cu and 50mg/L for nano-CuO to assess the kinetics of ion release from the particles (Fan et al. 2012; Kim et al. 2013).

2.3.3. Scanning electron microscope for algae

S. costatum exposed for 96 h were collected and centrifuged at 1509 × g for 10 mins for cell morphology with the SEM. SEM measurements were performed on a Hitachi S-4800 SEM with luminescence (Japan). The sediment was collected and kept at 4°C for 12 h with glutaraldehyde (2.5%) added, then washed three times with 0.1 mol/L phosphate buffer solution (PBS, pH = 7.4) and were gradient dehydrated with ethanol-water solutions of different concentrations (30%, 50%, 70%, 90% and 100%) in sequence. Each dehydration step lasted 20 min. The dehydrated samples were kept in tert-butanol at 4°C and freeze-dried, then were coated on the conducting resin and observed.

2.3.4. Analysis of intracellular amino acids

The type and amount of intracellular amino acids (IAA) measurements were performed on a Hitachi L-2000 high performance liquid chromatography (HPLC, Japan). Fourteen types of amino acids measured were aspartic acid (Asp), glutamic acid (Glu), serine (Ser), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), valine (Val), methionine (Met), phenylalanine (Phe), isoleucine (Ile) and leucine (Leu) respectively (Zhang et al. 2015).

The cultures were centrifuged (1509×g, 10 min) after 48 h exposure to collect algal cells. Then algal cells were washed with Miller-Q water three times to ensure no extracellular organic matter was left. The algal cells were disrupted using cell disruptor to ensure that intracellular organic matter (IOM) was released.

The samples were then centrifuged (4193×g, 15 min) to obtain supernatant containing IOM for total IAA analysis (Zhang, P.Y. et al. 2016). The supernatant, ascorbic acid and HCl (6 mol/L) were added into the ampoule bottles, which were then flame sealed under N_2 atmosphere. The samples were hydrolyzed at 110°C for 22 h then cooled. Milli-Q water was added to dissolve the dried hydrolysate for derivatization before analysis. A modified pre-column o–Phthaldialdehyde derivatization method (Zhang, P.Y. et al. 2016; Lindroth and Mopper 1979) was used before determining the samples with HPLC. Gradient elution separation was performed on HPLC for amino acid determination. The mobile phases were (A) methanol: acetonitrile: Milli-Q water = 1:1:1 (v/v/v) and (B) acetic acid buffer (0.05 M, pH = 7.2): tetrahydrofuran = 100:1 (v/v).

2.4. Statistical analysis

Statistical analysis was performed using SPSS 21. The normality of was checked by the Shapiro–Wilk's normality test ($p > 0.05$ in all treatments), and then homogeneity of variance for data was assessed with the Levene test ($p > 0.05$ in all treatments). One-way analysis of variance (ANOVA) with a least significant difference (LSD) post hoc-test ($p < 0.05$) was performed to test differences in *IR* and per-cell IAA content among different concentration groups in each copper material treatment. In the figures, values are presented as mean ± standard deviation (SD, shown as error bars), and the results of LSD´s test were performed as different small letters which means statistically significant differences ($p < 0.05$) exist between groups marked with different small letters.

Toxicity regression equations fitting and EC_{50} calculation were performed under a confidence interval of 95% with Probit analysis adapted (Wu et al. 2014; Feng et al. 2019). Inhibition ratio, concentration and sum were set as variables, and EC_{50} of different copper materials were calculated though Probit process with covariate transformed into log base 10. Probit model in the parameter estimate was the toxicity regression equations for different copper materials. Chi-square test was used to check the fitting degree of toxic regression equations. $\chi^2 < \chi_{0.05(3)}{}^2 = 7.815$ (df = 3) and $p > 0.050$ mean toxicology regression equation was well-fitted (χ means copper concentration transformed into log base 10). When $\chi^2 > \chi_{0.05(3)}{}^2 = 7.815$ (df = 3) and $p < 0.150$, heterogeneity factors were applied in the calculation of the confidence limits (details see Tab. S2).

3. Results

3.1. Algae responded to Cu^{2+}, nano-Cu and nano-CuO exposure

The exposure to Cu^{2+}, nano-Cu and nano-CuO had significant effects on algal growth. As shown in Fig. 1 (data in Tab. S1), the *IR* increased with the increasing concentrations and exposure time, which performed different levels according to the types of material.

For *N. closterium* treatment (Fig. 1A), the *IR* of Cu^{2+} exceeded 50% at the concentration of 0.7 mg/L, and reached 69.8% at the highest concentration of 1.0 mg/L. As for nano-Cu exposed, *IR* reached 59.6% at the concentration of 2.5 mg/L and 64.5% at the highest concentration of 5.0 mg/L. Even at the maximum concentration of 70 mg/L for nano-CuO, the *IR* was only 34.9%.

The *IR* of *S. costatum* increased with the increasing copper concentrations (Fig. 1B). *IR* exceeded 50% at the concentration of 0.5 mg/L for Cu^{2+}, and reached 87.7% at the highest concentration of 1.0 mg/L. *IR* reached a high level of 78.2% under 2.0 mg/L of nano-Cu. *IR* was only 40.5% under the maximum nano-CuO concentration of 50 mg/L. The significant differences ($p < 0.05$) in all treatments indicated that Cu^{2+}, nano-Cu and nano-CuO were toxic to *N. closterium* and *S. costatum*.

In order to compare the sensitivity of *S. costatum* and *N. closterium* to different forms of copper, 48 h toxicity regression equations and EC_{50} were calculated and shown in Table 1 (more details in Tab. S2). The EC_{50} of Cu^{2+} for *S. costatum* was 0.356 mg/L, less than 0.663 mg/L for *N. closterium*. Similarly, the EC_{50} of nano-Cu for *S. costatum* was 0.991 mg/L, less than 2.455 mg/L for *N. closterium*, indicating that *S. costatum* was more sensitive than *N. closterium* with a tendency for Cu^{2+} to be of more inhibitory than nano-Cu. EC_{50} of nano-CuO performed large errors because the inhibition ratios were less than 50% in all nano-CuO treatments, thus not shown.

Table 1
EC_{50} of nano-Cu and nano-CuO on *Skeletonema costatum* and *Nitzschia closterium*

Microalgae	Copper Material	EC_{50}(mg/L)
N. closterium	Cu^{2+}	0.663
	Nano-Cu	2.455
S. costatum	Cu^{2+}	0.356
	Nano-Cu	0.991

3.2. Cu^{2+} concentration in the cultures

Cu^{2+} dissolution in culture medium at the concentration of 5 mg/L for nano-Cu and 50mg/L for nano-CuO showed that ion dissolution rates of nano-Cu and nano-CuO seem to decline with time (Fig. S3). Cu^{2+} concentrations in the cultures of *S. costatum* at 96 h were presented in Fig. 2, showing a tendency that higher concentrations of Cu^{2+} were measured in seawater as more copper materials added. The result was also corroborated with Li (2015). When the concentration of nano-Cu was 5 mg/L, the concentration of Cu^{2+} in the culture medium was 0.68 mg/L, which was about the same level with 1 mg/L of Cu^{2+} added, and they both showed high inhibition ratios on *N. closterium* and *S. costatum*. The

concentration of Cu^{2+} was only 0.24 mg/L in seawater with 50 mg/L of nano-CuO added, which also indicated that nano-Cu was more likely to release Cu^{2+} than nano-CuO.

3.3. Cell morphology

Under nano-Cu stress condition, the changed morphology of *S. costatum* and broken siliceous thorns were observed in Fig. 3. Extracellular polymeric substances (EPS) released by algae were also observed (arrow a).

3.4. Effect of Cu^{2+}, nano-Cu and nano-CuO on algae-derived amino acids

IAAs at 48 h significant changed as shown in Fig. 4. Amino acid decreased gradually with the increasing exposure concentrations, which suggested that copper materials inhibited biosynthesis of total IAA. Large errors were observed in treatments with low cell density (for example, treatments under 1.0 mg/L of Cu^{2+}, 2.0 and 5.0mg/L of nano-Cu), thus not shown in the figure.

IAAs of *N. closterium* decreased from 3994.9 nmol/L to 3391.4 nmol/L and 2686.0 nmol/L (84.9% and 67.2% of the control) as exposure concentration of Cu^{2+} were 0.1 and 1.0 mg/L, respectively. The concentrations of IAA exposed to 5.0 mg/L of nano-Cu and 70 mg/L of nano-CuO were 1948.1 nmol/L (48.8% of the control) and 3038.3 nmol/L (76.1% of the control), respectively. Under nano-CuO exposure, no significant decline of IAA was detected in compared to Cu^{2+} and nano-Cu. The main composition of IAA found in algae were Gly, Glu, Ser and Ala with total content more than 50% of total IAA (Fig. 4).

For *S. costatum*, IAA also gradually decreased with the increasing concentrations of Cu^{2+}, nano-Cu and nano-CuO. The concentration of IAA decreased from 1284.77 to 534.02 nmol/L (41.6% of the control) as exposed to 1.0 mg/L of Cu^{2+} for 48 h. IAA reduced by approximately 53.5% for 1.0 mg/L of nano-Cu and 52.9% for 50 mg/L of nano-CuO, respectively. Gly, Glu, Ser and Ala accounted for more than 40% in total IAA of *S. costatum*. Like *N. closterium*, the proportion remained fairly stable.

To further investigate the effect of copper on IAA of microalgae, the changes of per-cell amino acids were analyzed and shown in Fig. 5 (data see Tab. S3). The IAA of single cells for *N. closterium* fluctuated between 9.83 and 20.14 nmol/L, and significant differences were observed among Cu^{2+} and nano-Cu treatments ($p < 0.05$) with a increasing trend. Meanwhile, it was slightly different from nano-CuO treatment with a modest rise as concentrations increased. However, no significant differences were observed in all *S. costatum* treatments (Fig. 5B, $p > 0.05$).

4. Discussion

4.1. Effect of Cu^{2+}, nano-Cu and nano-CuO on the growth of microalgae

In this research, it was found that the growth of phytoplankton was significantly inhibited when exposed to metal salt (Cu^{2+}), nano-metal (nano-Cu) and nano-metal oxide (nano-CuO) with a rising trend of inhibition ratios as exposure concentrations increased. Different microalgae responded differently to copper materials, and *S. costatum* was more sensitive than *N. closterium*. Copper is one of necessary trace elements in growth of phytoplankton (Leusch et al. 1995). Nevertheless, it has been proved to inhibit the photosynthesis of phytoplankton, reduce the amount of chlorophyll, and lead to changes in cell morphology and enzyme activity at high concentrations (Stampoulis et al. 2009; Lu et al. 2010; El-Kassas et al. 2017; Guo et al. 2019).

Li (2015) reported that nano-Cu (10-30nm) was toxic to *S. costatum* with a 96 h EC_{50} of 0.10 mg/L, which was less than our results. A possible explanation for this might be the different exposure time chosen. According to Joonas (2019), 72 h EC_{50} of nano-CuO (22–25 nm) to the diatom *Fistulifera pelliculosa* was 0.65 mg/L, which educed that *F. pelliculosa* was a more sensitive marine algae than the two diatoms used in our research. Details of comparison were listed in Table 2. We also found the toxicity of Cu^{2+}, nano-Cu and nano-CuO decrease in order. This finding supports evidence for previous observations that the ionic form of metals is more toxic than NPs (Batley et al. 2012; Bielmyer et al. 2006; Turan et al. 2019).

Table 2
List of EC_{50} about nano-Cu or nano-CuO on marine algae.

Species	Test material	Exposure time and endpoint	EC_{50} on compound basis, mg/L	EC_{50} on metal basis, mg Cu/L	Primary size (method used for determination), coating	Reference
Skeletonema costatum	Nano-Cu	96h EC_{50}	0.10	0.08	10–30 nm (provider), uncoated	Li et al. 2015
Fistulifera pelliculosa	Nano-CuO	72h EC_{50}	0.65	0.52	22–25 nm (provider), uncoated	Joonas et al. 2019
Skeletonema costatum	Nano-Cu	48h EC_{50}	0.99	0.79	10-30nm (provider), uncoated	This research
Nitzschia closterium	Nano-Cu	48h EC_{50}	2.46	1.96	10-30nm (provider), uncoated	This research

4.2. Inhibition mechanisms of nano-Cu and nano-CuO on microalgae

Toxicity mechanisms of NPs on algae include changing cell membrane structure or potentials, oxidizing protein, causing genetic mutation, interrupting energy transfer, inducing activated oxygen or releasing ions (Klaine et al. 2008). The results (Fig. 2) revealed that nano-Cu and nano-CuO could release Cu^{2+} into seawater. Both nano-Cu and comparable amount of Cu^{2+} showed considerable inhibition ratios on

microalgae, which suggested that the toxicity of nano-Cu was mainly due to Cu^{2+} released into water, and this finding was also proved by Li (2015) and Bondarenko (2012). Some researchers believed that ion release resulted in the toxicity of NPs (Jo et al. 2012; Kim et al. 2011). Wong (2010) conducted experiments on two diatoms and found that zinc ions release was nano-ZnO's main toxicity mechanism. Yuan (2013) declared that silver NPs released Ag^{+} into solution, which inhibited the growth of *Chlorella vulgari*. The results also suggest that nano-CuO hardly dissolved Cu^{2+} into seawater at low concentrations (0–10 mg/L) compared to nano-Cu, indicating that the possible transformation pathways of nano-Cu and nano-CuO into Cu^{2+} could be different. Nano-Cu underwent electrochemical corrosion with dissolved oxygen or other oxidants in seawater. Copper acted as an anode in the oxidation reaction (Eq. (2)) and released Cu^{2+} into seawater (Wang et al. 1997; Huang et al. 2017).

$$Cu \rightarrow Cu^{2+} + 2e^{-} \quad (2)$$

The covalent bonds of CuO are quite stable and not easy to be broken, which could hardly produce Cu^{2+}. A small amount of free H^{+} in seawater could combine with nano-CuO to produce Cu^{2+} according to reaction Eq. (3). The amount of Cu^{2+} produced by nano-CuO was much less than that by nano-Cu.

$$Cu + 2H^{+} \rightarrow Cu^{2+} + H_2O \quad (3)$$

This outcome is inconsistent with that of Wu (2020), who found no significant difference in ion concentrations between nano-Cu and nano-CuO dissolved in the water. This could mainly result from that they have chosen nano-Cu with a 1.4 nm oxidized copper outer shell (diameter < 50 nm) and nano-CuO (diameter < 50 nm) as test materials the similar characteristics. The oxidized copper shell outside of the nano-Cu might participate in the ion release at first, leading to the similar dissolved ion concentration with nano-CuO.

The concentration of 1 mg/L nano-CuO still inhibited the growth of *S. costatum* (inhibition ratio of 5.7%) with negligible dissolution of Cu^{2+}, indicating the toxicity of nano-CuO could not be mainly expressed by the dissolved fraction (Ye et al. 2017; Xiang et al. 2011; Nguyen et al. 2020). Cui et al. (2013) also found nano-CuO was of higher toxicity than comparable amount of Cu^{2+} on goldfish algae, and they attributed this to the special surface effect and quantum size effect of nano-CuO.

Nano-Cu or nano-CuO aggregated together and formed large-sized NPs, directly contacting with microalgae. Effects of nanomaterial can be determined as mechanical damage and shading effect (Zhao et al. 2016; Toh et al. 2016; Wang et al. 2016; Zhao et al. 2017). Physical damage of cell membranes with CuO NP exposure were also observed by Zhao et al. (2016), who found this exposure caused much stronger damage than the released Cu^{2+} ions did. NPs also covered the surface of microalgae, causing the shading effect which, in addition, hindered the microalgae to exchange substance and energy with the

surrounding environment, thus inhibiting the growth of microalgae (Tang et al. 2015; Zhang C. et al. 2016; Chen et al. 2017).

It was reported that many algae secreted EPS, especially in high cell density or under environmental stress due to light and nutrient restriction or excessive heavy metal, etc. (Staats et al. 2000; Passow 2002; Underwood et al. 2004). EPS could help microalgae adjust the interaction between NPs and cell membranes (Unrine et al. 2012; Zhao et al. 2019). It was hypothesized that EPS reduced the possibility of direct contact between NPs and cells through adsorbing NPs or adhering to the surface of cells which separated the NPs from outside (Zhou et al. 2016) and induces cell aggregation to reduce the specific surface area of algae. EPS could also adsorb part of Cu^{2+} to alleviate the adverse effects of released ions, and this need further research. However, the EPS was in small amount and in filamentous shape with minimal possibility of absorbing NPs and ions, but causing collisions between algae cells and EPS (Long et al. 2015). Thus, it was suggested that EPS hardly protect the algae under nano-CuO because the toxicity of nano-CuO was mainly caused by ion release, but EPS could partly alleviate the toxicity of nano-CuO because its toxicity was mainly caused by nanomaterial effects.

In conclusion, there are three ways which exerted influence on algae cells by nano-Cu and nano-CuO as schematic diagram (Fig. 6, taking *S. costatum* for example) shown: ① ion release; ② direct contact; ③ stimulating algae cells to secret EPS. Releasing ion was the main toxicity mechanisms for nano-Cu, while direct contact was for nano-CuO. The secretion of EPS could be the protective mechanism to some extent.

4.3. Effect of Cu^{2+}, nano-Cu and nano-CuO on algae-derived amino acids

According to this research, Gly, Glu, Ser and Ala were the main types of amino acids both in *N. closterium* and *S. costatum*, and the content of which dropped as more severe inhibition on the growth of algae. In contrast, the percentage of these four kinds of amino acids in the total amino acid content remained stable as concentrations increased (shown as line in Fig. 4), indicating the proportion was not affected by copper.

The addition of Cu^{2+}, nano-Cu and nano-CuO led to the decline of cell density and changes in per-cell IAA, which resulted in the significant decrease of total IAA. The difference in change of per-cell IAA may result from the characteristics of different algae. Microalgae could cope with copper by self-regulating, mainly performed as changes in types and content of proteins (Wong et al. 2010). Besides, it has been reported that organisms, especially monads, adapt to environmental stress by changing cell size or specific surface area (Xue et al. 2018). Toxic substances were possible to result in retard of chloroplast division and prevent the protoplast from dividing at low concentration (Li et al. 2019; Liu et al. 2016; Gu et al. 2017), thus leading to the increase in cell size by growing but not dividing (Chao and Chen 2001; Machado et al. 2014). In this situation, amino acid content in single cells increased, which corresponded to the *N. closterium* treated with Cu^{2+} and nano-Cu at high concentrations. As the concentration of toxic

substances increased, algal cells' physiological function (such as photosynthesis and respiration) was further attenuated (Li et al. 2019; Liu et al. 2016; M'Rabet et al. 2018). And the cells could no longer cope with heavy metals by changing the cell volume. The cell volume was equivalent to the control group or even shrank, so there was no significant difference in the per-cell amino acid content, which corresponded to the treatment group of *S. costatum* which was poorly resistant to copper. It could be concluded that the prime cause of changes in per-cell amino acid content mainly lies in the influence on the growth cycle of microalgae cells and the inhibitory effect on algal reproduction and division, which in turn affects the cell volume and size, thus making the single cell amino acid content change.

5. Conclusion

The toxicity of Cu^{2+}, nano-Cu and nano-CuO reduced in order, showing time and dose effects. Compared to the significant inhibition ratios by Cu^{2+} with 48 h EC_{50} ranging from 0.356 to 0.991 mg/L and nano-Cu from 0.663 to 2.455 mg/L, nano-CuO showed low inhibitory effects even at high concentrations, in which *S. costatum* was more sensitive than *N. closterium*. Inhibition of algae by nano-Cu and nano-CuO, substances with different dissolved mechanisms, was induced in different modes. Nano-Cu mainly inhibited the growth of marine phytoplankton by releasing Cu^{2+}. But nano-CuO mainly inhibited the growth of microalgae through NP effects, i.e., mechanical damage and shading effect. Causing collisions between algae and EPS, the EPS could alleviate nano-CuO's toxicity, but made no effects to protect algae from nano-Cu poison. The inhibition of heavy metal on phytoplankton could alter the output of algae-derived amino acids to the ocean by impacting on cell density and per-cell IAA which changed with the cell volume.

Declarations

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- Ethics approval and consent to participate

Not applicable

- Consent for publication

Not applicable

- Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

- Competing interests

The authors declare that they have no competing interests.

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- Authors' contributions

Wenqiu Huang: Term, Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing-Original Draft, Validation, Writing-Review & Editing.

Yuping Zhou: Validation, Methodology.

Ting Zhao: Investigation.

Liju Tan: Resources, Funding acquisition.

Jiangtao Wang: Resources, Writing-Review & Editing, Funding acquisition, Supervision.

All authors read and approved the final manuscript.

Abbreviations

EPS	extracellular polymeric substances
HDD	hydrodynamic diameter
IAA	intracellular amino acids
IOM	intracellular organic matter
IR	inhibition ratio
N closterium	*Nitzschia closterium*
NPs	nanopaerticles
S. costatum	*Skeletonema costatum*
SEM	scanning electron microscope

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Figures

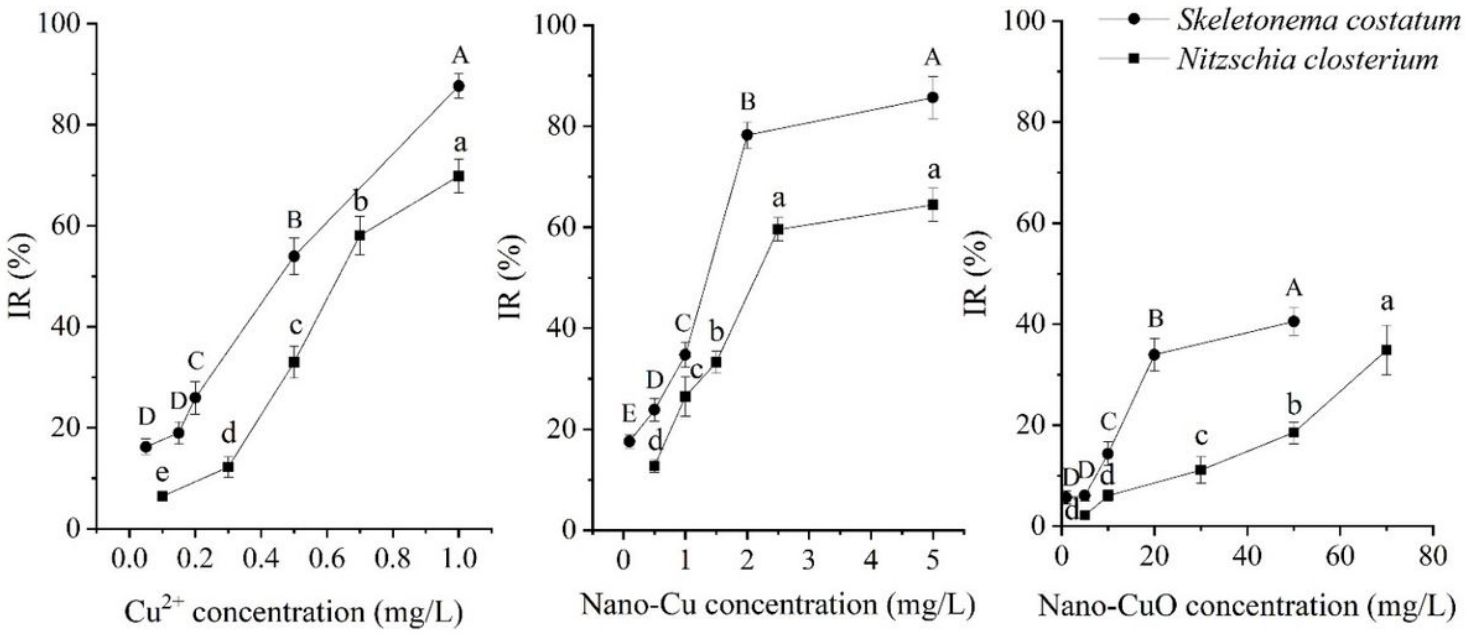

Figure 1

Inhibition ratios of microalgae after 48 h exposure. The different letters indicated statistically significant differences ($p < 0.05$) different concentration groups in each copper material treatment. The capital letters were used for S. costatum, and the small letters were used for N. closterium. Error bars: standard deviation.

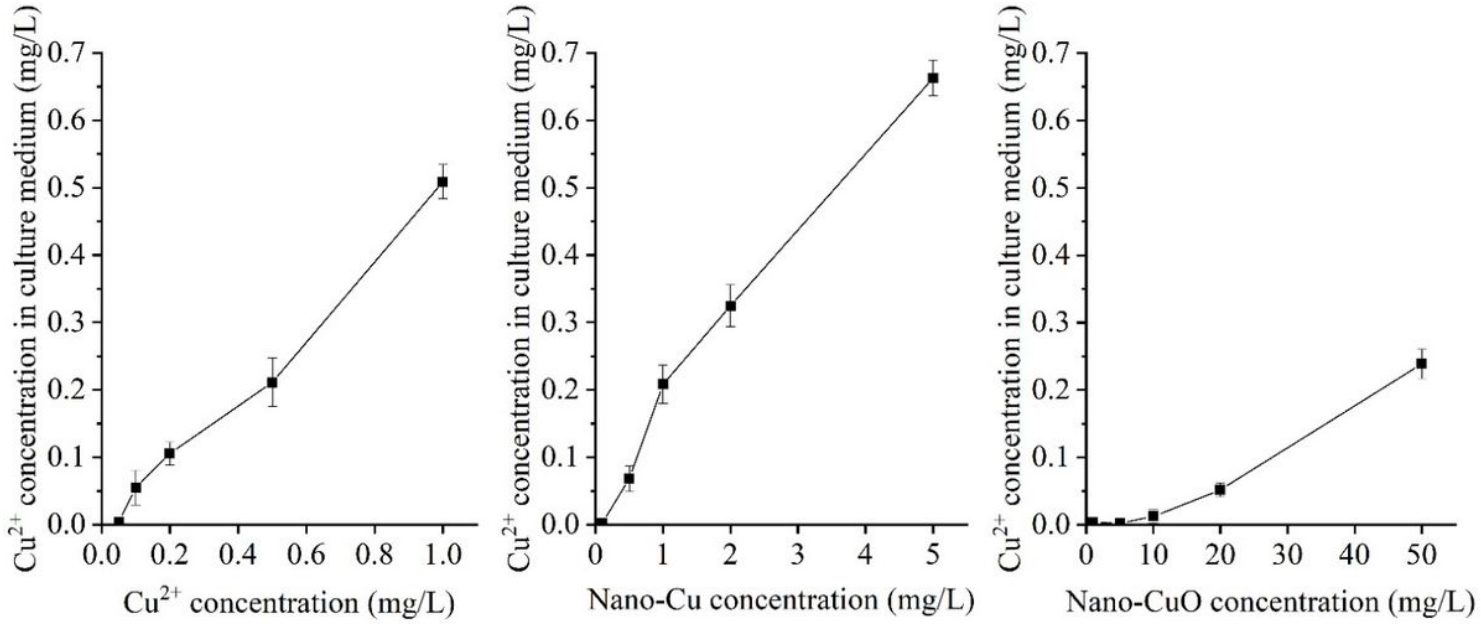

Figure 2

Cu2+ concentration in the culture medium of S. costatum. Error bars: standard deviation.

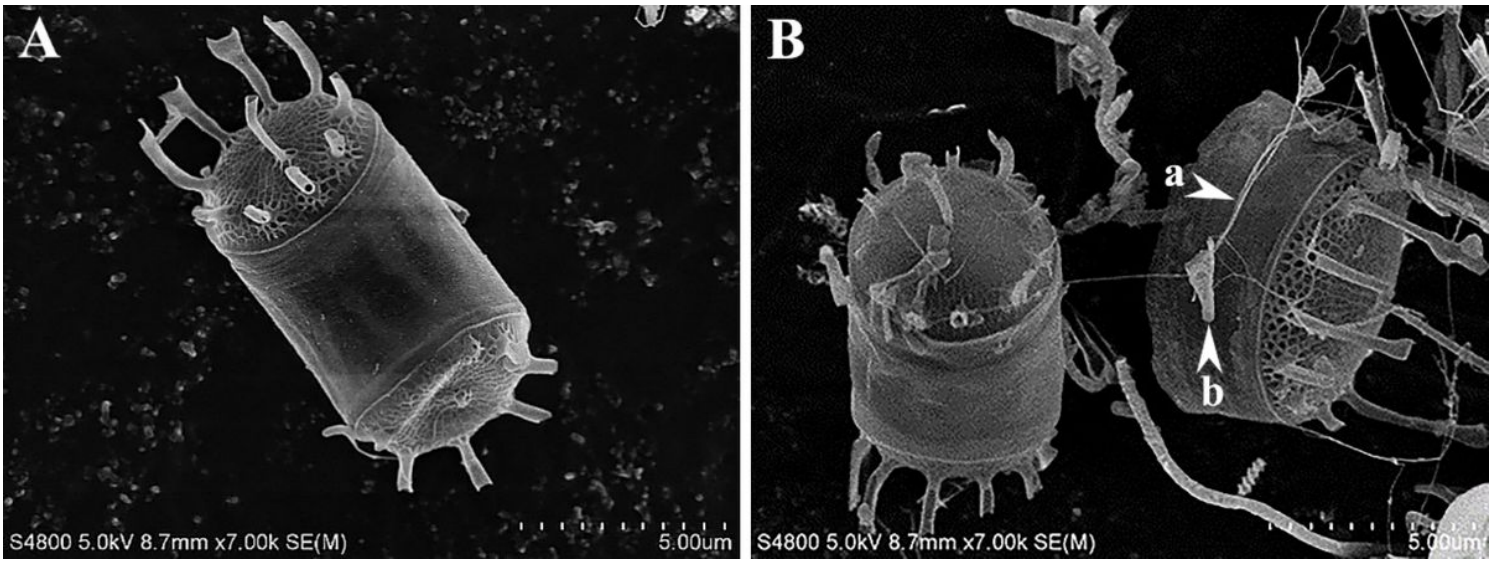

Figure 3

SEM images of S. costatum exposed to nano-Cu (A: Control, B: treatment with nano-Cu). Arrow a: extracellular polymeric substances. Arrow b: broken siliceous thorns.

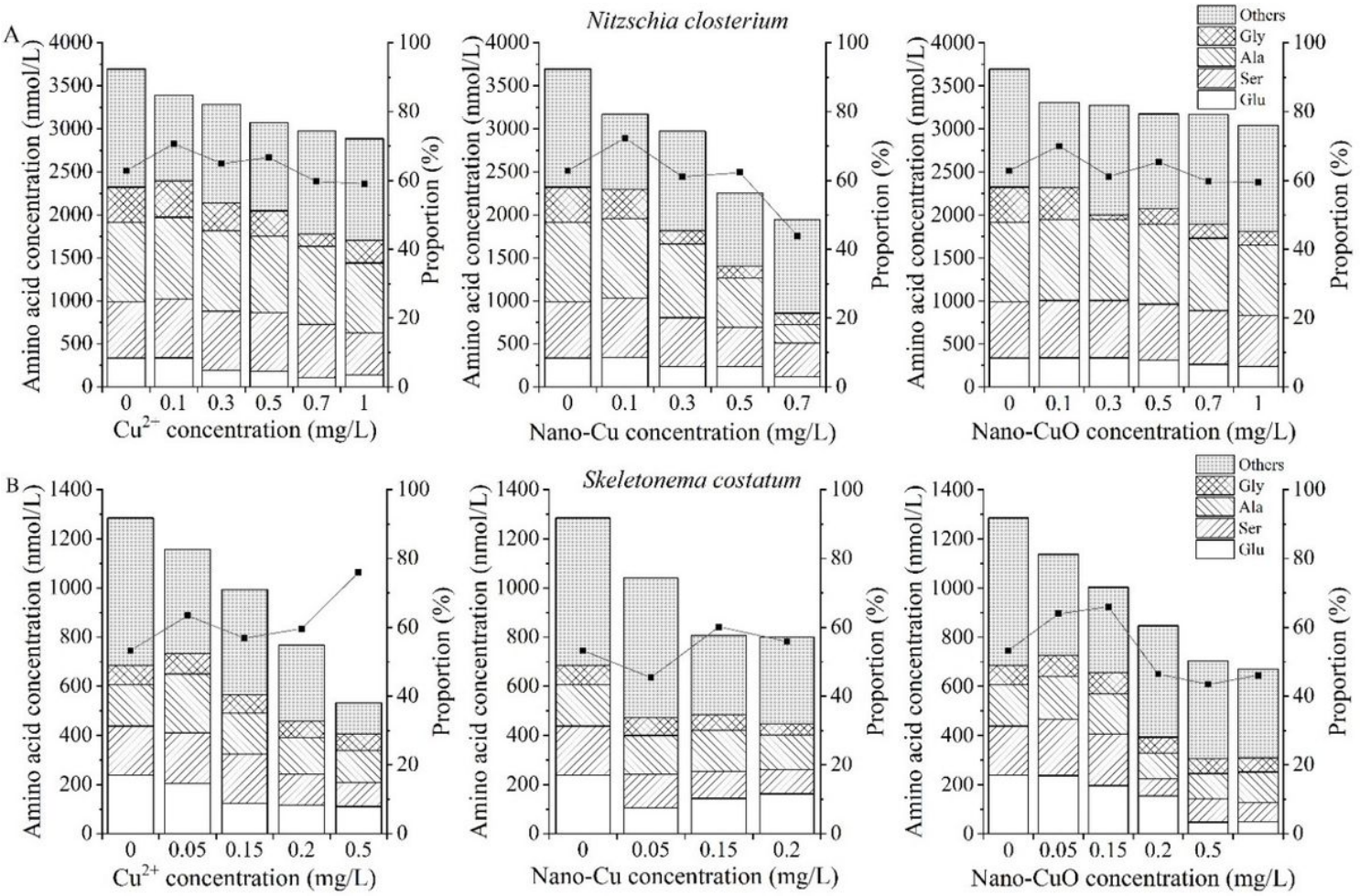

Figure 4

Intracellular amino acids composition and proportion for N. closterium (A) and S. costatum (B) exposed to Cu2+, nano-Cu and nano-CuO at 48 h. Left Y-axis and bar: intracellular amino acids concentrations. Right Y-axis and line: the proportion of four major amino acids in intracellular amino acids.

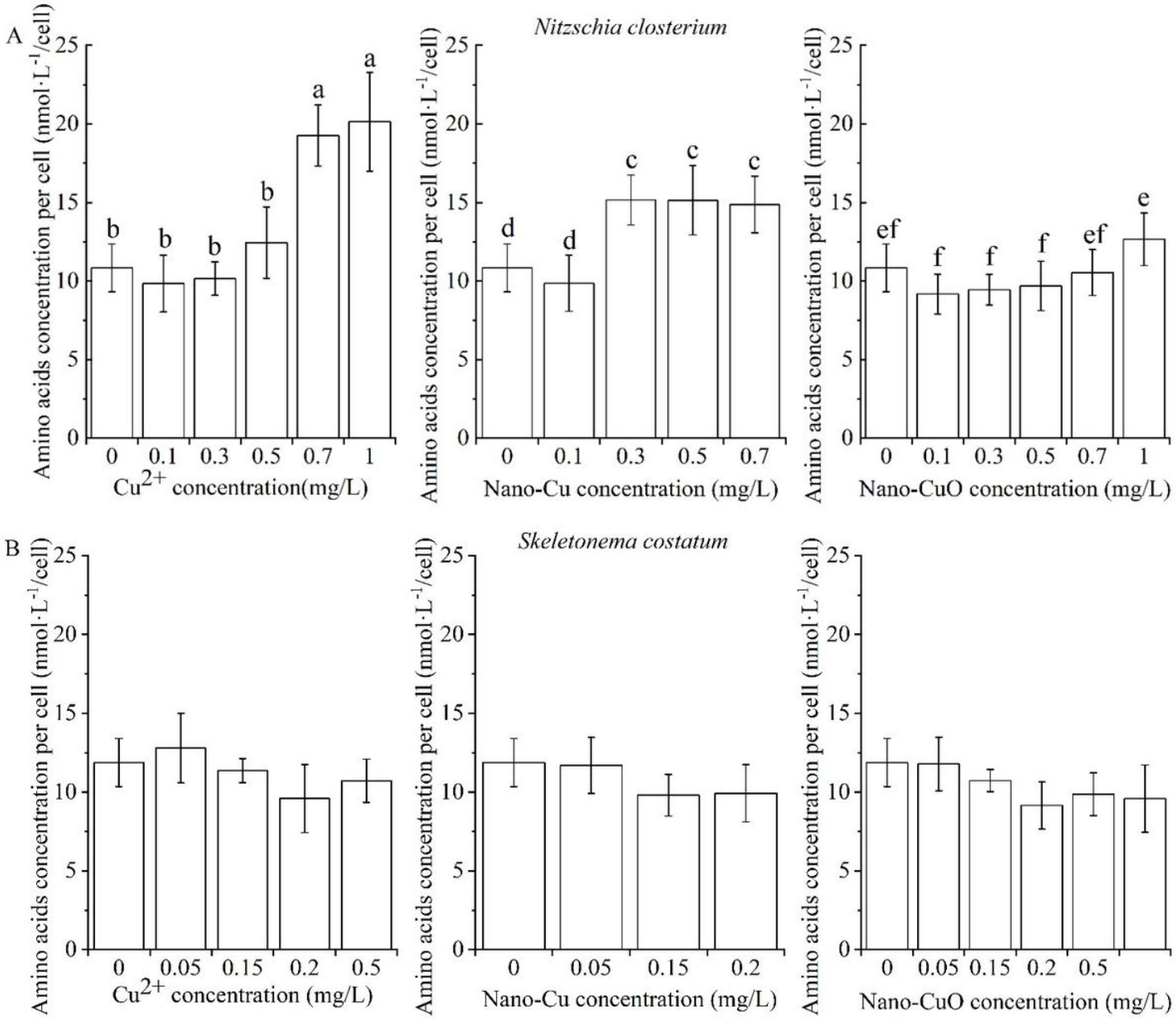

Figure 5

Per-cell intracellular amino acids concentration of N. closterium (A) and S. costatum (B). Different small letters indicated statistically significant differences ($p < 0.05$) among different concentration groups in each copper material treatment. Error bars: standard deviation.

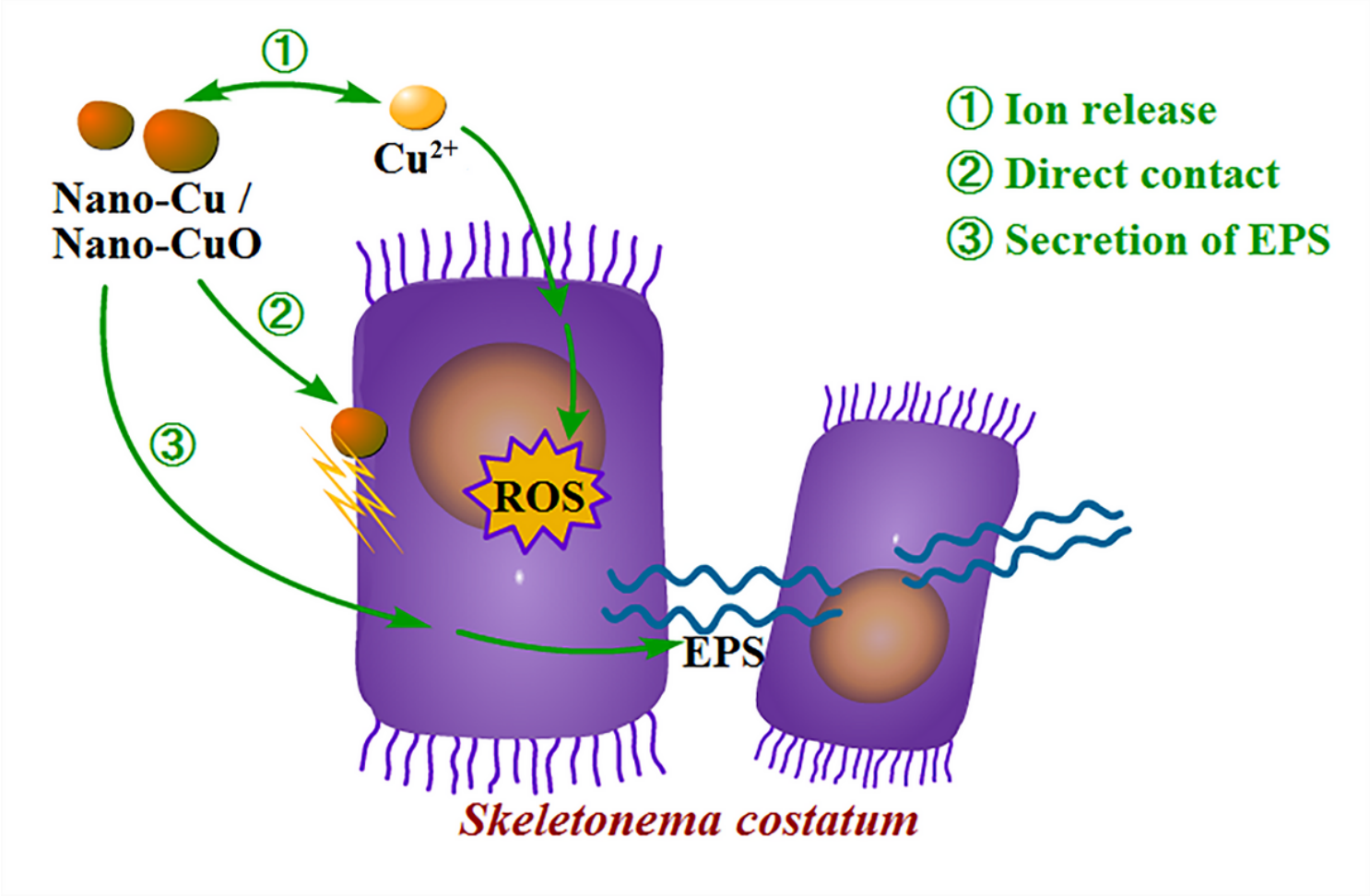

Figure 6

Schematic diagram of the way that nano-Cu and nano-CuO affect algae cells.

Supplementary Files

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