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Toxic Effects of Nano-CuO, Micro-CuO and Cu^{2+} on *Chlorella* sp.

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ABSTRACT

The 96 h acute toxic effects of nano-CuO (N-CuO), micro-CuO (M-CuO) and Cu^{2+} on *Chlorella* sp. were investigated in this paper. The results showed that toxicities decreased in an order of Cu^{2+}＞N-CuO＞M-CuO. The 96 h EC_{50} of Cu^{2+} on *Chlorella* sp. was 1.06 mg /L, and of N-CuO it was 74.61 mg /L, while no pronounced toxicity was observed when the concentration of M-CuO was lower than 160 mg/L. Further experiments were carried out in order to study the toxicity mechanism of nano-CuO on *Chlorella* sp.. The results of Cu^{2+} release from N-CuO showed less than 0.2 mg/L Cu^{2+} were released, so the release of Cu^{2+} was not responsible for the toxicity. Further experiments showed N-CuO inhibited formation of Chlorophyll A. Content of Chlorophyll A in the control group was 4.75 mg/10^8 cells, while it declined to 2.89 mg/10^8 cells for 160 mg/L N-CuO after 96 h, which indicated that N-CuO could inhibit photosynthesis of *Chlorella* sp.. Moreover, N-CuO condensed with algal cells. It affected the activity of SOD and POD, indicating that N-CuO could cause oxidant stress to *Chlorella* sp.. These may be the toxicity mechanism.

Keywords: Nano-CuO; Chlorella sp.; Toxic Effects; Photosynthesis; Oxidant Stress

1. Introduction

As a frontier technology, nano-science, as well as information science and life science, are the top three pillar sciences in contemporary society. It has been applied to broad areas such as materials, information, environment, life, national security and so on [1]. There are special physical and chemical properties lying in nano-materials, for example, small size effect, surface effect, macroscopic quantum tunneling effect, etc [2]. Distinguishing properties of nano-materials are observable in optics, electricity, magnetism, calorifics, mechanic, and chemical properties [3]. Nano-CuO (N-CuO) has been applied to catalysts, superconductors, thermoelectric materials, sensor materials, glass, ceramics and other areas. Also, it can be used as burning rate catalyzers. In a word, it is a kind of widely applied nano-material.

Algae, the primary producers, which are the initiate link of the food line of ecosystem and the origin of bioaccumulation, play a very essential role in the balance of the ecosystem. Highly sensitive, having short growth cycle, easy to be cultivated separately and owning observable toxic effects directly from a cellular level, algae are often used for risk assessment as the sensitive factors to environmental toxic substances. With relatively high resistance, *Chlorella* sp., which is one of the algae appeared early on the earth, is a species commonly used for assessment of toxic and harmful materials [4]. Nowadays, the toxic studies on N-CuO are still limited, so we carry on this study to investigate the toxic effects of N-CuO on *Chlorella* sp., aiming to assess the safety of nano-material, and provide evidence for standard use of nano-material.

2. Materials and Methods

2.1. Experimental Materials and Apparatus

N-CuO, with the particular size of 40 ± 10~20 nm, specific surface area of 80 m^2/g and purity of 99.9%, was supplied by Beijing Nachen S&T Ltd., which was produced in chemical vapor deposition method. The TEM analysis is shown in **Figure 1**;

M-CuO, analytical reagent,was bought from Sinopharm Chemical Reagent CO., Ltd, with average particular size of 30 μm measured via Laser Particle Size Analyzers in **Figure 2**;

$CuCl_2 \cdot H_2O$, analytical reagent, was bought from Tianjin Guangcheng Chemical Reagent CO., Ltd;

Other reagents were all analytical;

Chlorella sp. was obtained from the Fisheries College of Ocean University of China.

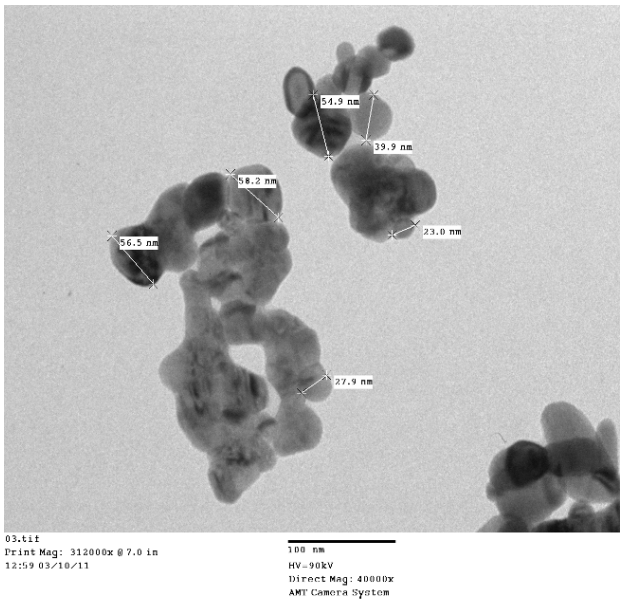

Figure 1. TEM image of N-CuO.

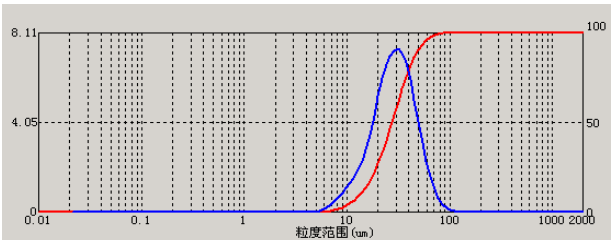

Figure 2. Particle size analysis of M-CuO.

Table 1. The improved f / 2 formula of medium.

Components	Stock concentration	Medium concentration
$NaNO_3$	75g/L	75mg/L
$NaH_2PO_4{\cdot}H_2O$	5g/L	5mg/L
$Na_2SiO_3{\cdot}9H_2O$	20g/L	20mg/L
Na_2EDTA	4.36g/L	4.36mg/L
$FeCl_3{\cdot}6H_2O$	3.16g/L	3.16mg/L
$CuSO_4{\cdot}5H_2O$	0.01g/L	0.01mg/L
$ZnSO_4{\cdot}7H_2O$	0.023g/L	0.023mg/L
$CoCl_2{\cdot}6H_2O$	0.012g/L	0.012mg/L
$MnCl_2{\cdot}4H_2O$	0.18g/L	0.18mg/L
$Na_2MoO_4{\cdot}2H_2O$	0.07g/L	0.07mg/L
Vitamin B1	0.1mg/L	0.1μg/L
Vitamin B12	0.5mg/L	0.5μg/L
Biotin	0.5mg/L	0.5μg/L

Experimental apparatus: GZX illuminating incubator (Ningbo Jiangnan Instrument Factory), KQ5200DE ultrasound distributor (Kunshan Ultrasound Instruments Co., LTD.), CKX41 inverted fluorescence microscope (Olympus), SCIENTZ-II D ultrasonic cell crusher (Ningbo Scientz Biotechnology Co., LTD), UV2600 ultraviolet and visible spectrophotometer (Shanghai Unico Instrument Co., LTD), Rise 2000 Laser particle size analyzer (Jinan Runzhi Technology Co., LTD.), TEM (JEM 1230, JEOL).

2.2. Experimental Methods

2.2.1. Release of Cu^{2+} From N-CuO

250 mL suspensions of 50 and 100 mg/L N-CuO were prepared with distilled water in 500 mL conical flasks. The pH is adjusted to 7.5 by NaOH. Then the flasks were put in the table of 20˚C, 150 r. After 24, 48, 72, 96, 120 , 144 h, 10 mL of the shaken up solution was taken from each flask, centrifuged 20 minutes at 4500 r and filtered by 150 nm filter membrane. The content of Cu^{2+} was measured by atomic absorption flame spectrometer.

2.2.2. Experiments of the Growth of Chlorella sp. Influenced by N-CuO, M-CuO and Cu^{2+}

We used the improved f / 2 formula of medium (Guillard, 1962). Components of the culture medium are shown in Table 2. One milliliter stock solution was added to one liter seawater which had been sterilized by high temperature and high pressure. After continuous training for three generations, 20 mL algae suspension of logarithmic phase was added to 200 mL culture medium for 96 h in 500 mL conical flasks. Initial algae density was about 6.0 × 10^5 cells/mL. Temperature of the illuminating incubator was 25˚C, illumination intensity was 5000 lux, and light: dark was 12 h :12 h. Initial pH was about 8.0.There were 3 samples in each group and the bottles were shaken by hand 3 times a day. Experimental equipment was sterilized for 20 minutes, at 121˚C using pressure steam sterilizer. Suspensions of 0 , 10 , 20 , 40 , 80 , 160 mg/L N-CuO and M-CuO were prepared and dispersed by ultrasound for 60 min. Concentrations of Cu^{2+} were 0.10 , 0.64 , 0.96 , 1.15 , 1.28 , 2.56 , 5.12 mg/L. Density of algae cells was measured by blood counting chamber at 0 , 24 , 48 , 72 , 96 h dividedly.

2.2.3. Experiments of the Toxicity Mechanism of N-CuO on Chlorella sp.

Cu^{2+} released from N-CuO: atomic absorption method; Chlorophyll a: spectrophotometry; SOD: nitroblue tetrazolium method; POD: guaiacol method; CAT: permanganate titration method; MDA: thiobarbituric acid method.

3. Release of Cu^{2+}

At pH 7.5, less than 0.17mg/L Cu^{2+} was detected in our experiments. As illustrated in **Figure 3**, small amount of

Cu^{2+} was released. It should be attributed to the aggregation of N-CuO and the slightly alkaline pH environment of the suspension.

4. Toxic Effects of N-CuO, M-CuO, and Cu^{2+} on Chlorella sp.

As illustrated by the growth curves in **Figure 4-Figure 6**, the toxicity ranking of copper materials on *Chlorella* sp. was: Cu^{2+}>N-CuO>M-CuO. The toxicity of Cu^{2+} on *Chlorella* sp. was pronounced. The linear interpolation suggested that 96 h EC_{50} value was 1.06 mg /L. The toxic effect of N-CuO was observed, and 96 h EC_{50} value was 74.61 mg /L . However, the toxic effect of M-CuO within the concentration of 160 mg/L was less pronounced. The toxicity of N-CuO to the algae increased with the concentration. A dose-effect relationship between the concentration of N-CuO and the toxicity to *Chlorella* sp. existed. Furthermore, studies showed that there was also a dose-effect relationship of N-ZnO and N-TiO_2 on *Chlorella* sp. [5].

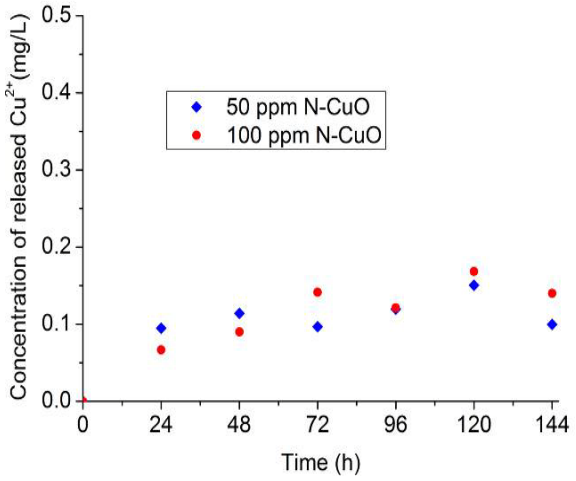

Figure 3. Cu^{2+} release from N- CuO.

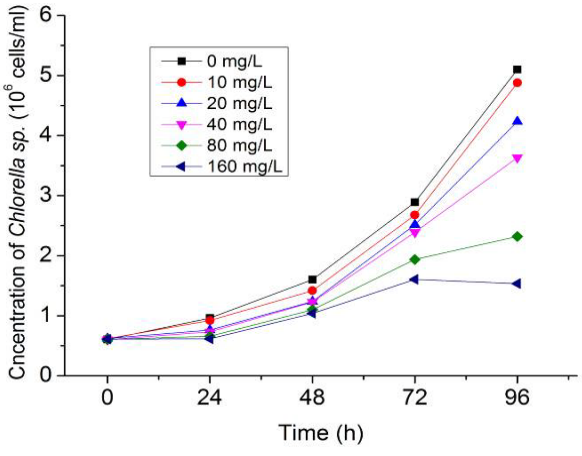

Figure 4. Growing effect of N-CuO on *Chlorella* sp.

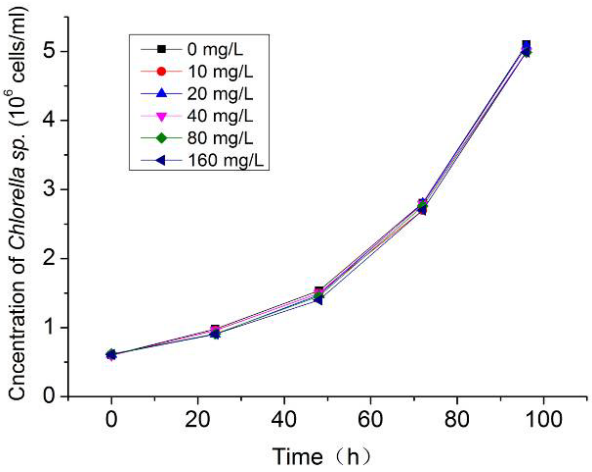

Figure 5. Growing efffect of M-CuO on *Chlorella* sp.

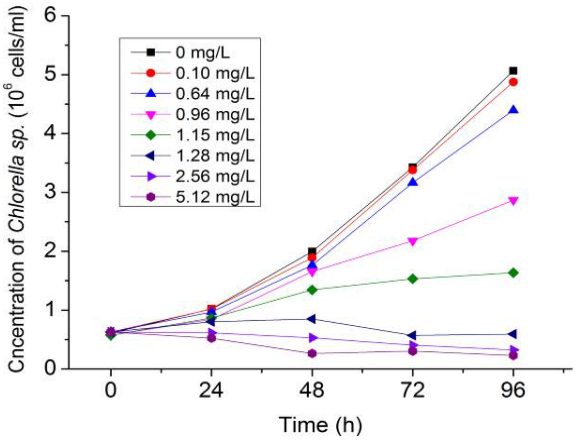

Figure 6. Growing efffect of Cu^{2+} on *Chlorella* sp.

5. The Mechanism of Toxicity of Nano-CuO on Chlorella sp.

5.1. The Influence of Cu^{2+}

Some studies attributed the toxicity of nanoparticles to the release of metal ions [6-10]. Miao discovered that it was the Ag^{+} released by N-Ag that resulted in the toxicity to *Thalassiosira*, while no toxicity was detected after the free Ag^{+} was infiltrated by membrane or was complexed with mercaptan when most N-Ag formed nonoxic aggregates which were larger than 0.22 μm in seawater[7]. However, there were studies indicating that the Ag^{+} released by N-Ag could not totally explain the toxicity of N-Ag on *Chlorella* sp. [8]. Aruoja and Franklin reported that M-ZnO and N-ZnO had similar toxicity to *Pseudokirchneriella subcapitata*, which is primarily attributed to the dissolved Zn^{2+} [9,10]. Aruoja discovered that Cu^{2+} was the main cause of the toxic effect of N- uO to *Pseudokirchneriella* [9]. But still some studies suggested that N-CuO induced *Chlamydomonas reinhardtii* to yield oxygen radicals ROS and the concentration of

ions alone cannot explain the toxicity of N-CuO[11].

Solubility tests of N-CuO showed that the aqueous solubility of it was highly pH-dependent. At pH 7.5, 100 mg/L N-CuO in distilled water released about 0.17 mg/L Cu^{2+}. Indicated by **Figure 6**, no pronounced toxic effect of 0.17 mg/L Cu^{2+} was found on *Chlorella* sp.. Still, the toxicity of N-CuO cannot be completely explained. In conclusion, the toxicity of N-CuO to *Chlorella* sp. is not caused by Cu^{2+} alone.

5.2. Condensation Effect

N-CuO aggregated with *Chlorella* sp. and precipitated, as illustrated in **Figure 7**. In addition, the shading effect of N-CuO on *Chlorella* sp. influenced the growth of it. Similarly, studies showed that N-Ag could directly influence the surface of *Chlorella* sp. cells and form large agglomeration. Though the 50nm Ag may not be able to enter the cells, but it can perform as the bridge for cells to connect with each other and accelerate the speed of cell-aggregating [12]. The aggregations of N-ZnO and anatase-TiO_2 can trap, catch and enclose cells of *Chlorella* sp. [5]. The toxicity of N-SiO_2 to *Scenedesmus* is probably caused by the absorption of nano-materials to the surface of algae cells [13]. The surface of *Chlorella* sp. and *Scenedesmus* absorb N-Al_2O_3 particles and lower the utilization rate of light, which can be the cause for inhibiting the growth [14]. Thus, the condensation effect of N-CuO on *Chlorella* sp. may be one of the probable explanations of its toxicity.

5.3. Inhibited Photosynthesis

Photosynthesis is an important influential factor of the growth of algae. Chlorophyll A is the material basis of photosynthesis, the content of which represents the growing condition of algae. **Figure 8** illustrates how the concentration of N-CuO influenced the Chlorophyll A content at 96 h.

As the concentration of N-CuO increased, the Chlorophyll A content in *Chlorella* sp. decreased. In control group, the Chlorophyll A content is 4.75 mg/10^8 cells and decreased to 2.89 mg/10^8 cells after being influenced by the 160 mg/L N-CuO for 96 h, indicating that N-CuO could influence the synthesis of Chlorophyll A in *Chlorella* sp. and inhibit the photosynthesis of *Chlorella* sp.

5.4. Oxidative Stress

Nanomaterials can cause oxidative stress to cells, yielding superoxide anions, hydroxyl radicals, hydrogen peroxides etc. Superoxide dismutase SOD is a kind of enzyme which can remove superoxide anion free radicals; peroxidase POD is a kind of enzyme which relies on hydrogen peroxides as the electron acceptor to catalyze and oxidize substrates. Thus, by measuring the activity of SOD and POD of algae cells, the extent that cells are being oxidative stressed can be indirectly measured.

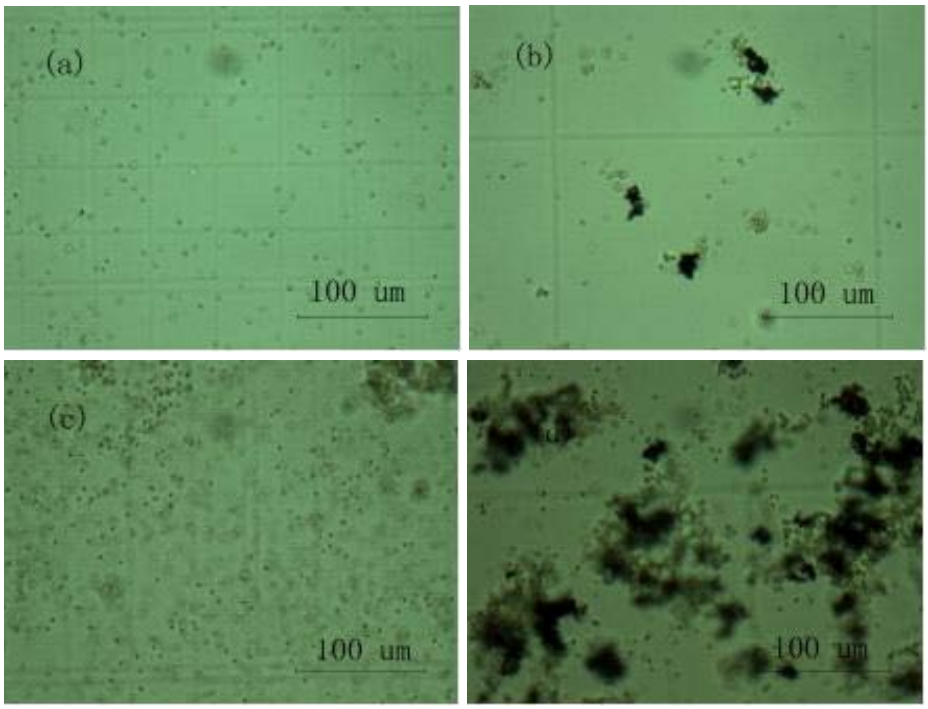

Figure 7. Light microscopic images of algal cells: control group (a), 80 mg/L (b),sediment of the control (c), sediment of 80 mg/L (d).

Figure 9 demonstrated that the impact of N-CuO on antioxidase activities of *Chlorella* sp. varied with the concentration of N-CuO. The activity of SOD was gradually enhanced as the concentration of N-CuO increased,. In the control group the SOD concentration was only 0.49 U/10^8 cells, and increased to 7.88 U/10^8 cells under the impact of 160 mg/L N-CuO for 96 h. This figure is 16 times higher than the one before, indicating that under the oxidative stress of N-CuO, the concentration of O_2^- increased, stimulating the activity of SOD to resist the stress caused by N-CuO. When the concentration of N-CuO was between 0 mg/L and 80 mg/L, the POD concentration increased from 0.48 U/(10^8 cells*min)to

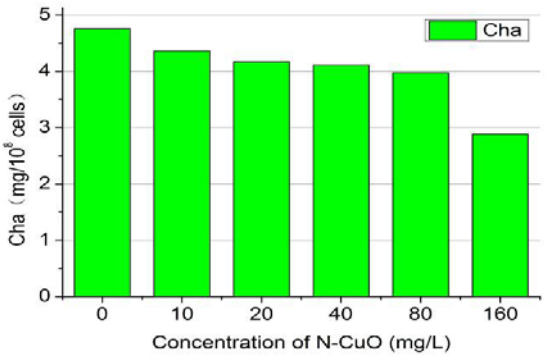

Figure 8. The influence of N-CuO on Chlorophyll A content in *Chlorella* sp.

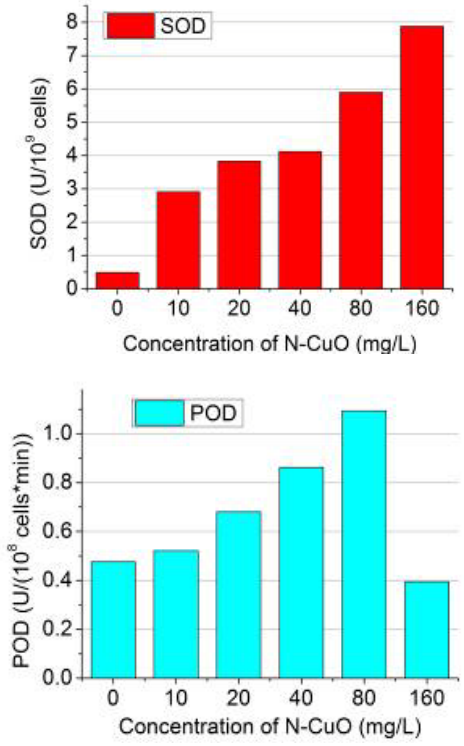

Figure 9. The impact of N-CuO on SOD and POD of *Chlorella* sp.

1.09 U/(10^8 cells*min), indicating the increased ability of cells to resist peroxide. When the concentration of N-CuO reached 160mg/L, the activity of POD declined to 0.39 U/(10^8 cells*min), one probable explanation of which might be that under the oxidative stress of high concentration of N-CuO, POD was destroyed, leading to the declined anti peroxidative ability.

6. Conclusion

The experiment demonstrates that N-CuO inhibits the growth of *Chlorella* sp. and the toxicity cannot be completely attributed to the released Cu^{2+}. N-CuO can absorb cells of *Chlorella* sp. and cause them to subside. It also inhibits the composition of Chlorophyll A in *Chlorella* sp. and thus influences the photosynthesis. The concentration of cells of *Chlorella* sp. was lower compared to control and the number of cells was smaller. The antioxidant enzyme system of algal cells is affected by N-CuO, indicating that N-CuO can cause oxidative stress. The results provide certain theory basis for evaluating the safety of N-CuO.

7. Acknowledgements

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