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Graphene oxide alleviates the ecotoxicity of copper on the freshwater microalga *Scenedesmus obliquus*

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ABSTRACT

The extensive industrial application of graphene oxide (GO), has increased its exposure risk to various aquatic organisms and its potential to affect the toxicity of other environmental pollutants. In this study, we investigated the combined toxicity of GO and copper on the freshwater microalga *Scenedesmus obliquus*, using the MIXTOX model. The effects of low concentration (1 mg/L) exposure to GO were investigated with environmentally relevant concentrations of copper by using a 12-d subacute toxicity test, with pre- and post-GO treatment. Results showed that there were significant antagonistic effects between GO and copper on *S. obliquus*, and GO was found to reduce ecotoxicity of copper even at low and environmentally relevant concentrations (1 mg/L).

1. Introduction

Graphene oxide (GO), an oxidized derivative of graphene, possesses interesting nanostructure characteristics, with unusual physical, chemical, electrical, and mechanical properties (Boukhvalov and Katsnelson, 2008). GO is used in a wide range of technological applications, such as electronics and biotechnology (Eda et al., 2008; Zhang et al., 2010) and the increase in production and application of GO structures, increases the level of exposure to humans and the natural environment around us. As such, the potential toxic effects of GO must be established.

Various studies have shown that GO exerts toxicological effects on various organisms (Jastrzębska and Olszyna, 2015), although the causative toxicological mechanisms remain unclear. For instance, when *Escherichia coli* were exposed to GO nanosheets, significant antibacterial activity was observed (Akhavan and Ghaderi, 2010; Hu et al., 2010; Liu et al., 2011). In addition a study by Ahme and Rodrigues (2013), found that the presence of GO in activated sludge significantly influenced bacterial metabolic activity, bacterial viability and the biological removal of nutrients.

We recently evaluated the ecotoxicological effects of GO on protozoa *Euglena gracilis*, establishing a 96 h median effective concentration (EC_{50}) of 3.76 ± 0.74 mg/L (Hu et al., 2015), with the cytotoxicity of GO toward cells such as human alveolar basal epithelial cells (A549), also having previously been reported (Hu et al., 2011).

In general, previous studies on the toxicological properties of GO have been performed in pure microbial cultures under controlled laboratory conditions. However, natural aquatic systems are more complex than these simplified experimental systems. High levels of organic and inorganic pollutants are increasingly being released into the aquatic environment due to industrial activities and GO may potentially interact with these pollutants affecting their activity or toxicity to aquatic organisms. Moreover, various pollutants are not always released into the environments simultaneously, which means that a wide range of different chemical combinations may occur over an extended period of time. Therefore, the combined toxicity of GO and other pollutant combinations and different exposure sequences should be investigated, to evaluate the potential impact of GO on the natural environment.

Copper (Cu^{2+}) is an essential micronutrient for plants and algae and can be commonly detected in the aquatic environments. Copper compounds have been widely used in industrial processes and agriculture for a long period of time, resulting in elevated copper concentrations in certain areas of the aqueous environment. Various studies have established that high levels of copper exposure can lead to acute toxicity (Flemming and Trevors, 1989; Gaetke and Chow, 2003), and there is evidence to show the significant risk of combined exposure to multiple heavy metals (Fernández and Beiras, 2001; Liu et al., 2007), or combinations of metals and organic pollutants (Gatidou and Thomaidis, 2007; Kim

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et al., 2006; Yang et al., 2008) on various organisms. The combined toxicity of nanomaterials and copper has however, shown some notable inconsistencies between studies. Fan et al. (2011) found that nano-TiO_2 significantly enhanced Cu^{2+} toxicity to *Daphnia magna* by increasing Cu^{2+} bioaccumulation, whereas in contrast, Rosenfeldt et al. (2015) reported that nano-TiO_2 reduced the Cu^{2+} toxicity on *Daphnia magna* depending on the crystalline phases (anatase, rutile, and the mixture) of the nanoparticles, suggesting that the decrease in toxicity was driven by the adsorption of copper to nanoparticle surfaces.

The purpose of this study was to investigate the combined effects of GO and Cu^{2+} on the freshwater microalga *Scenedesmus obliquus*, which dominates the phytoplankton community and is an established test model in ecotoxicity studies, based on growth inhibition assessment (Lewis, 1995). We also determine the influence of GO at an environmentally relevant concentration on the ecotoxicity of copper to *S. obliquus*.

2. Material and methods

GO nanosheets (Nanjing XFNANO Materials Tech Co., Ltd., China) were used and *S. obliquus* cultures were obtained from Nanjing University, China, grown, and maintained in 500 mL Erlenmeyer flasks containing 150 mL of HB-4 medium (A1) according to Kuang et al. (2003). Samples were characterized by atomic force microscopy (AFM, Veeco Multimode VIII, CEECO, USA) and transmission electron microscopy (TEM, JEM-1400, JEOL, Japan).

2.1. Acute toxicity assays

Growth inhibition tests were performed according to standard OECD Guideline 201 (OECD, 2006). The initial cellular concentration was 5×10^3 cells/mL. A GO stock dispersion was treated for 15 min by sonication (HN92-II D, Shanghai, China; 200 W, 10 s pulses, and 5 s intervals) and then added to 250 mL flasks with 100 mL of culture solution to achieve starting a GO concentration range of 0, 1, 2, 4, 8, 16, 32, and 64 mg/L for the GO toxicity test. For the copper toxicity tests, $CuCl_2 \cdot 2H_2O$ was added to the culture solution to form a starting Cu^{2+} concentration range of 0, 0.16, 0.32, 0.64, 1.28, 2.56, and 5.12 mg/L. A light/dark cycle of 12 h/12 h was used with a photon irradiation of 85–90 μmol/m^2 s, provided by cool white fluorescence lights. The flasks were shaken three times daily and cell numbers were determined in triplicate with a hemacytometer at 24 h intervals following a baseline of 96 h exposure to GO or Cu^{2+}. The 96 h EC_{50} was established via nonlinear regression using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Cells treated with 8 mg/L GO for 96 h were selected for investigation of the algal appearance under an optical microscope (Eclipse Ni, Nikon, Japan).

2.2. Combined toxicity test

For the combined toxicity test, starting cultures with a cell concentration of 5×10^3 cells/mL, were used and nominal combined concentrations of GO and Cu^{2+} were calculated based on expected toxic strengths of 0.5 (0.125+0.375; 0.25+0.25; 0.375+0.125), 0.75 (0.125+0.625; 0.25+0.5; 0.375+0.375; 0.5+0.25; 0.625+0.125), 1 (0.125+0.875; 0.25+0.75; 0.375+0.625; 0.5+0.5; 0.625+0.375; 0.75+0.25; 0.875+0.125), and 1.5 (0.75+0.75; 1+0.5; 0.5+1) toxic units ($TU_{GO}+TU_{Cu}$). The parameter c/EC_{50} denotes the toxic unit, where c is the concentration of a chemical in the mixture. The flasks were shaken three times daily and growth was determined with a hemacytometer, at 24 h intervals, over a of 96 h period. Data was analyzed using the MIXTOX model previously described by Jonker et al. (2005) with the conceptual mode of Independent Action (IA) used to derive the response patterns of algal growth and values of the parameter a below and beyond zero indicating synergism and antagonism, respectively.

2.3. Subacute toxicity test

The 12-d subacute toxicity test included 3 types of treatments. Treatment 1 used initial concentrations of Cu^{2+} of 0, 0.32, and 1.28 mg/L (i.e. 0, 5, and 20 μM). Treatment 2, used concentrations of Cu^{2+} similar to those applied in treatment 1, with GO added into each flask after 24 h of exposure to obtain a nominal concentration of 1 mg/L. In treatment 3, 1 mg/L GO was first added to the flask, and 24 h later, Cu^{2+} exposure was initiated as described in treatment 1. After a 12-d exposure period, cell numbers in each treatment were recorded.

For the chlorophyll assay, 5 mL cultures were collected at 12 d and centrifuged ($5198 \times g$ for 15 min). The resulting precipitate was re-suspended and extracted in 5 mL 90% acetone (dark at 4 °C for 24 h). The crude extracts were centrifuged ($5198 \times g$ for 15 min) and supernatants analyzed for optical density at 645 nm and 663 nm wavelengths. The concentrations of chlorophyll *a* and chlorophyll *b* were calculated according to the equations reported by Jeffrey and Humphrey (1975).

To determine the remaining copper concentrations in the culture, 5 mL of culture was collected after 12 d and centrifuged ($5198 \times g$ for 15 min). The supernatant was filtered (0.45 μm polysulfone filter) and acidized with HNO_3, allowing the copper concentration to be established by ICP-MS (iCAP Q, Thermo, USA). Parallel control treatments without any microalga culture present, were simultaneously performed and copper concentration were also determined.

In addition, we investigated both the extracellular and intracellular copper content according to a modified version of the method reported by Zhou et al. (2012). 30 mL of the algal culture was centrifuged ($5198 \times g$ for 10 min) and the concentrated algal cells and GO aggregates were re-suspended in 5 mL of 0.02 M EDTA and shaken immediately for 30 s to remove the extracellular copper adsorbed on to the cell walls and GO. After further centrifugation (10 min at $9432 \times g$), 250 μL of concentrated HNO_3 was added to supernatant (4.75 mL) and used to determine extracellular copper concentration. The precipitates were then ashed overnight in a muffle furnace (TDRG, Yixing, China) at 900 °C and dissolved in 8 mL 5% mol/L HNO_3 in order to determine the intracellular copper concentration by ICP-MS. Both extracellular and intracellular copper concentrations are presented as μg/cell.

2.4. Statistical analyses

The results are presented in terms of arithmetic means and their corresponding standard deviations ($n=3$ in all studies; $n=5$ for the growth inhibition test). Differences between treatments were tested for significance using one-way analysis of variance (ANOVA) with the software Origin 7.0. According to Tukey's multiple comparisons tests, $^{*}P<0.05$ and $^{**}P<0.01$ were considered as significant and highly significant, respectively.

3. Results and discussion

A visible homogenous GO dispersion was obtained through sonication of the culture medium and remained stable for over 3 days. GO samples were examined by TEM for shape and size determination, showing that the GO fragments were heterogeneous and ranged from between 1 and 10 μm in size (Fig. 1). The AFM image showed that the thickness of the sheets were ~0.7 nm