Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supporting Information

Single and combined nanotoxicity of ZnO nanoparticles and graphene quantum dots against the microalga *Heterosigma akishiwo*

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1. Morphological observation of nanoparticles

The nanoparticles suspensions of nZnO and GQDs were blending through ultrasonic for 30 min. The suspensions were dropped onto the surface of the copper mesh and dried for a few minutes. Then copper mesh was placed under microscope for morphological observation of nanoparticles.

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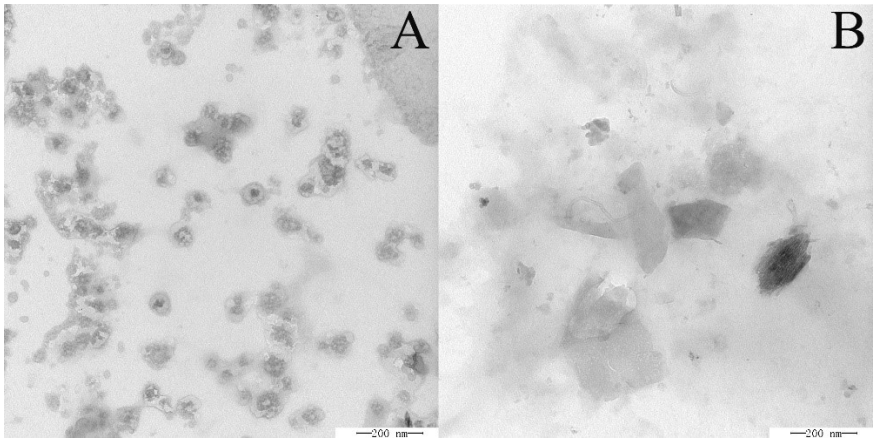

Fig. S1 TEM image of nZnO (A) and GQDs (B) dispersed in f/2 medium.[1]

2. Measurement of enzyme activities, total protein content

50 mL algal solution was collected on 4 d and then centrifuged (3000 rpm, 15 min). The algal cells were resuspension in normal saline water. The added volume of normal saline water was 9 times compared the weight of algal cells, and broke cells in ice water bath using ultrasonic cell crusher to obtain the 10% tissue homogenate. The homogenate would be diluted to different proportions for subsequent experiments.

2.1 The content of total protein

The content of total protein was determined using the assay kit from Nanjing Jiancheng Bioengineering Institute. The operation method was provided based on the principle that the brown-red coomassie brillian blue could bind with $–NH^{3+}$ located in a protein molecule and thus the solutions turned blue. The optical density of blue solution was related to the concentration of total protein.

Certain volume homogenate was diluted to 1% as sample suspension. Following the operation instruction in assay kit, 50 μL sample suspension, milli-Q or protein standard and the coomassie brillian blue was mixed and then let stand for 10 min at room temperature. The optical density (OD) of final solution was measured at wavelength of 595 nm using the microplate reader. The calculation formula was as follow.

$$total\ protein\ \left(mgprot{\cdot}mL^{-1}\right) = \frac{OD_{measured} - OD_{blank}}{OD_{standard} - OD_{blank}} \times 0.524\ \left(mgprot{\cdot}mL^{-1}\right)$$

where 0.524 was the concentration of protein standard.

2.2 The activity of superoxide dismutase

To investigate the change of the antioxidant enzyme systems in algal cells, the activity of superoxide dismutase (SOD) was determined using the assay kit from Nanjing Jiancheng Bioengineering Institute. In short, the method was provided based on the principle that WST-1 could react with the superoxide anion catalyzed by xanthine oxidase to produce water-soluble formazan dye. This reaction step could be inhibited by SOD. The enzyme activity of SOD could be calculated by WST-1 product.

According to the range of kit accurate determination and pre-experiment results, 4% tissue homogenate was used to determine SOD. Applied solution and working solution were prepared under the instruction of the manual. Milli-Q or 20 μL homogenate was mixed with a certain amount of applied solution and working solution, called control and measured group respectively. When working solution was replaced by the enzyme diluent, they were called control blank and measured blank. These solutions were blended, incubated at 37℃ for 20 min and then measured the absorbance at 450 nm using the microplate reader. One unit (U) of SOD activity was defined as the amount of enzyme per mL f/2 medium culture that inhibited the rate of reduction WST-1 by 50%. The calculation formula was as follow.

$$The\ activity\ of\ SOD\ \left(U{\cdot}mgprot^{-1}\right) = \left(1 - \frac{OD_{measured} - OD_{measured\ blank}}{OD_{control} - OD_{control\ blank}}\right) \div 50\% \times n \div total\ protein\ (mgprot{\cdot}mL^{-1})$$

where n was represented that diluted multiple.

2.3 The activity of adenosine triphosphatase

Adenosine triphosphate (ATP) can be decomposed into ADP and inorganic phosphorus under the influence of adenosine triphosphatase (ATPase). The activity of ATPase was calculated by determining the content of inorganic phosphorus. The content of ATPase was determined using the assay kit from Beijing Solarbio Science & Technology Co., Ltd.

According to the range of kit accurate determination and pre-experiment results, 4% tissue homogenate was used to determine K^+ Na^+ -ATPase. The procedure was divided into two steps: enzymatic reaction and fixed phosphorus reaction. Following the operation instruction in assay kit, sample homogenate was mixed with specific reagent, then incubated and centrifuged in steps of enzymatic reaction. The liquid supernatant was used to determinate the content of inorganic phosphorus. The fixed phosphorus reagent was mixed with supernatant, then blended, incubated at 40℃ for 10 min and measured the absorbance at 660 nm using the microplate reader. The calculation formula was as follow.

$$The\ activity\ of\ ATPase\ \left(U{\cdot}mgprot^{-1}\right) = \frac{OD_{measured} - OD_{control}}{OD_{standard} - OD_{blank}} \times 0.05\ \mu mol{\cdot}mL^{-1} \times 46.8 \div total\ protein\ (mgprot{\cdot}mL^{-1})$$

where 0.05 μmol mL^{-1} was the concentration of ATPase standard, 46.8 was the reaction system coefficient. The measured and control group were prepared with different reagents but both contained sample homogenate. The supernatant was replaced by milli-Q in blank group.

2.4 Malondialdehyde Content

The addition of nanomaterials causes lipid peroxidation in algal cells and the malondialdehyde (MDA) content reflects the level of lipid peroxidation.[2] The determination principle of MDA content is that MDA can react with thiobarbituric acid (TBA) to produce red-brown trimethoprim product at high temperature, and the product has the maximum absorption peak at 532nm. MDA content is calculated by absorbance according to the

following formula.

$$MDA\ Content(nmol{\cdot}mgprot^{-1}) = \frac{OD_{measured} - OD_{control}}{OD_{standard} - OD_{blank}} \times C\ standard \div total\ protein\ (mgprot{\cdot}mL^{-1})$$

References

1. X. Zhu, L. Tan, T. Zhao, W. Huang, X. Guo and J. Wang, Alone and combined toxicity of ZnO nanoparticles and graphene quantum dots on microalgae Gymnodinium, *Environ Sci Pollut Res Int*, 2022, DOI: 10.1007/s11356-022-19267-y.
2. Y. Hong, H. Y. Hu, X. Xie, A. Sakoda, M. Sagehashi and F. M. Li, Gramine-induced growth inhibition, oxidative damage and antioxidant responses in freshwater cyanobacterium Microcystis aeruginosa, *Aquat Toxicol*, 2009, **91**, 262-269.