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RESEARCH ARTICLE

Alone and combined toxicity of ZnO nanoparticles and graphene quantum dots on microalgae *Gymnodinium*

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Abstract

Investigation of ZnO nanoparticles (nano-ZnO) and graphene quantum dots (GQDs) toxicology on dinoflagellate *Gymnodinium* helps to understand the effects of different surface characteristic nanoparticles on marine algae. The growth and biological responses of the algae exposed to 1, 10, 20 mg L^{-1} nano-ZnO and GQDs in f/2 media were explored. Nano-ZnO showed slight effects on algal cells growth, while the growth inhibition rates of *Gymnodinium* increased as GQDs concentration increasing. Both nanoparticle treatments induced accumulation of reactive oxygen species and activated intracellular antioxidant defensive system, including SOD and ATPase, which were related to the two nanoparticles concentration. Under combined exposure of nano-ZnO and GQDs, the inhibitory effects decreased compared to the single GQDs and showed antagonistic effect. The addition of nano-ZnO could decrease the toxicity of GQDs due to aggregation and sedimentation interaction between nanoparticles. The morphologic change of the cells observed by SEM proved that nanoparticles adsorbed onto the cell surfaces and caused the cell shrinkage.

Keywords ZnO nanoparticle · Graphene quantum dots · Nanotoxicity · *Gymnodinium*

Introduction

Nanoparticles are defined as artificial particles with at least one dimension in the range of 1 to 100 nm. Nanoparticles can be divided into 6 types based on their chemical composition: metal oxide, zero-valent metals, carbon nanoparticles, quantum dots nanoparticles, organic polymers, and other NPs. Quantum dots (QD) are a kind of special nano materials with the three-dimensional particle sizes of 1 to 100 nm. Engineered nanomaterials with unique physical and chemical characteristics have been used in many fields, such as catalysts, cosmetics, and semiconductors. Nanoparticles could enter into the environment during production, transportation, consumption, and disposal (Barreto et al. 2021, Chen and Huang 2017, Wang et al. 2019). Many researches on biological effects showed that nanoparticles are toxic to bacteria, algae, fish, and mammals (Du et al. 2021; Griffitt et al. 2008; Khoshnamvand et al. 2021; Klaine et al. 2008; Wang et al. 2019). Microalgae, as the first level of the food chain and a vital part of ecosystems, are often used as the model organism for the study of nanotoxicity. In this paper, marine microalgae *Gymnodinium*, involved in red tides along the coastal areas, was chosen as the test species.

The toxicity of nanoparticles depends on characteristic and concentration of particles. Different nanoparticles have different toxic effects on algae. ZnO nanoparticle has a wide range of applications, especially in sunscreen, plastics, rubber, food additives, and fire retardants, etc. (Fazelian et al. 2020; Ma et al. 2013), and their production reached almost 1000 t/year all over the word (Piccinno et al. 2012). Many researches showed that nano-ZnO has negative effects on the growth of algal cells (Saxena and Harish 2019, Pereira et al. 2020; Saxena et al. 2021). Samei et al. (2019) showed that 0.7 mg L^{-1} of nano-ZnO could completely inhibit

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Raphidocelis subcapitata growth. Zhang et al. (2018) found that the inhibition ratio reached up to 56.8% in 10 mg L^{-1} nano-ZnO treatment at 48 h. Under 1 mg L^{-1} ZnO NPs exposure, the cell viability decreased about 25.8 ± 1.8% under visible light (Bhuvaneshwari et al. 2015).

Graphene quantum dots (GQDs) are graphene fragments with nano-diameter (< 100 nm) and many functional groups at the edge, such as epoxide (—O—), hydroxyl (—OH), and carboxyl (—COOH) (Golkaram and van Duin 2015, Zhou et al. 2019). GQDs, with larger specific surface area, richer edge, and basal functional group, are stable in aqueous solution and thus are widely used in fields such as photoelectric conversion, fluorescent probes, biomedical carriers, and polymer membranes (Lu et al. 2018). Many studies about the nanotoxicity of carbon materials on marine microalgae mainly focused on single- or multi-walled carbon nanotubes, C_{60}, and graphene oxide (Chen et al. 2016; Du et al. 2017; Glomstad et al. 2016; Hu et al. 2016; Zhao et al. 2018); however, there was only a small amount of work to study the toxic effects of GQDs on marine microalgae currently, which was one reason that GQDs were chosen as an experimental material. Zhang et al. (2019) reported that EC50 for the growth of *Chlorella vulgaris* was 70 μg mL^{-1} after 4 days for degradable carbon dots. Xiao et al. (2016) showed that EC50 of carbon quantum dots was 232.47 mg L^{-1} on the microalgae *Chlorella pyrenoidosa* at 96 h.

There are extensive researches about single nanoparticles toxicity on algae at present, but it is not sufficient to truly reflect the magnitude of nanotoxicity in the marine environment. Marine environment is a complex natural environment with different nanoparticles coexisting. Nanoparticles with high specific surface area have high affinity for other nanoparticles and algal cells in water. Nanoparticles could gather together each other, known as homoagglomeration, and could coalesce or clump together with other nanoparticles and adsorb onto cells formed NP-NP and NP-cell heteroagglomeration. These processes increase the complexity of the final toxicity for two nanoparticles, which may show different combined toxic effects compared to only one nanoparticle (Aruoja et al. 2015; Sendra et al. 2017). For example, nano-ZnO reduced the cell membrane damaging effect of nano-TiO_2 on *Escherichia coli* and nano-TiO_2 reduced the inhibitory effects of nano-ZnO on bacterial, which was caused by nanoparticle interactions and surface complexation reaction (Tong et al. 2015). Ye et al. (2018) reported that the joint effects of nano-ZnO and graphene oxide nanoparticles were additive to *Scenedesmus obliquus* and antagonistic to *Danio rerio*. The aggregation and sedimentation process decreased the chance of cell contacting and the possibility of particles entering cells (Navarro et al. 2008; Rodea-Palomares et al. 2011). Huynh et al. (2014) found that the aggregation of nano-Ag and hematite nanoparticles inhibited direct contact or close proximity between nano-Ag and bacterial cells. Thus, the combined toxicity of nanomaterials should be explored constantly. At present, the investigation about the combined toxicity of nano-ZnO and GQDs has not yet been found.

The objective of this research was to determine the toxic effects of nano-ZnO and GQDs on *Gymnodinium* in single and in combination. The effects on algae under nanoparticle treatment, including the growth inhibition and oxidative damage of cells, were investigated using short-term (4 days) acute toxicity tests. The extent of oxidative damage was reflected through the level of reactive oxide species and the relative enzyme activity change in the cell.

Materials and methods

Nanoparticles and chemicals

ZnO nanoparticles were purchased from Sigma-Aldrich with a purity of 99.9% and an advertised size of 50 ± 10 nm. The graphene quantum dots solution (4.5 g L^{-1}, particle size distribution 10 nm, black solution) was from the Key Laboratory of Marine Chemistry Theory and Technology, Ocean University of China (Lu et al. 2018; Zhou et al. 2019). Both actual size distributions of 5 mg L^{-1} nanomaterials in algal medium were examined by a Malvern Mastersizer 3000 (England, Malvern).

Microalgae cultures

Gymnodinium was provided by the Algal Center of Key Laboratory of Marine Chemistry Theory and Technology, Ocean University of China. The microalga was cultivated to exponential phase in sterile seawater with f/2 medium (*Supporting information*, Table S1-S3) in acid-cleaned 3-L Erlenmeyer flasks for subsequent experiments. The Erlenmeyer flasks were placed in a growth chamber with continuous illumination of 72 μmol photons m^{-2} s^{-1} and temperature of 20 ± 1 °C in a 12 h/12 h light/dark cycle. The seawater was filtered by a 0.45 μm membrane and sterilized at 120 °C under high pressure for 20 min in autoclave before experiment. All Erlenmeyer flasks were soaked with diluted HCl (10%) and washed several times with Milli-Q water before used.

Preparation of nano-ZnO and GQDs suspensions

Nano-ZnO stock suspensions were prepared through dispersing nano-ZnO powder into Milli-Q water to the final concentration of 500 mg L^{-1}. The graphene quantum dots solution (4.5 g L^{-1}) was diluted by Milli-Q water to 500 mg L^{-1}. The stock solution was diluted to a certain gradient concentration for subsequent toxicity assay.

Algal growth inhibition tests

The algal inhibition assays were carried out according to the OECD Guidelines 201. To investigate the toxicity of nano-ZnO and GQDs on *Gymnodinium*, 1, 10, and 20 mg L^{-1} nano-ZnO and GQDs were chosen as the added test concentration, referring to a large number of relevant researches and our preliminary experimental results (Du et al. 2019; Ma et al. 2013; Saxena et al. 2021; Yin et al. 2021; Zhang et al. 2016). The experiment of Zn^{2+} exposure was carried out as the supplementary experiment on toxicity effect of nano-ZnO. Zn^{2+} concentration was set as 0.5, 1, 2, 5, and 10 mg L^{-1}, respectively. In the toxicity tests, the exponential growing algae were exposed to nanomaterials in a 500-mL Erlenmeyer flasks. All the experiments were carried out in triplicate. The flasks were shaken twice a day to promote CO_2 dissolution and avoid the precipitation and adsorption of the algae to container walls and were randomized to avoid the influence of uneven illumination distribution.

The samples were collected at 0, 1, 2, 3, and 4 days to count algal cell density using a hemocytometer under a microscope (Leica, DM4000B). The specific growth rates (μ day^{-1}) were calculated as follows:

$$\mu(day^{-1}) = \frac{lnN_t - lnN_{t_0}}{t - t_0}$$

where N_t was the number of algal cells at time t (days) and N_{t_0} was the initial number of cells at time 0 days under the same nanoparticle concentration exposure.

The growth inhibition rates (*IR* %) were calculated according to the American Society for Testing and Materials (E1218-04e1, 2007) as follows:

$$IR(\%) = \left(\frac{\mu_0 - \mu_c}{\mu_0}\right) \times 100$$

where μ_c and μ_0 were the specific growth rates of test and control group on 1 day, respectively.

Detection of ROS production

Gymnodinium was incubated for 4 days in the culture medium with various nanoparticle exposure levels. The total intracellular ROS was detected using the cell permeable probe, 2′7’-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Hong et al. 2009; Saison et al. 2010; Stachowski-Haberkorn et al. 2013). The incubation was carried out at 37 °C for 1 h with DCFH-DA at a concentration of 15 μM. The DCF (an intracellular hydrolysate of DCFH-DA) was detected by a fluorescence spectrophotometer (Hitachi, F4600) with excitation (485 nm) and emission (522 nm) wavelength. Changes in ROS levels of treated samples were compared with the control and assessed using relative ROS levels that was calculated as follows:

$$\text{RelativeROSlevel}(\%) = \text{meanDCF}\frac{FI(testgroup)}{FI(control)} \times 100$$

where *FI* (test group) was the fluorescence intensity of the test group after nanoparticles exposure, and *FI* (control) was the fluorescence intensity of the control group.

Measurement of enzyme activities

The enzyme activity of superoxide dismutase (SOD) and adenosine triphosphatase (ATPase) was detected according to the instruction in the standard assay kit. SOD assay kits were purchased from the Nanjing Jiancheng Bioengineering Institute, China. The activity of ATP enzyme was also measured using the ATP assay kit (Beijing Solarbio Science & Technology Co., Ltd). The detailed steps were presented in *Supporting Information*.

Surface interaction of nanoparticles and algae

A scanning electron microscopy (SEM) was used to observe the surface interaction of nanoparticles and algal cells. After 4 days exposure under 1 mg L^{-1} nano-ZnO and GQDs treatment, 50-mL algae cultures were collected and centrifugated (3000 rpm, 10 min). After the supernatant was removed, the algal cells were fixed in 2.5% glutaraldehyde at 4 °C for 12 h. Then, the cells were washed through phosphate buffer solution (PBS, $pH = 7.4$, 0.1 M) for three times, centrifuged, and dehydrated by ethanol solution with the increased concentration of 30%, 50%, 70%, 80%, 90%, 95%, and100%. Finally, the sample was fixed with tert-butyl alcohol and freeze-dried for SEM observation.

The combined growth inhibition tests

To investigate the combined toxicity effects of nano-ZnO and GQDs on *Gymnodinium*, certain nanparticle concentrations were selected for assays. The final concentrations of nano-ZnO and GQDs combined nanomaterials were 0 mg L^{-1}, (5 + 20) mg L^{-1}, and (20 + 20) mg L^{-1}, respectively. The toxicity test of single nanoparticles with corresponding concentration of nano-ZnO (5, 20 mg L^{-1}) and GQDs (20 mg L^{-1}) was carried out at the same time to eliminate the possible influence of environmental factors. The steps and calculation formulas were the same as the aforementioned toxicity test.

Statistics

All experiments were conducted in triplicates. Results were represented as mean ± standard deviation. Analysis of one-way ANOVA was used to test the statistical significance of the single toxicity results by SPSS software version 24. *p*-values of less than 0.05 were considered statistically significant.

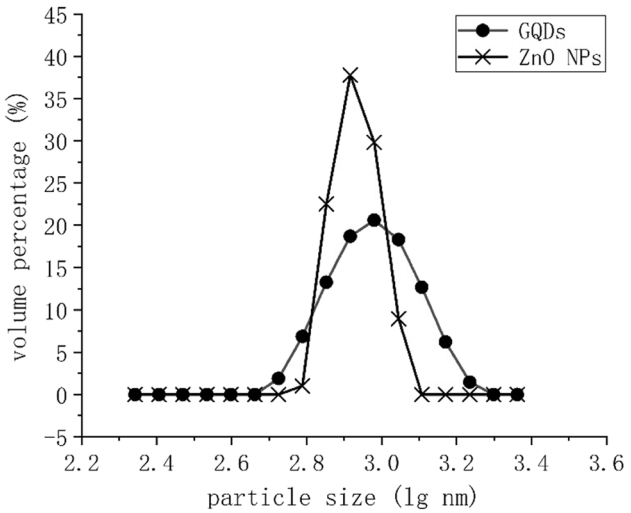

Fig. 1 The size distribution of 5 mg L^{-1} nano-ZnO and 5 mg L^{-1} GQDs in f/2 medium

Results

Behavior of nanomaterials in water

The zeta potential and the hydrodynamic size of nanoparticle suspensions were measured to investigate the behavior of the particles in culture medium. The hydrodynamic diameter of 5 mg L^{-1} ZnO nanoparticles ranged from 615 to 1110 nm in f/2 medium as shown in Fig. 1. The z-average of nano-ZnO was 862 nm, while the original average size was about 50 ± 10 nm. The original size of GQDs was about 10 nm and smaller than ZnO nanoparticle. GQDs (5 mg L^{-1}) formed larger aggregates with the average size of 948 nm and a wide size distribution between 531 to 1720 nm.

Zeta potential not only showed the changed of positive and negative charge on the particle surface but also was indicative for repulsive forces between different individuals. The higher the absolute zeta potential value, the more stable the nanoparticles are in aqueous solution (Wu et al. 2019). The zeta potentials of nano-ZnO and GQDs in seawater were − 3.24 mV and − 7.08 mV, respectively. Therefore, GQDs have better stability in aqueous solution.

Cytotoxicity of nanomaterials on Gymnodinium

The addition of nanomaterials to the culture medium led to cytotoxicity and growth inhibition to the microalgae *Gymnodinium.* The effects of nano-ZnO individual on algal growth over 4 d are shown in Fig. 2. The algal density increased with time, and there had no obvious significant difference

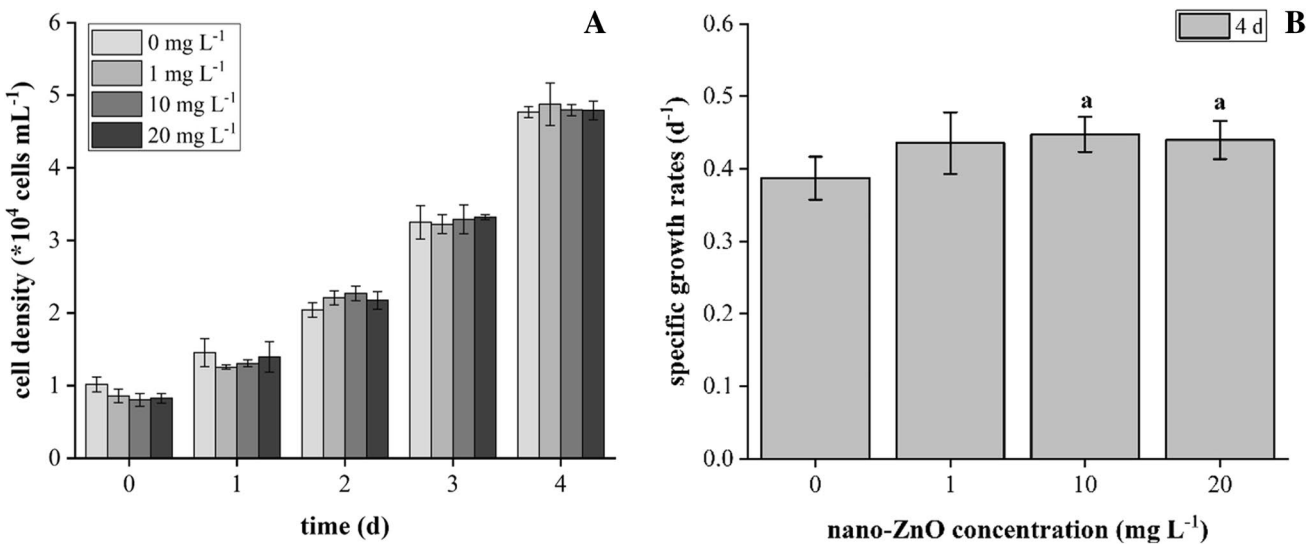

Fig. 2 Effects of different concentrations of nano-ZnO particles on microalgae cell density (**A**) and specific growth rates (**B**) over a period of 4 day. Values are reported as mean of 3 replicates ± standard deviation. Different lowercase letters indicated significant differences ($a < 0.05$ and $b < 0.01$) between the control and the tested concentration at the same time

between control and tested groups exposed to different nano-ZnO concentration on the same day. The specific growth rates increased obviously and reached to 15.6% compared to control group at 10 mg L^{-1} exposure on 4 day.

Zn^{2+} released by nano-ZnO had important contribution to the toxic effects of nano-ZnO (Aruoja et al. 2009; Li et al. 2017; Liu et al. 2018a). For further verification the toxicity of nano-ZnO, the exposure experiment of Zn^{2+} ions was conducted (Fig. 3). Here, 2 mg L^{-1} Zn^{2+} was the highest ion concentration released by nano-ZnO under the experimental concentration (20 mg L^{-1} nano-ZnO). Results showed that lower concentration (< 2 mg L^{-1}) of Zn^{2+} had negligible effects on the algae, and inhibition rate of 2 mg L^{-1} Zn^{2+} was only 9.0%. However, the inhibition effects of Zn^{2+} increased significantly when its concentration was over 2 mg L^{-1}, and IR reached 75.4% at 10 mg L^{-1} of Zn^{2+}.

The microalgal growth was inhibited under different GQD concentration exposure compared to ZnO nanoparticle (Fig. 4). The cell density of test groups was lower than the control group and the specific growth rate decreased. The inhibition rate reached 15.3% at 10 mg L^{-1} GQDs on 4 day.

Oxidative stress assessment

As algal cells were exposed to nanoparticles, reactive oxygen species (ROS) were produced, including ·OH, H_2O_2, and O^{2-} that were harmful for the growth of cells (Fan et al. 2018; Zhao et al. 2020). The relative ROS level related to the concentration and style of nanoparticles is shown in Fig. 5. The relative level of reactive oxidative free radicals increased as the concentration of two nanoparticle increased. The relative ROS level of GQDs was generally higher than that of nano-ZnO at the corresponding concentration, showing higher oxidative stress under GQDs exposure. Under 1 mg L^{-1} nano-ZnO or GQDs exposure, the ROS level reached 146.2/mgprot^{-1} and 215.7/mgprot^{-1} and the relative ratio compared to control group reached 2.3 and 3.4 times, respectively.

Related enzyme activity assessment

ROS induced by nanoparticle leads to subsequent oxidative stress response (Liang et al. 2020). The SOD activity was no significant difference compared to the control when *Gymnodinium* was exposed to different concentrations of nano-ZnO (Fig. 6A). However, SOD activity decreased significantly under 1 mg L^{-1} GQD treatment and then gradually increased (Fig. 6B). Exposed to 1 mg L^{-1} nanoparticle, the enzyme activity of SOD reached 309.4 U mgprot^{-1} for nano-ZnO and 129.3 U mgprot^{-1} for GQDs. K^+Na^+-ATPase activity of algal cell changed in different trend after the interaction between cell and two nanoparticle. The activity slightly increased to 1348.5 U mgprot^{-1} under 1 mg L^{-1} nano-ZnO exposure but decreased significantly to 887.4 U mgprot^{-1} under 1 mg L^{-1} GQD treatment compared to the control group, respectively (Fig. 6C and D).

The combined growth effects

Five and 20 mg L^{-1} nano-ZnO and 20 mg L^{-1} GQDs were chosen for joint toxic experiments. To exclude the influence of different batches, the toxicity assay of

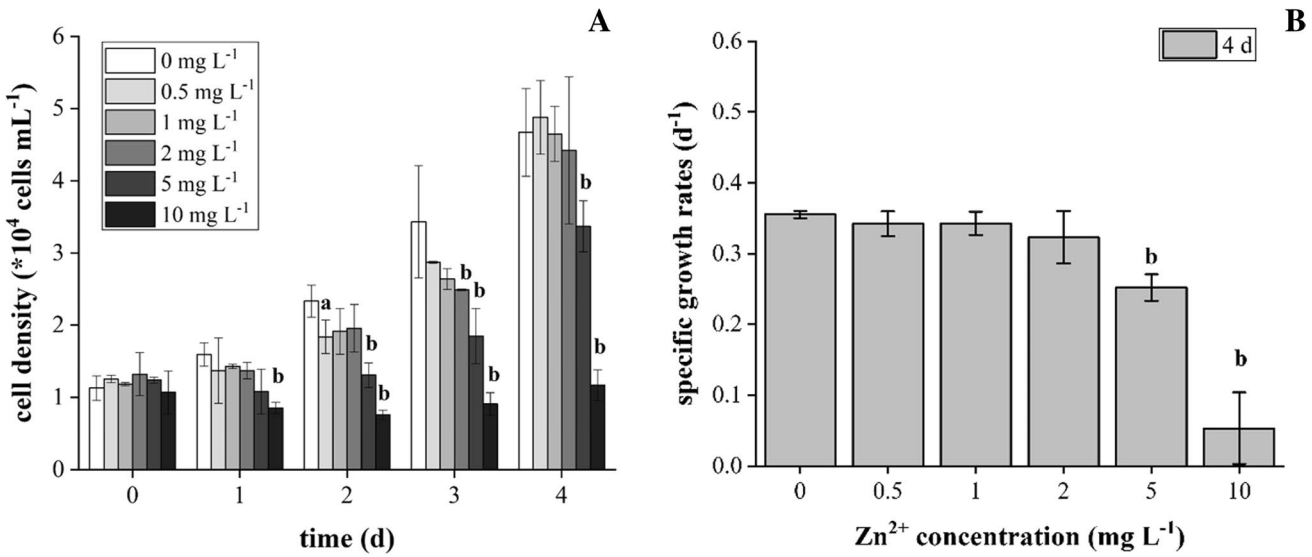

Fig. 3 Effects of different concentrations of Zn^{2+} on microalgae cell density (**A**) and specific growth rates (**B**) over a period of 4 day. Values are reported as mean of 3 replicates ± standard deviation. Different lowercase letters indicated significant differences ($a < 0.05$ and $b < 0.01$) between the control and the tested concentration at the same time

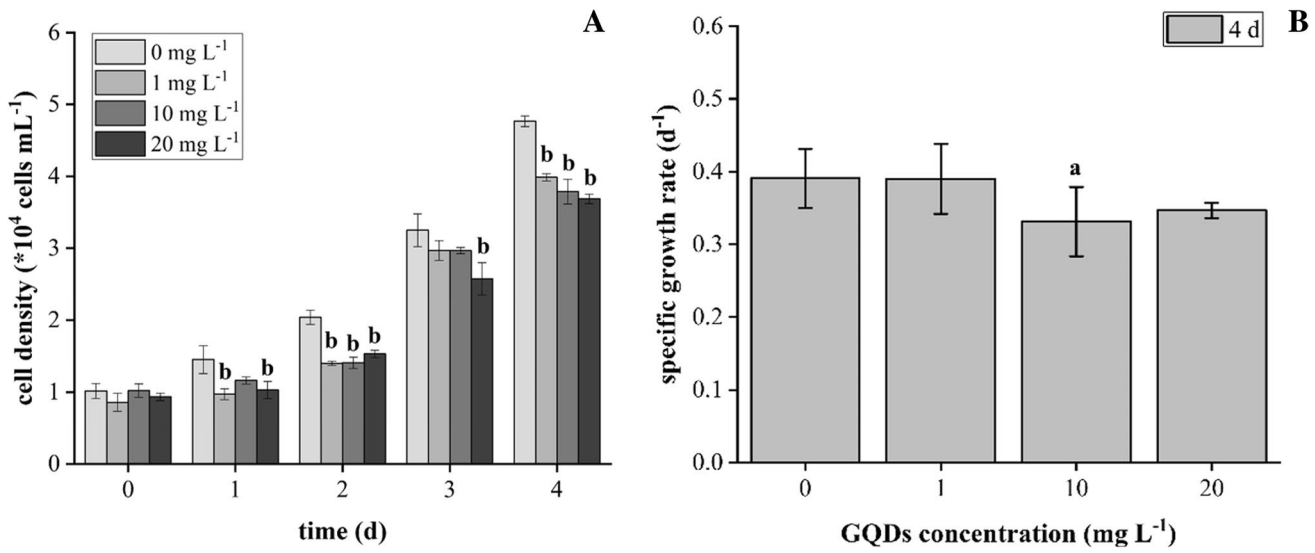

Fig. 4 Effects of different concentrations of GQDs particles on microalgae cell density (**A**) and specific growth rates (**B**) over a period of 4 day. Values are reported as mean of 3 replicates ± standard deviation. Different lowercase letters indicated significant differences ($a < 0.05$ and $b < 0.01$) between the control and the tested concentration at the same time

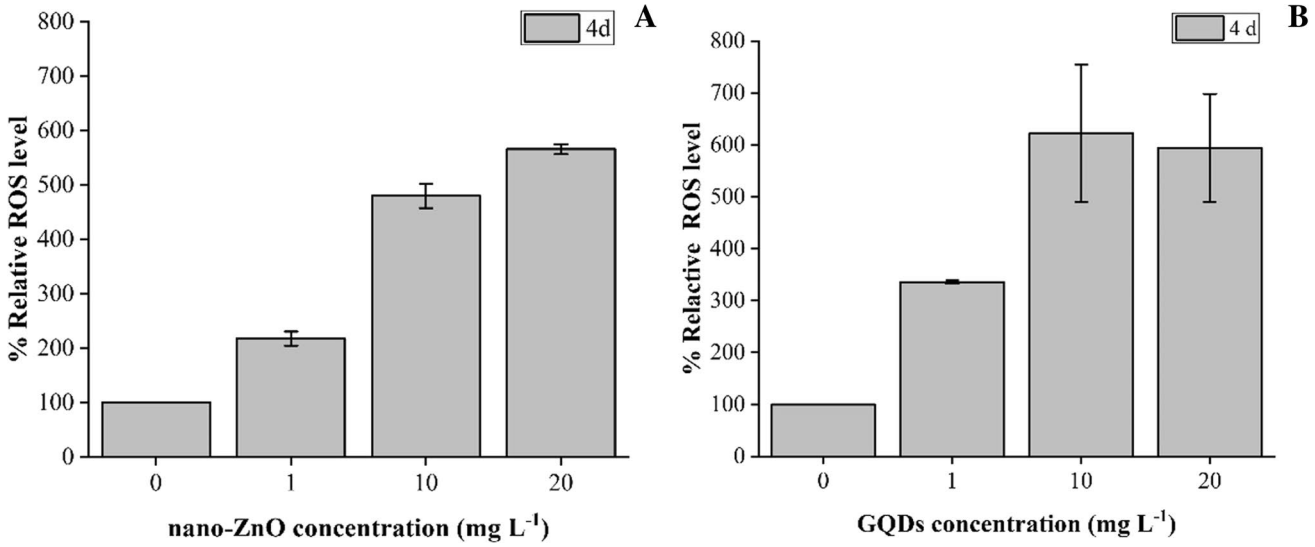

Fig. 5 Relative ROS level of *Gymnodinium* exposed to varying concentrations of nano-ZnO (**A**) and GQDs (**B**) on 4 day. Values were reported as mean of 3 replicates ± standard deviation

corresponding concentrations of single nanoparticles was re-ran simultaneously.

The combined growth inhibitory effects on *Gymnodinium* over a period of 4 day are shown in Fig. 7. The cell density and specific growth rate decreased obviously in the presence of GQDs whatever in single or in combination. The specific growth rates of two nanoparticle co-exposure were significantly higher than that of only GQDs treatment, suggesting that inhibitory effects decreased. For example, IR reached 5.4% for 20 mg L^{-1} nano-ZnO and 30.0% for 20 mg L^{-1} GQDs, but only 19.7% for (20 + 20) mg L^{-1} nano-ZnO + GQDs.

Surface interaction of nanoparticles and algae

SEM provided an intuitionistic and clear method to investigate the morphologies change of algal cells exposed to nanoparticles. The interaction between *Gymnodinium* cells and nanoparticles formed heteroaggregation led to cell membrane shrinkage as shown in Fig. 8. Nano-ZnO of 1 mg L^{-1}

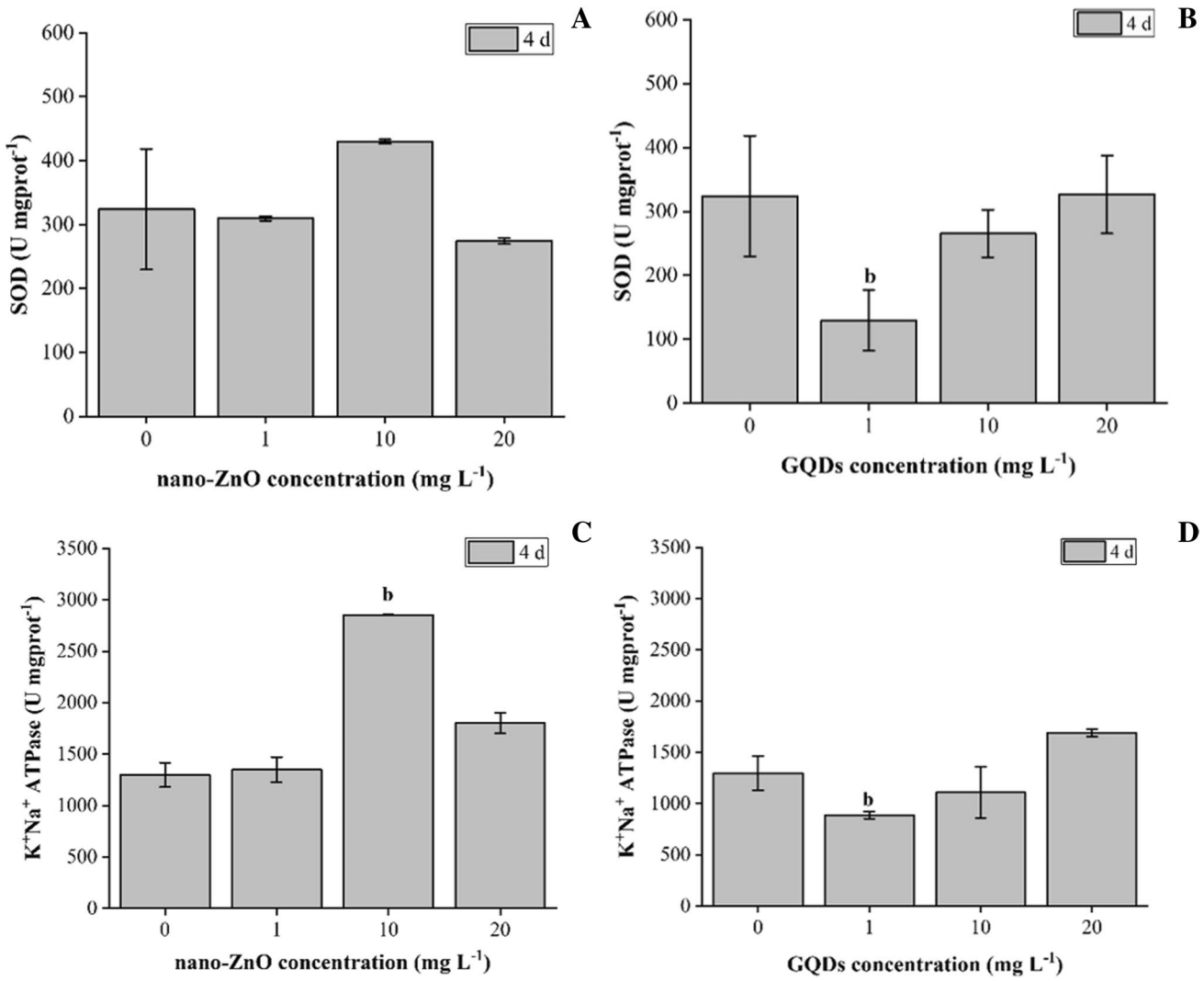

Fig. 6 The antioxidant defensive systems response of *Gymnodinium* exposed to different concentration of nano-ZnO or GQDs. **A** and **B** The SOD activity; **C** and **D** K^+Na^+-ATPase. Values are reported as mean of 3 replicates ± standard deviation. Different lowercase letter indicated significant differences ($a < 0.05$ and $b < 0.01$) between the control and the tested concentration

lower concentration absorbed onto the cell surface less, but GQDs could envelop algal cells due to their unique morphology and physiochemical characteristics. These adsorption and wrapped effects had a negative influence on cells and damaged the smoothness and integrity of the membrane.

Discussion

Physicochemical characterization of nanoparticles

The characterization of nanoparticles in the cultural medium was essential for their toxicity on algae. The potential physicochemical property of nanoparticles, such as agglomeration state and surface charge, changed under the influence of environmental factors (Jiang et al. 2008). Nanoparticles were agglomerated as soon as they were introduced into the aqueous solution to form homoaggregation with larger diameter compared to the priliminary particle size. Fazelian et al. (2020) showed that hydrodynamic diameter of nano-ZnO was 745.9 ± 49.4 nm while the optical diameter was 10–30 nm. Zhao et al. (2015) reported that nano-TiO_2 with the initial size of 15 nm aggregated to form irregularly shaped micro-sized particle. In this paper, the average hydration diameters of nano-ZnO and GQDs were 862 nm and 948 nm in medium, respectively.

Sedimentation of nanoparticles was related to the zeta potential that resprented the electrostatic repulsive forces between particles (Aruoja et al. 2015; Tursunay et al. 2021). The zeta potential value of nano-ZnO and GQDs was close. The nanoparticle suspension was stable and the amount of suspended particles changed little with time

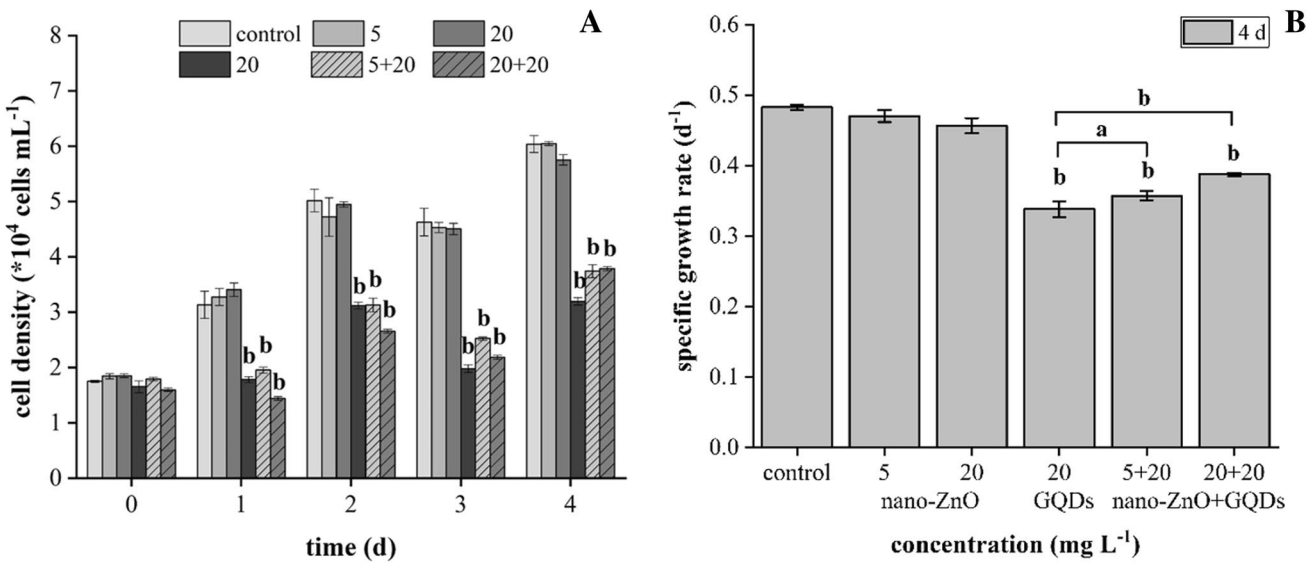

Fig. 7 The combined growth effects of nano-ZnO (5, 20 mg L^{-1}) and GQDs (20 mg L^{-1}) over the period of 4 day. **A** The cell density; **B** the specific growth rate. Values are reported as mean of 3 replicates ± standard deviation. Different lowercase letter indicated significant difference ($a < 0.05$ and $b < 0.01$) between the control and the tested concentration

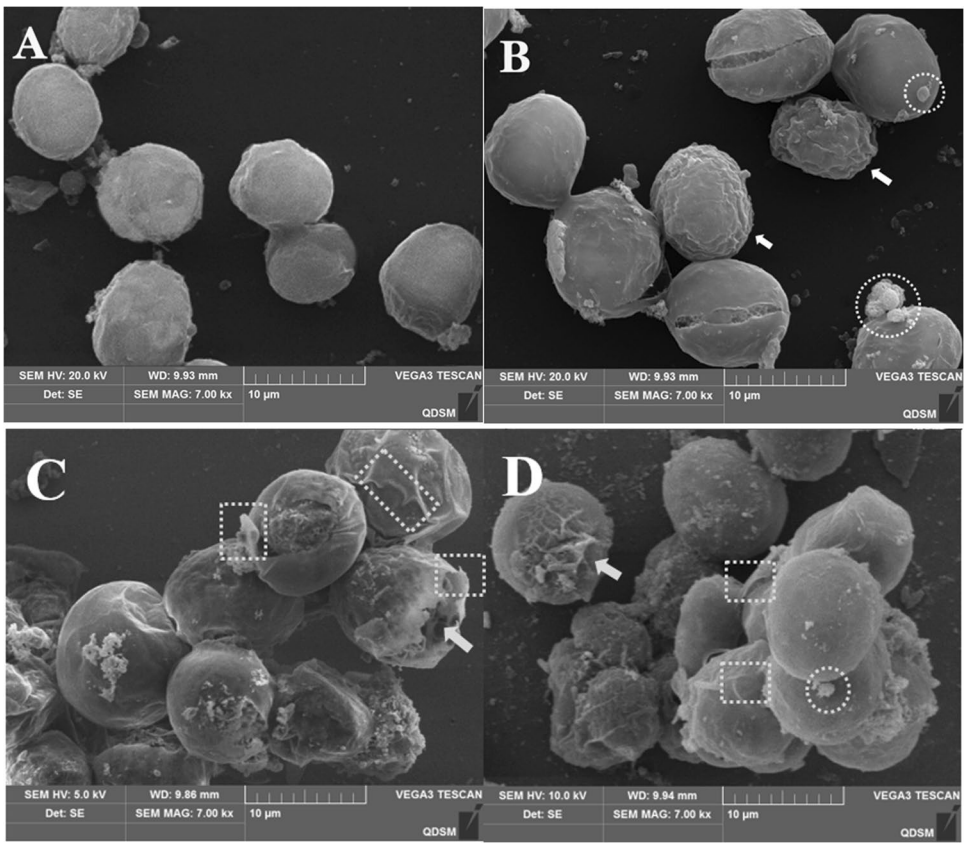

Fig. 8 Surface interaction of nano-ZnO or GQDs and algae on 4 day. **A** Control group; B 1 mg L^{-1} nano-ZnO; **C** 1 mg L^{-1} GQDs; **D** (1 + 1) mg L^{-1} nano-ZnO + GQDs. The circle represents the particles of nano-ZnO, the rectangle represents the particles of GQDs, the hexagon represents the aggregation of two nanoparticles, and the arrows represent shrinkage or damage of cell membranes

(Fig. S2). The deposition was enhanced at high nano-ZnO concentration (e.g., 10 mg L^{-1} nano-ZnO) by the effects of surface electrical double layer and more much aggregation (Jiang et al. 2008); however, GQDs had better stability in solution due to the hydrophilic functional groups on the surface (Golkaram and van Duin 2015, Liu et al. 2018b; Zhou et al. 2019).

Effects of nanoparticles on the growth of Gymnodinium

Nanoparticles influenced the growth of algae, showing varied changes of cell density. Nano-ZnO exposure did not product significant inhibit effects, while GQDs showed stronger dose- and time-dependent toxic effects. Nanoparticle of metal oxide could dissolve and release metal ions in aqueous solution, which made great contributions to nanotoxicity (Liu et al. 2018a; Muna et al. 2018; Zhang et al. 2016). Aruoja et al. (2009) reported that the toxicity of nano-ZnO with lower concentrations on *Pseudokirchneriella subcapitata* was attributed solely to solubilized Zn^{2+}. Miller et al. (2010) found that free Zn^{2+} released by nano-ZnO made major contribution to toxic effects on phytoplankton. However, it was also reported that released Zn^{2+} ions cannot fully explain the toxicity of nano-ZnO. Du et al. (2019) showed that higher growth inhibition was observed under 1 mg L^{-1} nano-ZnO exposure than under 0.71 mg L^{-1} Zn^{2+} (the corresponding dissoluble Zn^{2+} concentration of 1 mg L^{-1} nano-ZnO). To further explore nano-ZnO toxic effects, Zn^{2+} toxicity exposure was carried out, which showed that the influence of low concentrations Zn^{2+} was also marginal on cell growth.

Gymnodinium, a dinoflagellate, has cytoderm and flagellum on cell surface. Nanoparticles could adsorb onto the cytoderm, and only particles which were smaller than the bore diameter on cell surface could get into the cell (Navarro et al. 2008). Li et al. (2012) and Zhao (2012) found that nano-TiO_2 with average of 40 nm entered *Gymnodinium* cell and affected the algal photosynthesis. The average hydrodynamic size of tested nanoparticles was too large in this study. In the image of SEM, nano-ZnO adsorbed onto the surface and the cell only shrunk but did not damage under nano-ZnO exposure as shown in Fig. 8B. Zn^{2+} was an important trace element in cell growth and could form metal chelator protein (e.g., carbonic anhydrase and AKP). We inferred that the slight Zn^{2+} toxic effects were related to the formation of metal chelator protein and activation of the antioxidant defensive system. Gunawan et al. (2013) reported that the increasing nano-ZnO dosage did not result in further growth inhibiting effects on microalgae *Chlamydomonas reinhardtii* by reason of the accumulation of vesicular zinc in polyphosphate bodies and the formation of metal chelator protein to scavenge the excess cellular zinc. Certainly, the specific mechanism of negligible toxicity on *Gymnodinium* shown by nano-ZnO still needs to be further explored.

Exposure to various concentrations of GQDs appeared significant toxic effects on *Gymnodinium* compared to nano-ZnO. Carbon nanomaterials, releasing no ions into the culture medium, caused cell toxicity by nanoparticles effects (such as mechanical damage, oxidative stress, and wraparound effects). Physical interactions could damage the cell structure, and wraparound effects influenced the cell photosynthetic system and signal transformation (Akhavan & Ghaderi 2010; Wang et al. 2013). GQDs with unique liminated structure wrapped the cell and caused direct physical damage to algal cells (Figs. 8C and S1) (Zhao et al. 2017) and GQDs of small particle size can easily access to cell inducing excessive ROS production.

Oxidative stress response of Gymnodinium to nanoparticles

Reactive oxygen species (ROS) generation was considered as an important mechanism of cell death. In the normal state of algae cells, the production and elimination of reactive oxygen free radicals are always in a dynamic equilibrium state. When cell suffered environmental stress from the added nanoparticles, the equilibrium state was destroyed and produced a lot of free radical and ROS. Many studies about the oxidative stress of nanoparticles on marine microalgae suggested that the production of ROS was the main mechanism of toxic action and gradually increased with the nanoparticle concentration (Huang et al. 2016; Long et al. 2012; Oukarroum et al. 2018). ROS level exposed to GQDs was higher than that to nano-ZnO, which may be related to the surface functional groups of GQDs. Epoxide (—O—) was the dominant functional group of graphene surface and could form oxidative species (e.g., hydroxyl groups) in the presence of water molecules (Golkaram and van Duin 2015). Meanwhile, GQD particles with the unique size and shape were accessible to cell to induce ROS free radical production.

The antioxidant defensive system was activated to remove excess ROS in algae and protect organisms (Du et al. 2021; Huang et al. 2021). SOD, an antioxidant enzymens, was the first line of defensing against ROS and disproportionating $O_2^{\cdot-}$ into H_2O_2. Then, H_2O_2 was finally turned into harmless H_2O and O_2 in the presence of other antioxidant enzymes. Our results indicated that the activities of SOD enhanced slightly to eliminate oxidative radicals when algae were exposed to nano-ZnO, triggered by the elevated production of superoxides. The elimination of excess ROS was the combined action of a variety of enzymes, such as CAT, POD, GPx, maintaining the cell growth (Chen et al. 2015; Wang et al. 2008). When the algal cells were treated by GQDs particle, the activity of SOD enzyme was slumped at 1 mg/L as shown in Fig. 6B. The ROS level was higher than that of nano-ZnO, which suggested that ROS could not be eliminated through SOD since the decreased activity of SOD. This may be another reason that the toxicity of GQDs was stronger than nano-ZnO. ROS were a signal in cell to regulate enzyme activity, which led to the increasing enzyme activity and regain of activity of SOD with the increasing

exposure concentration (Dat et al. 2000; Lei et al. 2013; Zhao et al. 2020).

Nanoparticle exposure and excess ROS production influenced the cell viability and physiological function (Zhang et al. 2021). K^+Na^+ ATPase was an ion-regulated protease on the cell membrane and played an important role in maintaining the balance of membrane potential and the osmotic pressure inside and outside the cell. K^+Na^+ ATPase activity increased to keep the cell function and ion equilibrium when cells were exposed to nanoparticle (Yao et al. 2020). Sawosz et al. (2013) reported that the gene expression of K^+Na^+-ATPase upregulated in chicken embryos to accelerate muscle cell growth after nano-Ag was injected. The increased ATPase activity also helped to catalyze the hydrolysis of ATP to produce more energy to maintain the normal function and activity of cells. Under GQD treatment, the change of ATPase was similar to that of SOD and obvious inhibition appeared at 1 mg L^{-1} exposure, which would impact the normal function and activity of cells.

Combined growth inhibitory effects

Ocean as a complex environment contained many kinds of nanoparticles. The combined growth experiments were carried out to preliminarily understand the combined toxicity of the two nanoparticles. Under the combined exposure of nano-ZnO and GQDs, the specific growth rate increased and inhibition effects decreased compared to the alone GQDs exposure. Among the test, nano-ZnO had tiny inhibition effects on *Gymnodinium*. The combined toxicity of two nanoparticles on microalgae showed antagonistic effects. The existence of nano-ZnO and GQD nanoparticle interactions, including aggregation, sedimentation and so on, caused the difference between the combined toxic effects and the two single toxicity. As shown in SEM of Fig. 8D, nano-ZnO and GQDs were aggregated to form heteroaggregation and adsorbed onto the cell surface, which was less damaged to the cell compared to GQDs exposure (Fig. 8C). Aggregation and sedimentation of nanoparticles decreased the chance of cell contact (Navarro et al. 2008). Zhao et al. (2018) reported that the formation of GO-Al_2O_3 heteroaggregation suppressed GO-induced algal membrane damage and reduced nanotoxicity. Li et al. (2017) showed that the sedimentation resulted in a shorter availability of the bulk aggregates to swimming algae and settled out at the bottom of the wells, mitigating toxic effects. The aggregated nanoparticles could directly impact the uptake of nanoparticles on algae cells surface and indirectly affect the exposure concentration of nanoparticles to algae by the deposition effects (Hu et al. 2018). The settlement action of two nanoparticles aggregation was stronger than that only one particle shown in Fig. S2, thus reducing the possibility of contact with cells and decreasing the growth inhibition effects.

Conclusions

The biological response of *Gymnodinium* exposed to single and combined nanoparticles of nano-ZnO and GQDs was evident. Nano-ZnO had no negative effect on the algae growth, while the growth inhibition of GQDs increased as GQDs exposure concentration increasing. This phenomenon was likely due to the difference of nanoparticle characterization, such as particle shape and surface functional group. Both nanoparticles induced the production of excess reactive oxide species and activated the cellular antioxidant defensive system. SOD and ATPase activity induced by nano-ZnO increased to eliminate the excess ROS, while they were too low to preclude the oxidative damage as exposed to GQDs. The combined growth inhibition effects of the two nanoparticles showed an antagonistic effect. Nano-ZnO exposure decreased the toxic effects of GQD particles, which may be related to aggregation and sedimentation of two nanoparticles.

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Declarations

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