EPA Public Access

Author manuscript

Environ Toxicol. Author manuscript; available in PMC 2021 January 01.

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Published in final edited form as:

Environ Toxicol. 2020 January ; 35(1): 87–96. doi:10.1002/tox.22845.

Toxicity of ZnO/TiO₂-conjugated carbon-based nanohybrids on the coastal marine alga *Thalassiosira pseudonana*

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Abstract

Increasing consumption of metal-oxide nanoparticles (NPs) and carbon-based nanomaterials has caused significant concern about their potential hazards in aquatic environments. The release of NPs into aquatic environments could result in adsorption of NPs on microorganisms. While metal-oxide NP-conjugated carbon-based nanohybrids (NHs) may exhibit enhanced toxicity toward microorganisms due to their large surface area and the generation of reactive oxygen species (ROS), there is a lack of information regarding the ecotoxicological effects of NHs on marine diatom algae, which are an indicator of marine pollution. Moreover, there is scant information on toxicity mechanisms of NHs on aquatic organisms. In this study, four NHs (ie, ZnO-conjugated graphene oxide [GO], ZnO-conjugated carbon nanotubes [CNTs], TiO₂-conjugated GO, and TiO₂-conjugated CNT) that were synthesized by a hydrothermal method were investigated for their toxicity effects on a *Thalassiosira pseudonana* marine diatom. The in vitro cellular viability and ROS formation employed at the concentration ranges of 50 and 100 mg/L of NHs over 72 hours revealed that ZnO-GO had the most negative effect on *T. pseudonana*. The primary mechanism identified was the generation of ROS and GO-induced dispersion that caused electrostatic repulsion, preventing aggregation, and an increase in surface areas of NHs. In contrast to GO-induced dispersion, large aggregates were observed in ZnO/TiO₂-conjugated CNT-based NHs. The scanning electron microscopy images suggest that NHs covered algae cells and interacted with them (shading effects); this reduced light availability for photosynthesis. Detailed in vitro toxicity effects and mechanisms that cause high adverse acute toxicity on *T. pseudonana* were unveiled; this implied concerns about potential hazards of these mechanisms in aquatic ecosystems.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

Keywords

carbon nanotube; graphene oxide; metal oxides; nanohybrids; *Thalassiosira pseudonana*; toxicity

1 | INTRODUCTION

The use of nanoparticles (NPs) is increasing and becoming widespread in various fields, such as medicine, electronics, water purification, cosmetics, and manufacturing.¹ Among NPs, metal-oxide NPs such as ZnO and TiO₂ are the most abundant NPs in water.² The production of nanohybrids (NHs) consisting of metal-oxide NPs and carbon-based nanomaterials has increased because NHs can be applied to improve surface area and catalytic efficiency of NHs.³ The recent growth of the nanotechnology industry has raised concerns about the fate and potential hazards of NPs in water ecosystems, since NPs could end up in significant accumulation in the aquatic environment through their manufacturing process, product use, and disposal.^{4–7}

Properties of NPs in aquatic systems may exhibit different behaviors through aggregation and precipitation and interact with aquatic microorganisms such as algae.^{8,9} In aquatic environments, algae play a critical role in producing basic nourishment in all water bodies and in self-purification of organic contaminants in natural waters.^{10,11} Studies have revealed the toxicity effect of metal-oxide NPs on algae, and the reactive oxygen species (ROS) generation of NPs was suggested as a primary toxicity mechanism.^{12–15} In a recent study, the generation of ROS was also found to be one of key mechanisms of carbon nanomaterials' toxicity to microbes.¹⁶ Few studies have examined combined effects of different nanomaterials on the biodegradation of contaminants in the environment.¹⁷ The ROS generated by TiO₂ and ZnO NPs may oxidize organic contaminants and can cause fatal damage to microorganisms by disrupting or damaging cell DNA or structures.¹⁸ Given that different types of NPs are released into water, released metal-oxide NPs can react or interact with other NPs and form NHs due to the NPs' large surface area and high reactivity. Besides, the toxicity effect of NPs in aquatic systems has been made for single-NP types of nanomaterials rather than NHs that consist of two different types of NPs.^{18–22} To the best of our knowledge, in the recent literature, there is no study on the toxicity of NHs to marine microalgae.

Given various responses of algae species to exposure to the heterogeneous nature of NPs^{23,24} and algae as a primary source of food chains to higher consumers (animals/humans), it is imperative to explore the potential ecotoxicity of NHs on algae species. Thus, this study aims to (a) investigate in vitro ecotoxicity effects of NHs (ZnO-GO, TiO₂-GO, ZnO-CNT, and TiO₂-CNT) on *Thalassiosira pseudonana*, a species of marine-centric diatoms chosen as a model for diatom physiology studies widely distributed throughout entire marine food chains²⁵; (b) explore the toxicity mechanisms caused by the selected NHs; and (c) specifically link how differently characterized NHs influence algal growth inhibition, reactive oxidative stress, and cell morphology changes.

2 | MATERIALS AND METHODS

2.1 | Materials

Graphene oxide was purchased from ACS Material (CA; GO; a single-layer of more than 99% purity, 0.26 g/cm³ bulk density, 1–5 µm diameter, and 0.8–1.2 nm thickness according to Hummers' method). A multiwalled carbon nanotube (MWCNT) was supplied by Sigma-Aldrich (MO; MWCNT; more than 98% purity, 6–13 nm [O.D.] × 2.5–20 µm [L]). Chemicals such as zinc nitrate hexahydrate (Zn[NO₃]₂ 6H₂O; >98% purity), ZnCl₂ (>99.99% purity) for ZnO nanocomposite synthesis and a titanium oxysulfate solution (TiOSO₄; ~15 wt% and 99.99% purity) for TiO₂ nanocomposite synthesis were obtained from Sigma-Aldrich.

2.2 | Fabrication of four types of NHs

A hydrothermal process was used for the synthesis of four types of NHs (see Supporting Information for an additional synthesis procedure). A literature method²⁶ was used for synthesis of TiO₂-GO. The TiO₂-CNT NH was synthesized by applying the method of Huang et al.²⁷ The synthesis of the ZnO-GO NH was fabricated according to the method described by Fu et al.,²⁸ and ZnO-CNT was synthesized using the method described by Wang et al.²⁹

2.3 | Physicochemical properties of NHs

Characterization (physicochemical properties) of the four types of NHs was performed using field emission scanning electron microscopy (FE SEM; Philips XL-30 FEG), energy dispersive spectroscopy, JEOL 1400 transmission electron microscopy (TEM), and Perkin Elmer Frontier Fourier Transform-Infrared Spectroscopy (FT-IR; MA). First, in regard to SEM preparation, cultures were placed in 2% glutaraldehyde in a phosphate-buffered saline (PBS). Samples were rinsed three times for 5 minutes each in the PBS buffer, then dehydrated in a graded series of ethanol to 100%, and dried in hexamethyldisilazane.

After being outgassed overnight, samples were coated with Pd in a sputter coater and imaged using an XL-30 field emission SEM. High-resolution TEM (HR-TEM) images were taken using a Philips CM-10 TEM (Eindhoven, Netherlands) at the Miller School of Medicine TEM Core at the University of Miami. Aqueous samples were then drop-casted onto a carbon grid and let to air-dry before being scanned. The vibrational frequencies of the chemical bonds were measured to identify the different functional groups in nanocomposites using an FT-IR analysis. A pellet of the sample powders was measured with Perkin Elmer Frontier FT-IR (MA) after the sample powders were ground and compressed in the 600–4000 cm⁻¹ range.

2.4 | Cell viability analysis with *T. pseudonana*

The in vitro toxicity evaluation of the NHs on *T. pseudonana* was carried out as follows. The algal cell in the marine medium was obtained by the University of Texas at Austin. According to the information provided by the supplier, the marine diatom (unicellular and brackish with centric cells) has shells made of silica. Artificial seawater containing an #2 medium was used for culturing algal cells, and incubation at 12 hours:12 hours (light:dark)

cycle was completed with a Verilux VT 10 (5000 lx white light). Guillard's method was used for making artificial sea water,³⁰ and UV absorbance was measured to determine the algal growth using a Beckman Coulter DU 720 spectrophotometer. Five points of calibration indicated the peak absorbance of *T. pseudonana* at 670 nm (linear with $R^2 = .9991$). The diatom cells were exposed to four different NH suspensions in culture tubing under the same temperature and light and incubated for 72 hours. Algal cells were measured with UV absorbance at 0, 24, 48, and 72 hours. The growth inhibition rate of algae was calculated to measure the extent of toxicity of NHs compared to that of the control.

2.5 | ROS detection

Photocatalytic activity of the algal cell was measured by fluorescence intensity using a Spectramax Gemini EM Microplate Reader (Molecular Devices, CA). A DCF-DA (2',7'-dichlorofluorescein diacetate) cellular-ROS-detection assay kit (Abcam, Inc., Toronto, ON, Canada) was used to dye hydroxyl and peroxy within the diatomic cells. Cells were exposed to four different NH suspensions for 0, 24, 48 hours, and 72 hours under illuminated conditions using a lamp. At each time point, cells were collected in a conical tube and washed by centrifugation in PBS. The washed cells were suspended in the diluted DCF-DA solution and incubated at 37°C for 30 minutes in the dark. After they were washed by centrifugation with PBS buffer, the cells were transferred into a clear-bottom 96-well microplate. The detected DCF by fluorescence spectroscopy indicated a maximum excitation at 485 nm and emission spectra at 535 nm in the presence of the buffer. The photocatalytic activity of different NHs was examined with antioxidant *N*-acetyl cysteine (NAC), a ROS scavenger, to evaluate the toxicity mechanism between NHs and cells.³¹ NAC prevents toxicity caused by ROS through directly scavenging ROS or promoting production of antioxidant enzymes.³² Our hypothesis is that the addition of NAC reduces the ROS generation of NHs. To measure this, cells were pretreated with 0.5 and 2 mM of NAC, and fluorescence intensity was measured at 72 hours.

2.6 | Statistical analysis

All experimental conditions were run in triplicate. Based on Student's *t* test, which compares the means of two groups using correlation and regression, the Graph Pad Prism 3.0 (Graph Pad Software, Inc., San Diego, CA) was used to estimate *P* values; all the experimental values were compared to their corresponding control values and had statistical significance at $P < .05$.

3 | RESULTS AND DISCUSSION

3.1 | Physicochemical properties of NHs

The formation of metal oxides (ZnO and TiO₂) and their compounding with the CNT and GO were verified using SEM and TEM (Figure 1). As illustrated in Figure 1A,B, aggregated ZnO NPs were dispersed irregularly on GO, with clusters covering the surface of GO sheet plates, suggesting enhancement of dispersion by GO sheets. When TiO₂ was conjugated with GO, aggregated clusters were observed between GO sheets (Figure 1C,D). Byrappa et al.³³ argued that CNTs act as substrates for the ZnO and TiO₂ NPs, thus causing dispersion of metal-oxide NPs onto the CNTs. However, they found metal-oxide NPs loaded on CNTs

to have more aggregation with each other compared to those loaded on GO sheets. ZnO NPs were observed to have spherical shapes on CNT (Figure 1F), and TiO₂ NPs had rod shapes (Figure 1H). Thus, TiO₂ NPs on CNTs formed more aggregation clusters than those due to ZnO NPs on CNTs (Figure 1H). According to a recent study, whereas a fixed aggregation structure of ZnO NPs on ZnO-CNT is found, TiO₂ NP in TiO₂-GO is found to have a rod shape and self-aggregation.³⁴ Experimental results on the stability of ZnO-conjugated NHs (ZnO-CNT and ZnO-GO) in comparison to ZnO NPs (Supporting Information) also confirm dispersion and stability from ZnO-conjugated NHs compared to ZnO NPs alone, which are in the order of ZnO-GO > ZnO-CNT > ZnO.

3.1.1 | Analysis of Raman spectra—The G band at 580 cm⁻¹ displayed on the Raman spectrum of graphene-based materials indicates the C—C vibrational mode, and the D band peak at 1350 cm⁻¹ suggests that carbon is disordered or a graphitic structure is flawed.^{35,36} The peaks of the D band in CNT-based NHs exhibit more significance compared to that of the D band in the GO-based material, indicating better stability in the structure of GO-based NHs compared to that of CNT-based nanocomposites.³⁶ The presence of the anatase TiO₂ polymorph was confirmed with peaks at 398, 519-, and 641 cm⁻¹, and E2 and E1 (LO) modes of the wurtzite ZnO structure were verified at 430 and 580 cm⁻¹ peaks.³⁷

3.1.2 | Analysis of FT-IR spectroscopy—Fourier transform-infrared (FT-IR) spectra of bare GO, ZnO-GO, TiO₂-GO, bare CNT, ZnO-CNT, and TiO₂-CNT NHs were examined to characterize the compounds' functional groups (Figure 2). In Figure 2A, the IR peaks at 1725, 1150, and 1180 cm⁻¹ are associated with oxygen-containing functional groups on C=O, C—OH, and C—O stretching vibrations, respectively. The C=C bond is indicated in the peak at 1630 cm⁻¹, and the IR peak around 650 cm⁻¹ is associated with the Zn—O structure. The oxygen functional groups confer high hydrophilicity on GO, which produces its solubility and potentially high mobility in water.

The significant decrease in the intensity of the O—H peak for GO in ZnO-GO and remarkable elimination of oxygen functional groups in TiO₂-GO composites were consistent with those in the literatures.^{38,39} The GO was further reduced as indicated by the lower peaks in ZnO-GO because of the attachment of ZnO NPs onto the GO nanosheet.⁴⁰ The stretching vibration of Ti—O—Ti and Ti—O—C bonds was found from the dramatic peaks at 500 to 900 cm⁻¹.³⁶ However, as revealed in Figure 2B, no distinct peaks were found in the CNT spectrum at 500 to 4000 cm⁻¹.⁴¹ There were several peaks (O—H, C—H, C=O, C=C, and C—O) from the spectrum of ZnO-CNT to TiO₂-CNT, with the peaks at 1300 cm⁻¹ (Ti—OH) and 550 cm⁻¹ (Ti—O—Ti).^{34–42}

3.2 | Toxicity effect of NHs on algae

Figure 4 illustrates the growth inhibition of the *T. pseudonana* algae when the marine diatomic algae was exposed to four types of NHs at two different concentrations of NHs (50 and 100 mg/L) over 24, 48, and 72 hours. Given the accumulation of NPs present in the environmental media, high particle concentrations of NHs were applied in this study. There are various entry points for NPs into the environment, including direct application to an

environment, wastewater treatment plant effluent, and wastewater treatment sludge. There is a large range for NPs present through environmental media.

According to a recent study⁴³ that shows the predicted environmental concentrations of highly produced and used NPs (eg, Ag, TiO₂, ZnO, and carbon-based) in the environment, the concentrations of TiO₂, ZnO, and Ag NPs present are estimated up to 10 mg/L in surface water and their concentrations increase in wastewater treatment sludge, showing 100 to 2000 mg/kg for TiO₂ NPs. Given that there is no appropriate treatment for the removal of NPs in wastewater treatment facilities, water distribution system would serve a reservoir for the accumulation of NPs. Besides, as the global production and use of NPs increases, amount of NPs release into the environment will also increase and be gradually accumulated in water, resulting in significant concentrations of accumulated NPs.

The growth inhibition was estimated based on the following²³:

$$\text{Growth inhibition (\%)} = \left(1 - \frac{\text{UV} \cdot \text{abs}_{\text{concentration at 6 hours}}}{\text{UV} \cdot \text{abs}_{\text{control}}} \right) \times 100 \quad (1)$$

The growth inhibition of *T. pseudonana* increased over increasing exposure time from 24 to 72 hours, and the extent of growth inhibition was more apparent at the high concentration of NHs and dependent on the types of NHs. For instance, the growth inhibition rate of *T. pseudonana* was in the order ZnO-GO (45.5%) > ZnO-CNT (42.5%) > TiO₂-GO (11.2%) > TiO₂-CNT (1.3%). In regard to the concentration effect of NHs on the growth inhibition of *T. pseudonana*, the highest inhibition rate of 45.5% NHs was observed with ZnO-GO at 50 mg/L, and at a higher concentration of NHs at 100 mg/L, the growth inhibition rate of *T. pseudonana* increased, especially for the ZnO-based NH-exposed suspensions (eg, ZnO-GO, ZnO-CNT, TiO₂-GO), in the order ZnO-GO (75.5%), ZnO-CNT (47.5%), TiO₂-GO (33.2%), and TiO₂-CNT (1.3%), respectively.

Several studies have noted that ZnO NPs are more toxic than TiO₂ NPs because of the higher solubility of ZnO NPs than that of TiO₂ NPs.^{44–46} The primary attribute of toxicity appears to be due to the production of ROS from NPs, which is responsible for cell damage by such oxidative stress.^{12,14,21} Researchers^{47–49} reported ROS generation from NPs to be increased as NP concentration increased and size decreased. The correlation between the toxicity of NPs on algal cells and reduced cell viability by a NP concentration increase was noted in several studies,^{15,50–52} with proposed mechanisms including physical interaction between algae and NPs, ROS generation, and the shading effect (aggregation). NPs can damage or enter the cell wall by passing through or attaching to cells.

As surface area increases, NP attachment on cell membranes can be increased by electrostatic force, thereby causing damage to the cell membranes.⁴⁷ The shading effect reduces light availability for photosynthesis of algae, so that aggregation of NPs can affect the shading by blocking or attaching to the surface of the cells.⁵¹ Thus, the size of aggregation can also be a key factor in determining the effect of NPs on cells due to NPs' surface area and reactivity.²⁰ Notably, ZnO- and TiO₂-cojugated GO (ie, ZnO-GO and TiO₂-GO) exhibited higher growth inhibition than ZnO- and TiO₂-combined CNT (eg, ZnO-CNT

and TiO₂-CNT) at both concentrations of NHs, with no toxicity effect on algae at both concentrations of 50 and 100 mg/L from the TiO₂-CNT NH (Figure 2).

A similar observation when there was more toxicity of GO on algal cells compared to CNT was observed in a recent study by Zhao et al,⁵³ possibly due to GO's hydrophilic surface and strong electrostatic repulsion between GO sheets. Lv et al⁵⁴ reported that GO could be more toxic than other carbon nanomaterials due to its enhanced dispersibility and high hydrophilicity, whereas CNTs are hydrophobic nanomaterials that are likely to form more aggregates. A study by Lv et al⁵⁴ revealed the toxicity mechanism of GO on *Daphnia magna* as oxidative stress from GO accumulation after 24 hours exposure of *D. magna* to GO NPs. Other suggested explanations were high hydrophilicity of GO and faster GO uptake/depuration that occurred in daphnia than those in other carbon nanomaterials,⁵⁴ thereby preventing aggregation by dispersion of NPs on GO, with a subsequent increase in NP surface and reactivity. A study by Hu et al²⁰ reported that the aggregation and the following sedimentation of TiO₂ NPs reduced their adverse effect on microalgae and suggested that other factors such as ionic strength and NP concentration influenced aggregation and sedimentation rates.

The toxicity effect of single metal-oxide NPs (ZnO or TiO₂) on *T. pseudonana* was compared with toxicity effects from GO-conjugated TiO₂ or ZnO NPs. The results suggest higher toxicity on exposure of *T. pseudonana* to metal-oxide NPs (either ZnO or TiO₂)-conjugated with GO in comparison to the toxicity, when *T. pseudonana* is exposed to single type of NPs. This is possibly due to dispersion effect by GO, rendering NPs to have high surface area and to increase the ROS generation. For instance, the growth inhibition rate of *T. pseudonana* was in the order of ZnO-GO (77%) > ZnO (42%) > TiO₂-GO (23.4%) > TiO₂ (4.3%). Notably, ZnO-based NHs showed higher toxicity than TiO₂-conjugated NHs, because of higher solubility of ZnO NPs than that of TiO₂ NPs, influencing the ROS generation. Soluble ZnO NPs was more efficient than insoluble TiO₂ in stimulating ROS production.⁵⁵ In terms of the toxicity mechanism, the possibility of the effect of dissolved ZnO or released Zn ions on the observed high toxicity by ZnO-conjugated NHs was examined, and results (Figures S2 and S3) indicate negligible effect of Zn ions released from ZnO-conjugated NHs. Additionally, given the neutral pH of ZnO NH suspension, the effect of dissolved ZnO on toxicity is also trivial due to the suspension pH near the IEP of 7.6.

3.3 | Assessment of ROS generation

When nanocomposites absorb light energy, charge separation is induced, and nanocomposites produce pairs of e⁻ and h⁺. The excited electrons are transferred from the valence band (VB) to the conduction band (CB), and leave holes in the VB. This is followed by the generation of ROS by oxidation and reduction reactions of facilitated pairs of e⁻ and h⁺.⁵⁶ When metal-oxide NPs are combined with other nanocomposites (GO and CNT), the separated charges are transferred to each other, thereby enhancing suppression of the recombination of electron-hole pairs and increasing photocatalytic activity of nanocomposites by adsorption of NHs.⁵⁷ The ROS generation measured by the fluorescence intensity rate (%) after exposure of algae to four types of NHs was in the ascending order

ZnO-GO > ZnO-CNT > TiO₂-GO > TiO₂-CNT, which was consistent with observation in the toxicity effect of algae (Figure 3).

The generation of ROS from ZnO-GO-exposed algae was significantly higher than that of the other types (ie, CNT, TiO₂-GO, and TiO₂-CNT) of NH-exposed algal cells. A previous study suggested that the distribution of metal oxide on GO can enhance ROS generation because the distribution of NPs on GO can increase the surface area of NHs.⁵³ Thus, hydroxyl radicals were generated even under the absence of ultraviolet light by metal-oxide NPs.⁴⁴ Wang et al⁵⁸ reported that the toxicity of graphene and ionic liquids may be driven by few mechanisms, such as adsorption, stability, and oxidative stress. Especially, the physicochemical interaction of graphene by the liquids' sharp edges was important to toxicity on algae in aquatic environments.

As illustrated in Figure 3, the increase of ROS correlated to the concentration of NHs and exposure time. ROS generation from NHs was increased over exposure time and increasing concentration of NHs. However, there was a slight decrease in ROS generation observed over 72 hours exposure to algae at 50 mg/L of ZnO-GO, possibly due to reduced photoactivity from aggregation and the shading effect over long-term exposure of NHs to algae at a low ZnO-GO concentration (50 mg/L). It appears that reduced surface area and reactivity from aggregation of NHs decreased the photoactivity of NHs.

A study by Ko et al⁵⁹ revealed that the exposure of Ag-GO nanocomposites to algae resulted in the inhibition of microorganism activities through oxidative stress and cell-membrane damage induced from GO and Ag-GO. In this study, 80% of ROS was generated by GO within 1 hour, whereas ROS was not detected in 24 and 48 hours because of aggregation and precipitation of GO. Applying a scavenger (NAC) suppressed the ROS generation and the extent of suppression was increased with higher concentration of NAC (0.5 vs 2 mM), indicating that the ROS generation was a primary toxicity mechanism and the maximum capacity of NAC increased with increasing NAC concentrations.^{32,60} Different types of nanomaterials (ZnO NPs, TiO₂ NPs, GO, and CNTs) may induce oxidative stress by producing ROS, causing DNA damage and attaching to cell surfaces, which leads to toxic effects in the cells by disrupting metabolic biomolecules.⁶⁰ Several studies reported that NHs, which are a combination of metal-oxide NPs and carbon-based nanomaterials, have more photocatalytic activity than single-NP types of NPs.^{61,62} This is because the long distance of shuttling electrons between metal-oxide NPs and carbon-based nanomaterials leads to blocking of the recombination of photo-generated e⁻ and h⁺ pairs by electron transfer to each other.⁶³

Results on the ROS generations from single type of NPs vs ZnO or TiO₂-conjugated GO NHs were consistent with the toxicity effect of four types of NPs (ZnO, ZnO-GO, TiO₂, TiO₂-GO) on *T. pseudonana*, in which the ROS generation was in the ascending order of ZnO-GO > ZnO > TiO₂-GO > TiO₂. Results suggest the dispersion effect by GO increases the active surface area of NPs,⁶¹ and a recent study⁶⁴ indicated higher ROS production from TiO₂-GO than that from TiO₂ NPs, which is consistent with results presented in this study. Overall, results of the fluorescence intensity test exhibited that toxicity of NHs combined

with ZnO NPs is higher than that of TiO₂ NP-based NHs. These results are consistent with those obtained by other researchers.^{44,45,65}

Compared to ZnO NPs, suspensions of TiO₂ NPs were of remarkably lower toxicity to algae. Several studies reported that the kinetics and aggregation of ZnO NPs and TiO₂ NPs on algae were highly dependent on pH and ionic strength.^{45,66,67} As observed in this study, no influence of TiO₂-CNT on both cell growth inhibition and ROS intensity was observed over 72 hours exposure of TiO₂-CNT to algae, which was attributed to the aggregation of the CNT due to its hydrophobic nature, thereby promoting self-association and aggregation, as noted by other studies.⁶⁸

3.4 | Analysis of SEM images on algal cells

The interaction between algae and four types of NHs was assessed using SEM image analysis. As illustrated in Figure 4, the SEM images of NHs after exposure to *T. pseudonana* for 72 hours indicate that algal cells were directly clumped and attached to NHs. GO sheets formed aggregation and attachment to the algae and exhibited significant catalytic effects,⁶⁹ through the interaction of nanocomposites with algal cells, disrupting the algal cell wall by oxidative degradation of lipids resulting in cell damage.⁷⁰ A recent study by Zhao et al.⁵³ indicated that there were direct physical damages to algal cells with sharp edges and corners induced by GO, although GO did not penetrate into algal cells because of the protection of cell walls.

Other studies that employed SEM images to analyze the interaction between algal cells and CNTs reported severely aggregated CNTs,^{71–73} which visualized the CNTs' exposure to algal suspension—interaction of CNTs with algal cells by attaching to the outer cell wall of algae. As revealed in Figure 4, significant morphology changes with algal cell structure were observed, and aggregated NHs were attached to the surface of cells when NHs were exposed to algae. Shading effects of GO or the CNT and the resultant reduction of light for algal photosynthesis were reported in several studies^{45,74,75} in which GO or CNT agglomeration was around the algal cells, and such shading effects resulted in decreasing chlorophyll to algal cells due to nanocomposites' adsorption on cell surfaces. According to these studies,^{45,74} the growth inhibition was correlated with the shading due to carbon nanomaterials and their agglomeration around algal cells.

According to a study by Schwab et al.,⁷⁶ well-dispersed CNTs also had a stronger shading effect than high CNT concentration, and they affected ROS generation.⁷⁶ In this study, experimental results indicated that well-dispersed CNTs absorbed over six times more light than agglomerated CNTs. Consequently, both shading and agglomeration of algal cells with GO and CNTs could cause the toxicity effects and ROS generation. Another study reported that the toxicity of algae was not influenced by photocatalytic activity of GO, whereas algal growth inhibition was correlated with the shading effect due to carbon nanomaterials and their agglomeration with algal cells.⁶⁰ As discussed earlier, the shading effect that was related to NP aggregation could reduce a specific area and active sites of the NP surface, so that this aggregation may prevent chlorophyll from absorbing light and inhibit algae growth.

4 | CONCLUDING REMARKS

The in vitro assessment of synthesized NHs on *T. pseudonana* indicated higher toxicity of NHs on *T. pseudonana* over increasing NH concentration and exposure time. The toxicity significance was in the order ZnO-GO > ZnO-CNT > TiO₂-GO > TiO₂-CNT. Microscopic image analysis indicated that GO-based NHs formed less aggregation clusters and dispersed well compared to CNT-based NHs. Well-dispersed NHs may have more reactive sites and large surface areas. Possible mechanisms inducing toxicity of NHs on organisms are 3-fold. First, the generation of ROS by NHs following the induction of electron-hole pairs appears to be a primary toxicity mechanism. As evidenced in a series of experiments, the generation of ROS was most profound from ZnO-GO, which was consistent with the extent of growth inhibition of algal cells (Figure 5).

Second, cell-membrane damage caused by NH attachment may contribute to the toxicity of NHs, especially ZnO NP-conjugated NHs. The NHs containing ZnO NPs exhibited higher toxicity effects to algae than those containing TiO₂ NPs because of the high solubility of ZnO NPs in water, due to the hydrophilic affinity of GO nanosheets, which increase dispersions of metal-oxide NPs on their surface. A significant dispersion of metal-oxide NPs on the GO sheet could induce higher oxidation and toxicity through prevention of NP aggregation. Last, the shading effect of NHs by preventing the photosynthesis of algae is one of toxicity mechanisms. As revealed in the SEM images, there were aggregated NHs attached to or around the algae surfaces, thus causing shading effects. Our key findings on the toxicity kinetics and underlining mechanisms of the NH toxicity are encouraging in that designing and manufacturing NHs in a safe and eco-friendly manner can not only minimize harm to the marine environments but also improve understanding of the ultimate fate and transport of NPs, since scant information is available on the NH toxicity and toxicity mechanisms of a marine aquatic species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This research was supported by the U.S. Environmental Protection Agency through the Office of Research and Development (AWARD: EP 17Z000237). The views expressed in this article are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. We thank Dr. Minseo Park and Mr. Min Khanal at Auburn University for Raman spectroscopy analysis.

Abbreviations:

CNT	carbon nanotube
GO	graphene oxide
MWCNT	multiwalled carbon nanotube
NPs	nanoparticles
<i>T. pseudonana</i>	<i>Thalassiosira pseudonana</i>

TiO₂-CNT	synthesized TiO ₂ -CNT nanocomposite
TiO₂-GO	synthesized TiO ₂ -GO nanocomposite
ZnO-CNT	synthesized ZnO-CNT nanocomposite
ZnO-GO	synthesized ZnO-GO nanocomposite

REFERENCES

1. Piccinno F, Gottschalk F, Seeger S, Nowack B. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. *J Nanopart Res.* 2012;14:1109.
2. Minetto D, Volpi Ghirardini A, Libralato G. Saltwater ecotoxicology of Ag, Au, CuO, TiO₂, ZnO and C₆₀ engineered nanoparticles: an overview. *Environ Int.* 2016;92–93:189–201.
3. MamathaKumari M, Praveen Kumar D, Haridoss P, DurgaKumari V, Shankar MV. Nanohybrid of titania/carbon nanotubes—nanohorns: a promising photocatalyst for enhanced hydrogen production under solar irradiation. *Int J Hydrogen Energy.* 2015;40:1665–1674.
4. Hotze EM, Phenrat T, Lowry GV. Nanoparticle aggregation: challenges to understanding transport and reactivity in the environment. *J Environ Qual.* 2010;39:1909. [PubMed: 21284288]
5. Chen M, Zhou S, Zhu Y, et al. Toxicity of carbon nanomaterials to plants, animals and microbes: recent progress from 2015-present. *Chemosphere.* 2018;206:255–264. [PubMed: 29753288]
6. Love SA, Maurer-Jones MA, Thompson JW, Lin YS, Haynes CL. Assessing nanoparticle toxicity. *Annu Rev Anal Chem (Palo Alto Calif).* 2012;5:181–205. [PubMed: 22524221]
7. Suresh AK, Pelletier DA, Doktycz MJ. Relating nanomaterial properties and microbial toxicity. *Nanoscale.* 2013;5:463–474. [PubMed: 23203029]
8. Baek S, Joo SH, Blackwelder P, Toborek M. Effects of coating materials on antibacterial properties of industrial and sunscreen-derived titanium-dioxide nanoparticles on *Escherichia coli*. *Chemosphere.* 2018; 208:196–206. [PubMed: 29870909]
9. Joo SH, Knecht M, Su C, Seo S, Lawrence R. Influence of siloxane on the transport of ZnO nanoparticles from different release pathways in saturated sand. *RSC Adv.* 2016;6:100494–100503. [PubMed: 32095232]
10. Cardinale BJ. Biodiversity improves water quality through niche partitioning. *Nature.* 2011;472:86–89. [PubMed: 21475199]
11. Wilkie AC, Mulbry WW. Recovery of dairy manure nutrients by benthic freshwater algae. *Bioresour Technol.* 2002;84:81–91. [PubMed: 12137274]
12. Hou J, Wu Y, Li X, Wei B, Li S, Wang X. Toxic effects of different types of zinc oxide nanoparticles on algae, plants, invertebrates, vertebrates and microorganisms. *Chemosphere.* 2018b;193:852–860. [PubMed: 29874759]
13. Middepogu A, Hou J, Gao X, Lin D. Effect and mechanism of TiO₂ nanoparticles on the photosynthesis of *Chlorella pyrenoidosa*. *Ecotoxicol Environ Saf.* 2018;161:497–506. [PubMed: 29913418]
14. Morelli E, Gabellieri E, Bonomini A, Tognotti D, Grassi G, Corsi I. TiO₂ nanoparticles in seawater: aggregation and interactions with the green alga *Dunaliella tertiolecta*. *Ecotoxicol Environ Saf.* 2018;148: 184–193. [PubMed: 29055202]
15. Suman TY, Radhika Rajasree SR, Kirubakaran R. Evaluation of zinc oxide nanoparticles toxicity on marine algae *Chlorella vulgaris* through flow cytometric, cytotoxicity and oxidative stress analysis. *Ecotoxicol Environ Saf.* 2015;113:23–30. [PubMed: 25483368]
16. Chen M, Sun Y, Liang J, et al. Understanding the influence of carbon nanomaterials on microbial communities. *Environ Int.* 2019;126: 690–698. [PubMed: 30875562]
17. Chen M, Qin X, Zeng G. Biodegradation of carbon nanotubes, graphene, and their derivatives. *Trends Biotechnol.* 2017;35:836–846. [PubMed: 28063621]
18. Peng X, Palma S, Fisher NS, Wong SS. Effect of morphology of ZnO nanostructures on their toxicity to marine algae. *Aquat Toxicol.* 2011; 102:186–196. [PubMed: 21356181]

19. Freixa A, Acuna V, Sanchis J, Farre M, Barcelo D, Sabater S. Ecotoxicological effects of carbon based nanomaterials in aquatic organisms. *Sci Total Environ*. 2018;619:328–337. [PubMed: 29154051]
20. Hu J, Wang J, Liu S, et al. Effect of TiO₂ nanoparticle aggregation on marine microalgae *Isochrysis galbana*. *J Environ Sci (China)*. 2018;66: 208–215. [PubMed: 29628089]
21. Rajput VD, Minkina TM, Behal A, et al. Effects of zinc-oxide nanoparticles on soil, plants, animals and soil organisms: a review. *Environ Nanotechnol Monitor Manag*. 2018;9:76–84.
22. Wang Y, Zhu X, Lao Y, et al. TiO₂ nanoparticles in the marine environment: physical effects responsible for the toxicity on algae *Phaeodactylum tricornutum*. *Sci Total Environ*. 2016;565:818–826. [PubMed: 27060054]
23. Galletti A, Seo S, Joo SH, Su C, Blackwelder P. Effects of titanium dioxide nanoparticles derived from consumer products on the marine diatom *Thalassiosira pseudonana*. *Environ Sci Pollut Res Int*. 2016;23: 21113–21122. [PubMed: 27596588]
24. Karimi M, Sadeghi R, Kokini J. Human exposure to nanoparticles through trophic transfer and the biosafety concerns that nanoparticle-contaminated foods pose to consumers. *Trends Food Sci Technol*. 2018;75:129–145.
25. Armbrust EV, Berges JA, Bowler C, et al. The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science*. 2004;306:79–86. [PubMed: 15459382]
26. Stengl V, Bakardjieva S, Grygar TM, Bludska J, Kormunda M. TiO₂-graphene oxide nanocomposite as advanced photocatalytic materials. *Chem Cent J*. 2013;7:41. [PubMed: 23445868]
27. Huang J, Zang J, Zhao Y, Dong L, Wang Y. One-step synthesis of nanocrystalline TiO₂-coated carbon nanotube support for Pt electrocatalyst in direct methanol fuel cell. *Mater Lett*. 2014;137: 335–338.
28. Fu H, Jiang Y, Ding J, et al. Zinc oxide nanoparticle incorporated graphene oxide as sensing coating for interferometric optical microfiber for ammonia gas detection. *Sens Actuators B*. 2018;254: 239–247.
29. Wang X, Xia B, Zhu X, Chen J, Qiu S, Li J. Controlled modification of multiwalled carbon nanotubes with ZnO nanostructures. *J Solid State Chem*. 2008b;181:822–827.
30. Guillard RR. Culture of Phytoplankton for Feeding Marine Invertebrates. Boston, MA: Springer; 1975.
31. Chen TH, Lin CC, Meng PJ. Zinc oxide nanoparticles alter hatching and larval locomotor activity in zebrafish (*Danio rerio*). *J Hazard Mater*. 2014;277:134–140. [PubMed: 24424259]
32. Ma H, Wallis LK, Diamond S, Li S, Canas-Carrell J, Parra A. Impact of solar UV radiation on toxicity of ZnO nanoparticles through photocatalytic reactive oxygen species (ROS) generation and photo-induced dissolution. *Environ Pollut*. 2014;193:165–172. [PubMed: 25033018]
33. Byrappa K, Dayananda AS, Sajan CP, et al. Hydrothermal preparation of ZnO:CNT and TiO₂:CNT composites and their photocatalytic applications. *J Mater Sci*. 2008;43:2348–2355.
34. Upasani P, Sreekumar TV, Gaikar VG, Jha N. Preparation of ZnO nanoribbon–MWCNT composite film and its application as antimicrobial bandage, antibacterial filter and thermal IR camouflage material. *Bullet Mater Sci*. 2017;40:865–876.
35. Perreault F, de Faria AF, Nejati S, Elimelech M. Antimicrobial properties of graphene oxide nanosheets: why size matters. *ACS Nano*. 2015;9:7226–7236. [PubMed: 26091689]
36. Karaolia P, Michael-Kordatou I, Hapeshi E, et al. Removal of antibiotics, antibiotic-resistant bacteria and their associated genes by graphene-based TiO₂ composite photocatalysts under solar radiation in urban wastewaters. *Appl Catal Environ*. 2018;224:810–824.
37. Zamiri R, Rebelo A, Zamiri G, et al. Far-infrared optical constants of ZnO and ZnO/Ag nanostructures. *RSC Adv*. 2014;4:20902–20908.
38. Liu LP, Yang XN, Ye L, et al. Preparation and characterization of a photocatalytic antibacterial material: graphene oxide/TiO₂/bacterial cellulose nanocomposite. *Carbohydr Polym*. 2017;174:1078–1086. [PubMed: 28821030]
39. Rajeswari R, Prabu HG. Synthesis characterization, antimicrobial, anti-oxidant, and cytotoxic activities of ZnO nanorods on reduced graphene oxide. *J Inorg Organometal Polym Mater*. 2017;28:679–693.

40. Rajaura RS, Sharma V, Ronin RS, et al. Synthesis, characterization and enhanced antimicrobial activity of reduced graphene oxide–zinc oxide nanocomposite. *Mater Res Express*. 2017;4:025401.
41. Nguyen VH, Shim J-J. Green synthesis and characterization of carbon nanotubes/polyaniline nanocomposites. *J Spectrosc*. 2015; 2015:1–9.
42. Yuan C, Hung CH, Yuan CS, Li HW. Preparation and application of immobilized surfactant-modified PANi-CNT/TiO₂ under visible-light irradiation. *Materials (Basel)*. 2017;10:877.
43. Maurer-Jones MA, Gunsolus IL, Murphy CJ, Haynes CL. Toxicity of engineered nanoparticles in the environment. *Anal Chem*. 2013;85: 3036–3049. [PubMed: 23427995]
44. Adams LK, Lyon DY, Alvarez PJ. Comparative eco-toxicity of nanoscale TiO₂, SiO₂, and ZnO water suspensions. *Water Res*. 2006;40: 3527–3532. [PubMed: 17011015]
45. Aruoja V, Dubourguier HC, Kasemets K, Kahru A. Toxicity of nanoparticles of CuO, ZnO and TiO₂ to microalgae *Pseudokirchneriella subcapitata*. *Sci Total Environ*. 2009;407:1461–1468. [PubMed: 19038417]
46. Dasari TP, Pathakoti K, Hwang H-M. Determination of the mechanism of photoinduced toxicity of selected metal oxide nanoparticles (ZnO, CuO, Co₃O₄ and TiO₂) to *E. coli* bacteria. *J Environ Sci*. 2013; 25:882–888.
47. Hou J, Wang L, Wang C, et al. Toxicity and mechanisms of action of titanium dioxide nanoparticles in living organisms. *J Environ Sci*. 2018a;75:40–53.
48. Lei C, Zhang L, Yang K, Zhu L, Lin D. Toxicity of iron-based nanoparticles to green algae: effects of particle size, crystal phase, oxidation state and environmental aging. *Environ Pollut*. 2016;218: 505–512. [PubMed: 27449531]
49. Oukarroum A, Bras S, Perreault F, Popovic R. Inhibitory effects of silver nanoparticles in two green algae, *Chlorella vulgaris* and *Dunaliella tertiolecta*. *Ecotoxicol Environ Saf*. 2012;78:80–85. [PubMed: 22138148]
50. Bhuvaneshwari M, Iswarya V, Archana S, et al. Cytotoxicity of ZnO NPs towards fresh water algae *Scenedesmus obliquus* at low exposure concentrations in UV-C, visible and dark conditions. *Aquat Toxicol*. 2015;162:29–38. [PubMed: 25770694]
51. Li F, Liang Z, Zheng X, Zhao W, Wu M, Wang Z. Toxicity of nano-TiO₂ on algae and the site of reactive oxygen species production. *Aquat Toxicol*. 2015;158:1–13. [PubMed: 25461740]
52. Xia B, Chen B, Sun X, Qu K, Ma F, Du M. Interaction of TiO₂ nanoparticles with the marine microalga *Nitzschia closterium*: growth inhibition, oxidative stress and internalization. *Sci Total Environ*. 2015; 508:525–533. [PubMed: 25483108]
53. Zhao J, Cao X, Wang Z, Dai Y, Xing B. Mechanistic understanding toward the toxicity of graphene-family materials to freshwater algae. *Water Res*. 2017;111:18–27. [PubMed: 28040538]
54. Lv X, Yang Y, Tao Y, et al. A mechanism study on toxicity of graphene oxide to *Daphnia magna*: direct link between bioaccumulation and oxidative stress. *Environ Pollut*. 2018;234:953–959. [PubMed: 29665635]
55. Kocbek P, Teskac K, Kreft ME, Kristl J. Toxicological aspects of long-term treatment of keratinocytes with ZnO and TiO₂ nanoparticles. *Small*. 2010;6:1908–1917. [PubMed: 20677183]
56. Yuan Yao GL, Ciston S, Lueptow RM, Gray KA. Photoreactive TiO₂/carbon nanotube composites: synthesis and reactivity. *Environ Sci Technol*. 2008;42:4952–4957. [PubMed: 18678032]
57. Qi K, Cheng B, Yu J, Ho W. Review on the improvement of the photocatalytic and antibacterial activities of ZnO. *J Alloys Compd*. 2017;727:792–820.
58. Wang Z, Zhang F, Wang S, Peijnenburg W. Assessment and prediction of joint algal toxicity of binary mixtures of graphene and ionic liquids. *Chemosphere*. 2017;185:681–689. [PubMed: 28728125]
59. Ko K, Kim MJ, Lee JY, Kim W, Chung H. Effects of graphene oxides and silver-graphene oxides on aquatic microbial activity. *Sci Total Environ*. 2018;651:1087–1095. [PubMed: 30266053]
60. Nogueira PF, Nakabayashi D, Zucolotto V. The effects of graphene oxide on green algae *Raphidocelis subcapitata*. *Aquat Toxicol*. 2015; 166:29–35. [PubMed: 26204245]
61. Chen Y-L, Zhang C-E, Deng C, Fei P, Zhong M, Su B-T. Preparation of ZnO/GO composite material with highly photocatalytic performance via an improved two-step method. *Chin Chem Lett*. 2013;24:518–520.

62. Wang YW, Cao A, Jiang Y, et al. Superior antibacterial activity of zinc oxide/graphene oxide composites originating from high zinc concentration localized around bacteria. *ACS Appl Mater Interfaces*. 2014;6: 2791–2798. [PubMed: 24495147]
63. Zhang K, Zhang FJ, Chen ML, Oh WC. Comparison of catalytic activities for photocatalytic and sonocatalytic degradation of methylene blue in present of anatase TiO₂-CNT catalysts. *Ultrason Sonochem*. 2011;18:765–772. [PubMed: 21146437]
64. Hu Z, Huang Y, Sun S, et al. Visible light driven photodynamic anticancer activity of graphene oxide/TiO₂ hybrid. *Carbon*. 2012;50:994–1004.
65. Kumar A, Pandey AK, Singh SS, Shanker R, Dhawan A. Engineered ZnO and TiO₂ nanoparticles induce oxidative stress and DNA damage leading to reduced viability of *Escherichia coli*. *Free Radic Biol Med*. 2011;51:1872–1881. [PubMed: 21920432]
66. Krol A, Pomastowski P, Rafinska K, Railean-Plugaru V, Buszewski B. Zinc oxide nanoparticles: synthesis, antiseptic activity and toxicity mechanism. *Adv Colloid Interface Sci*. 2017;249:37–52. [PubMed: 28923702]
67. Pagnout C, Jomini S, Dadhwal M, Caillet C, Thomas F, Bauda P. Role of electrostatic interactions in the toxicity of titanium dioxide nanoparticles toward *Escherichia coli*. *Colloids Surf B Biointerfaces*. 2012;92:315–321. [PubMed: 22218337]
68. Koh B, Cheng W. Mechanisms of carbon nanotube aggregation and the reversion of carbon nanotube aggregates in aqueous medium. *Langmuir*. 2014;30:10899–10909. [PubMed: 25144606]
69. Zhang Y, Meng T, Shi L, et al. The effects of humic acid on the toxicity of graphene oxide to *Scenedesmus obliquus* and *Daphnia magna*. *Sci Total Environ*. 2018;649:163–171. [PubMed: 30173026]
70. Souza JP, Venturini FP, Santos F, Zucolotto V. Chronic toxicity in *Ceriodaphnia dubia* induced by graphene oxide. *Chemosphere*. 2018; 190:218–224. [PubMed: 28992473]
71. Pikula KS, Zakharenko AM, Chaika VV, et al. Effects of carbon and silicon nanotubes and carbon nanofibers on marine microalgae *Hetero-sigma akashiwo*. *Environ Res*. 2018;166:473–480. [PubMed: 29957500]
72. Rhiem S, Riding MJ, Baumgartner W, et al. Interactions of multiwalled carbon nanotubes with algal cells: quantification of association, visualization of uptake, and measurement of alterations in the composition of cells. *Environ Pollut*. 2015;196:431–439. [PubMed: 25467692]
73. Zhang L, Lei C, Chen J, Yang K, Zhu L, Lin D. Effect of natural and synthetic surface coatings on the toxicity of multiwalled carbon nanotubes toward green algae. *Carbon*. 2015;83:198–207.
74. Wei L, Thakkar M, Chen Y, Ntim SA, Mitra S, Zhang X. Cytotoxicity effects of water dispersible oxidized multiwalled carbon nanotubes on marine alga, *Dunaliella tertiolecta*. *Aquat Toxicol*. 2010;100: 194–201. [PubMed: 20673592]
75. Wang J, Zhang X, Chen Y, Sommerfeld M, Hu Q. Toxicity assessment of manufactured nanomaterials using the unicellular green alga *Chlamydomonas reinhardtii*. *Chemosphere*. 2008a;73:1121–1128. [PubMed: 18768203]
76. Schwab F, Bucheli TD, Lukhele LP, et al. Are carbon nanotube effects on green algae caused by shading and agglomeration? *Environ Sci Technol*. 2011;45:6136–6144. [PubMed: 21702508]

FIGURE 1.

Physicochemical properties of nanohybrids. A, SEM and TEM images; B, Raman analysis of four types of nanohybrids; C, FT-IR analysis of four different types of NHs.⁸ FT-IR, Fourier transform-infrared; NH, nanohybrid; SEM, scanning electron microscopy; TEM, transmission electron microscopy

FIGURE 2.

Toxicity effect of four different types of NHs on *Thalassiosira pseudonana*, A, growth inhibition at 50 mg/L and, B, growth inhibition at 100 mg/L over 72 hours. NH, nanohybrid

FIGURE 3.

The fluorescence intensity of *Thalassiosira pseudonana* exposed to four different types of NHs for 72 hours. NH, nanohybrid

FIGURE 4.

SEM images of four different types of NHs after exposure to *Thalassiosira pseudonana* for 72 hours. NH, nanohybrid; SEM, scanning electron microscopy

FIGURE 5.

A, Growth inhibition of algae by a single type of NPs (ZnO or TiO₂) vs nanohybrids; B, the fluorescence intensity of *Thalassiosira pseudonana* exposed to nanomaterials for 72 hours.

NP, nanoparticle