Environmental Chemistry and Ecotoxicology 3 (2021) 85–90

Contents lists available at ScienceDirect

Environmental Chemistry and Ecotoxicology

journal homepage: www.keaipublishing.com/en/journals/environmental-chemistry-and-ecotoxicology/

# Evaluation of toxicity of zinc oxide nanorods on green microalgae of freshwater and marine ecosystems

Ana Claudia O. de Almeida [a], Lais F. dos Santos [a], Denice S. Vicentini [b], William G. Matias [b], Silvia P. Melegari [a,*]

[a] Center for Marine Studies, Federal University of Parana - UFPR, Campus Pontal do Paraná, Av. Beira-Mar, 83255-976 Pontal do Paraná, PR, Brazil
[b] Department of Sanitary and Environmental Engineering, Federal University of Santa Catarina - UFSC, Campus Universitário Trindade, 88040-970 Florianópolis, SC, Brazil

ARTICLE INFO

*Article history:*
Received 8 October 2020
Received in revised form 6 January 2021
Accepted 29 January 2021
Available online 4 February 2021

*Keywords:*
Chronic toxicity
*Desmodesmus subspicatus*
Oxidative stress
*Tetraselmis sp.*
Zinc oxide nanorods

ABSTRACT

Zinc oxide (ZnO) nanomaterials (NMs) are widely used in the manufacture of several commercial products like foods, packaging, cosmetics, medicines and healthcare formulations and anti-fouling paints. These NMs can pollute water bodies when they become bioavailable. In this context, this study investigated the toxicity of ZnO nanorods (NRs) on green microalgae from freshwater and marine ecosystems, to better understand the behavior of this NM on each environment. Two green microalgae species, *Desmodesmus subspicatus* (freshwater) and *Tetraselmus* sp. (marine), were evaluated by chronic toxicity tests and oxidative stress induction by the enzymatic activity of catalase (CAT). The exposition assays were performed using three different concentrations of ZnO NRs (0.1, 1.0, and 10 mg/L, and a negative control). ZnO NRs significantly affected the growth rate of both tested chlorophytes. The chronic toxicity test showed LOEC (Lowest Observed Effect Concentration) levels of 10 mg/L (72 h) for *D. subspicatus* and 1.0 mg/L (24 h) for *Tetraselmis* sp. It was observed NOEC (No Observed Effect Concentration) levels of 1.0 mg/L to *D. subspicatus* was (at 72 h) and of $<0.1$ mg/L for *Tetraselmis* sp. (at 24 h). In the enzymatic activity tests of *D. subspicatus* exposed to ZnO NRs, the CAT activity caused significant changes at the concentration at 10 mg/Lof ZnO NRs when compared to the control test, but for *Tetraselmis* sp. no change was observed in CAT activity. These results indicate that *D. subspicatus* was more sensitive to the effects of ZnO NRs at the concentration of 10 mg/L after 72 h, while oxidative stress of this alga was also observed at the same concentration. The results of this study show the importance of further investigating the toxicological effects of ZnO NRs on green microalgae from distinct aquatic environments and of evaluating the toxicological response of these microalgae in culture media.

## 1. Introduction

Investigation of the toxicity of nanomaterials (NMs) is increasing due to the large production and incorporation of these materials in many products. The increase of these consumer products causes NMs' release and bioavailability in the environment. Zinc oxide (ZnO) NMs are widely used in the manufacture of anti-fouling paints as well as skincare products. ZnO NMs have excellent ultraviolet (UV)-absorbing properties and transparency to visible light, making these NMs excellent sunscreen agents [1]. Additionally, the biocidal activity gives ZnO NMs high potential for applications in products such as foods, packaging, cosmetics, medicines and healthcare formulations [2].

Due to the large production of consumer goods containing ZnO NMs and the possibility of their bioavailability in the environment, global interest in studying the toxicity of these NMs is increasing. There is rising awareness of the need to prevent the environment from suffering harmful effects from these pollutants, especially in aquatic ecosystems [3,4].

These emerging contaminants can become available in the environment, thus becoming pollutants by the release of Zn ions to the solution [5]. Zinc is widely distributed throughout the aquatic environments and is an essential trace element required by most living organisms for their growth and development [6]. Ionic $Zn^{2+}$ species can be found in natural surface waters at usually concentration below 10 μg/L [7]. The bioavailability of $Zn^{2+}$ ions in seawater vary widely in coastal and oceanic waters

* Corresponding author at: Laboratory of Ecotoxicology, Center for Marine Studies, Federal University of Parana - UFPR, Campus Pontal do Paraná, Av. Beira-Mar, 83255-976 Pontal do Paraná, PR, Brazil.
*E-mail address:* silvia.melegari@ufpr.br (S.P. Melegari).

Production and hosting by Elsevier on behalf of KeAi

http://dx.doi.org/10.1016/j.enceco.2021.01.003

with concentrations of 0.006 to 22 μg/L [6]. This wide range of Zn concentration is due several factors like pH, salinity, temperature, depth, circulation, upwelling and others [6].

However, the toxic effects in aquatic environments of the use of Zn-contain NMs need to be further investigated since these environments are the ultimate receivers of all anthropogenic pollution. Furthermore, the biocidal activity of ZnO NMs is due to the formation of hydrogen peroxide on the surface of the particles, which are responsible for inducing oxidative stress [8].

The ecotoxic effects of ZnO NMs have been well reported in literature regarding several trophic levels: primary producers [9–13], primary consumers [8,14–20], secondary consumers [21–23], and decomposers [24–27]. The main mechanism of action for ZnO NP toxicity in aquatic ecosystems reported in these studies include Zn ions' dissolution, NM internalization, and induction of oxidative stress by the generation of reactive oxygen species (ROS). The uptake of ZnO NMs through the skin, mucous membranes, and cell membranes is made easier due to their small size [28]. Within cells, ZnO NMs can trigger several toxicity mechanisms, including oxidative stress of certain organelles, resulting in a redox imbalance, leading to dysfunction and cell death [29,30].

The oxidative stress due to ROS generation is one of the effects that can be observed in various organisms. The balance of antioxidant enzymes contributes to the response of organisms to stress as a way of protecting cells. Adequate responses to environmental changes are crucial for organisms' growth and survival. Despite this, the molecular and biochemical mechanisms that govern these responses are still poorly understood. ROS can act as damaging or as signaling molecules that activate multiple defense responses [31]. These functions can be achieved when cell levels of ROS are well controlled in both production and consumption, [30–32]. The level and type of ROS are determining factors of the type of response.

Microalgae can regulate these ecosystems because they exist on a large scale and are primary producers [32]. Microalgae are generally employed as indicators of aquatic pollution by chemical substances [33]. Chlorophytic microalgae are good bioindicators of water quality due to their place in the trophic chain and predictable response to various environmental changes [34] Therefore, they are widely used in environmental toxicology studies [35,36].

In this context, this study investigated the toxic effects of ZnO nanorods (NRs) on green microalgae from freshwater and marine ecosystems, to better understand the behavior of this NM in each environment. We also aimed to contribute to more accurate assessment of the potential toxicological risk, as there are no regulations regarding the potential risk of ZnO NMs in aquatic environments. Therefore, we evaluated the toxicity of ZnO NRs on two different chlorophytes: the freshwater green microalga *Desmodesmus subspicatus* and the marine green microalga *Tetraselmis sp*.

## 2. Material and methods

### 2.1. Microalgal strains and cultures

The *D. subspicatus* strain was kindly provided by the Environmental Laboratory of Joinville Regional University (UNIVILLE), located in Joinville, Santa Catarina, Brazil. Algal cells were cultivated through the methods described by ABNT [37] and OECD [38], with some modifications. The culture medium used was LC-Oligo, prepared with ultrapure water, and the culture was kept under continuous illumination (6500 lx), aeration and stirring (150 rpm; Color Squid White Magnetic Stirrer - IKA, Germany), at controlled 20 ± 2 °C into an incubator (LT 320 TP – LimaTec, Brazil). 80% of the culture was discarded weekly and renewed by a new sterile culture medium.

The *Tetraselmis* sp. strain (CCMP908 from Bigelow Laboratory for Ocean Sciences) was kindly provided by the Engineering and Malacoculture Laboratory (LEMAQUI) of Federal University of Paraná (UFPR), located in Pontal do Paraná, Paraná, Brazil. Algal cells were cultivated by the methods described by ABNT [39], with some adaptations. The culture medium used was F/2 Guillard (1975), prepared with filtered seawater, with salinity adjusted to a minimum of 30 (30‰), and the cell cultures were also kept in continuous illumination (6500 lx), aeration and stirring (150 rpm; Color Squid White Magnetic Stirrer - IKA, Germany), at controlled 20 ± 2 °C into an incubator (LT 320 TP – LimaTec, Brazil). 80% of the culture was discarded weekly and renewed by a new sterile culture medium.

### 2.2. Synthesis and characterization of ZnO NRs

ZnO NRs were synthesized as described by Gonçalves et al. [17] and Melegari et al. [8]. The size and shape of ZnO NRs were determined by transmission electron microscopy (TEM; JEM 1011 MET-100 kV, JEOL, Japan). The ZnO NR suspension (1 g/L) in ultrapure water was prepared by placing droplets of the suspension on a CCu grid (300 mesh) and drying in a desiccator under vacuum for 24 h.

Characterization of the ZnO NR suspension was performed in cultures using LC-Oligo (freshwater medium) and F/2 Guillard (saltwater medium) by measurement of hydrodynamic diameter (HD) and Zeta potential (Pz). The Pz was determined by the electrophoretic mobility approach using a NanoBrook 90Plus PALS analyzer (Brookhaven Instruments, USA). The stability of the particles in both media was determined by measuring HD using dynamic light scattering. The concentration of ZnO NRs used in the suspension was 500 mg/L. These analyses were performed to verify the behavior and stability of the ZnO NR suspension in the diluent medium employed in the toxicity tests.

### 2.3. Chronic toxicity assays

The chronic toxicity assays were developed according to guidelines of ABNT and OECD [37–39]. The chronic toxicity tests were performed for both microalgae (*D. subspicatus* and *Tetraselmis* sp). Three different test concentrations of ZnO NRs (0.1, 1.0, and 10 mg/L, final concentration in the exposition test) were used, plus the negative control (microalgae only in culture medium). All toxicity tests for concentrations of ZnO NRs were conducted in triplicate. ZnO NR stock suspension was prepared at a concentration of 10,000 mg/L in both culture media. The ZnO NR stock suspension was homogenized with an ultrasound probe (Labsonic U - 500 W - B. Braun, Germany) for 5 min at 80% power. From the ZnO NR stock suspension, serial dilutions were performed in the test concentration range directly in the algal exposure vial. The vessels were incubated in a shaker incubator for 3 days at 20 ± 2 °C and the same luminosity as the growth culture, continuously stirred at 150 rpm, in an orbital shaker-incubator (LT-600 – LimaTec, Brazil). The cell density was measured at 24, 48, and 72 h after exposure to ZnO NRs with a UV–Vis spectrometer at a wavelength of 680 nm for *D. subspicatus* and 425 nm for *Tetraselmis* sp. To validate the results of each toxicity test, it was considered a minimum of growth rate to the negative control of 0,9 $d^{-1}$. Two calibration curves were previously fitted in the UV–Vis spectrometer (EEQ9011I.UV-B – Edutec, Brazil), correlating cell density (measured by counting in a Neubauer chamber under an optical microscope) and absorbance, to perform the chronic assay with cell density measurement. This method was adapted from Valer and Glock [40]. The resulting data were submitted to analysis of variance (ANOVA) as recommended by the US EPA [41]. Orthogonal two-way ANOVA with the Tukey post hoc test was employed to evaluate significant differences between cell density and ZnO NR concentrations after the three exposure times. A level of $p < 0.05$ was accepted as statistically significant. To assure homogeneity of variance and normal distribution, cell concentrations were transformed to log(x + 1). The results were statistically analyzed and NOEC (No Observed Effect Concentration), and LOEC (Lowest Observed Effect Concentration) were defined.

### 2.4. Enzymatic activity

CAT enzymatic activity was measured through its catalytic activities, and the results of these determinations are expressed in terms of the amount of activity present in a given volume or mass of the sample. This method was based on Melegari et al. [42], with adaptations. The specific

activity in this experiment is presented as the activity per protein weight unit (U/mg protein). All exposure and negative control microalgal suspensions were recovered after 72 h of exposure by centrifugation at 15 min at 10 °C. The supernatant was discarded, and the cell pellet was resuspended in 500 μL of PBS buffer 0.1 M, pH 7. The recovered microalgal suspension was stored in a freezer at −80 °C until the enzymatic activity was quantified. The protein in the microalgal suspension was quantified by the Bradford method [43]. To plot the calibration curve, 100 μL of the protein standard (bovine serum albumin, BSA, range of 3.12–100 mg/L) was added to 900 μL of Bradford's reagent, and the absorbance was measured at the wavelength of 595 nm in the UV–Vis spectrometer. CAT enzymatic activity of *D. subspicatus* and *Tetraselmis* sp. algae after 72 h of exposure to NR ZnO were evaluated using the molar absorption coefficient of CAT. For CAT, the activity was evaluated through the degradation of hydrogen peroxide at a wavelength of 240 nm. CAT has a known molar absorption coefficient of 0.0436 $mM^{-1}.cm^{-1}$. 1 μg of protein from each sample of exposed microalgae was added to 100 μL of hydrogen peroxide at 200 mM, and the volume was completed to 1 mL with PBS buffer 0.1 M, pH 7. After 30 s of mixing the reagents, the measurement was performed in a quartz cuvette with 1 cm optical length. The kinetic of measurement at 240 nm was done every 10 s for 60 s. From these results, it was possible to determine the CAT activity of each sample. The resulting data were submitted to ANOVA and a confidence level of $p < 0.05$ was accepted as statistically significant.

## 3. Results and discussion

### 3.1. Characterization of ZnO NRs

TEM images of ZnO NRs can be seen in Fig. 1. The images show that the tested NMs presented rod morphology and average lengths ranging from 50 to 100 nm and diameters from 20 to 30 nm, confirming the nanometric dimensions of the material tested. For additional data on physical-chemical characterization of ZnO NRs (e.g., crystalline system -hexagonal, mean of crystallite size, and surface area) please refer to the previously published manuscripts of Gonçalves et al. [17] and Melegari et al. [8]. The same ZnO NRs were used in these ecotoxicity tests.

Table 1 presents the results of ZnO NR characterization in the culture medium. The results indicate that ZnO NRs in the F/2 medium, with salinity of 29‰, presented normal pH for marine water of 8.02 [39]. In this medium, ZnO NRs had large hydrodynamic diameter due to the higher ionic strength present in the saline medium [17]. The LC-Oligo medium had a recommended pH of 7.42 [37], and the hydrodynamic diameter for ZnO NRs in suspension was smaller than in the saline medium. In both cases, the ionic strength of the media affected the original size of ZnO NRs and promoted agglomeration of this NM in both suspensions [8]. The large hydrodynamic size will affect the bioavailability of ZnO NRs to the exposed organism. Consequently, the response of microalgae to chronic toxicity caused by ZnO NR exposure will change.

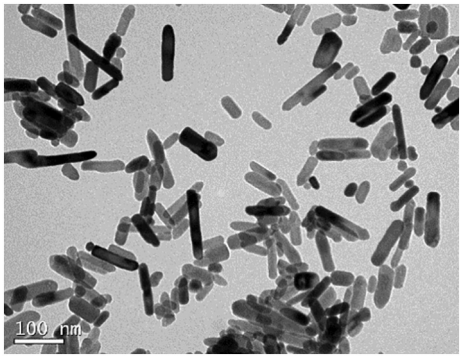


**Fig. 1.** TEM images of ZnO NR, showing the morphology compatible with NRs on nanoscale.

**Table 1**
pH, HD, and Pz values for ZnO NRs (500 mg/L) on F/2 and LC-Oligo culture media.

| Medium | pH | HD (nm) ± SD | Pz (mV) ± SD |
|---|---|---|---|
| LC-Oligo | 7.42 | 840.98 ± 111.50 | −7.54 ± 1.81 |
| F/2 | 8.02 | 1834.93 ± 405.15 | 18.94 ± 3.61 |

Regarding the stability of ZnO NRs in the culture media, in general both suspensions were not stable, since the Pz values were not close or higher than +/− 30 mV. When comparing the suspensions in both tested media, ZnO NRs presented higher stability in the F/2 medium (18.94 ± 3.61 mV) than in the LC-Oligo medium (−7.54 ± 1.81 mV) (Table 1). This behavior can be attributed to the higher ionic strength of the F/2 medium.

The characterization of ZnO NRs in the culture media helps to better understand how the toxicological behavior of NM is altered. In general, the toxicity of ZnO NM is related to the hydrodynamic size of NM in suspension and its release of Zn ions to the aqueous medium [8]. The behavior of ZnO NRs in the evaluated suspensions in our study reflects the toxicological response of the aquatic organism, since NM agglomeration results in larger hydrodynamic size and consequently smaller surface area available to release Zn ions to the solution. Additionally, the presence of high concentrations of divalent cations in the tested culture medium, such as $Ca^{2+}$ and $Mg^{2+}$, causes greater agglomeration than monovalent cations [44].

### 3.2. Chronic toxicity

The protocols recommended by ABNT [37,39] and OECD-201 [38] were considered for the evaluation of data on chronic toxicity. As mentioned previously, three different concentrations of ZnO NRs (0.1, 1.0, and 10 mg/L, final concentration in the exposition flask) were used, plus the negative control (microalgae only in culture medium). All the concentrations were tested in triplicate. Only tests with a minimum of growth rate of 0,9 $d^{-1}$ on the negative control were consider to the ANOVA analysis. The results obtained from the exposure of the microalgae *D. subspicatus* and *Tetraselmis* sp. to ZnO NRs are presented in Fig. 2 and Table 2.

A total of 15 chronic toxicity tests were performed for *D. subspicatus,* exposed to three different concentrations of ZnO NRs, plus negative control (all in triplicate), at three different exposure times (24, 48, and 72 h). For these tests, ZnO NR suspensions were prepared in LC-Oligo medium (composed of several ions diluted in ultrapure water).

In these assays, although there was a significant interaction between treatment and time ($p < 0.001$), the time factor explained only 40% of cell density, and the influence of this factor on cell density was different between concentrations. At a concentration of 10 mg/L, there were no significant differences in cell density with time (Tukey test, $p > 0.05$; Fig. 2), indicating that exposure to ZnO NRs at this concentration inhibited the increase in cell density at all times tested. However, for the other concentrations (concentrations ≤ 1 mg/L), significant increases in cell density were seen after 48 h of exposure.

Based on our results, it is possible to state that for *D. subspicatus,* the chronic responses at 48 and 72 h were LOEC of 10 mg/L and NOEC of 1 mg/L ($p < 0.05$). Since this toxicological effect is already different within 48 h, this exposure time was considered for the effects of LOEC and NOEC (Table 3).

A total of seven chronic toxicity tests were performed for *Tetraselmis* sp. exposed to three different concentrations of ZnO NRs, plus the negative control (all in triplicate), for three different exposure times (24, 48, and 72 h). In these tests, ZnO NR suspensions were prepared in F/2 medium (composed of several ions diluted in filtered seawater).

The cell density varied significantly (ANOVA, $p < 0.001$) between the control and concentrations with algae exposure times, without significant interaction between them. While time explained 49% of the variability in cell density, concentration accounted for 19%. Cell density was significantly different between the control and all tested concentrations of ZnO

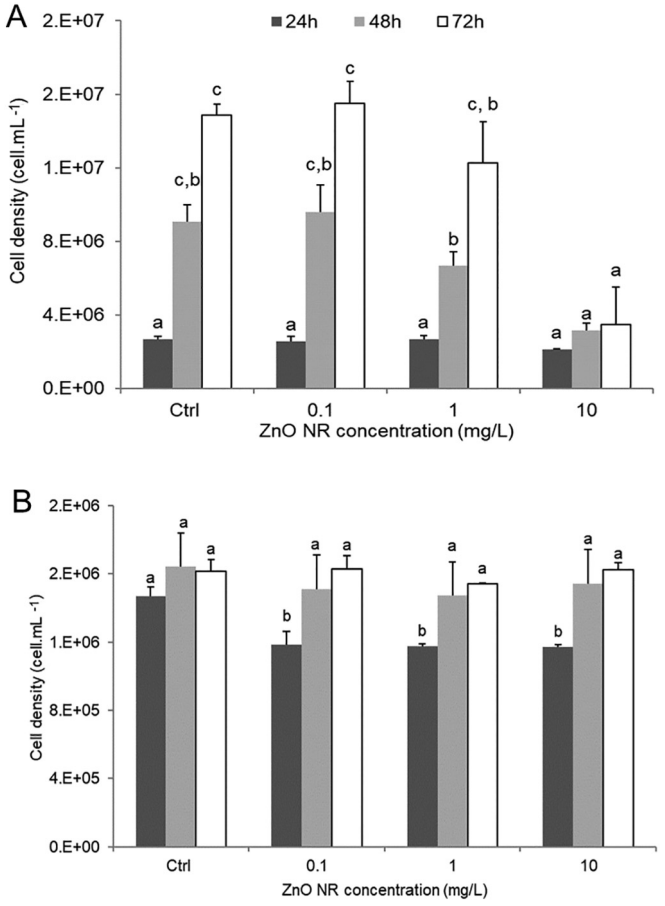


**Fig. 2.** Chronic toxicity assays, with dose response of (A) *D. subspicatus* and (B) *Tetraselmis* sp. microalgae to ZnO NRs after different exposure times (24, 48, and 72 h), where different letters indicate significantly different groups according to the Tukey test ($p < 0.05$).

NRs after 24 h of exposure (Tukey test, $p < 0.05$; Fig. 2). At times of 48 h and 72 h, the differences were not significant (Tukey test, $p > 0.05$; Fig. 2).

In summary, the algal growth rate was significantly different between the control and the ZnO NR treatment at 24 h of exposure. At 48 h and 72 h, the differences were not significant. With this, it is possible to state that for *Tetraselmis* sp., the chronic responses were LOEC of 0.1 mg/L and NOEC <0.1 mg/L at 24 h ($p < 0.05$; Table 3). This result can be also indicative that ZnO NRs can cause acute effects (6 h of exposition) to *Tetraselmis* sp. However, further investigations need to be conducted to confirm that hypothesis.

The results summarized in Table 3 indicate that in absolute terms (without considering the time variable), ZnO NRs were more toxic to the microalga *Tetraselmis* sp., with CEO values of 0.1 mg/L. However, more detailed analysis needs to be carried out to determine the toxicological effects of ZnO NRs on green microalgae, considering the behavior of ZnO NRs in the culture media, the aquatic nature of green microalgae, and the exposure time.

Considering the chemical composition of the F/2 medium (*Tetraselmis* sp.), a previous study found that high ionic strength influenced ZnO NR agglomeration [17], which was confirmed in our analysis (Table 1). Seawater has a strong tendency to enhance the toxic effects of NMs on marine organisms, in comparison with fresh water, due to the high ionic strength and alkaline pH, which can alter the physical-chemical properties of NMs [45,46].

ZnO NRs were toxic at a concentration of 10 mg/L to *D. subspicatus* at 48 and 72 h (Fig. 2), while *Tetraselmis* sp. suffered significant effects after exposure for 24 h. These different responses of green microalgae from two different aquatic ecosystems highlights the behavior of NMs in the culture media

**Table 2**
Two-way ANOVA for the effect of the concentration (Con) and time factors on the cell density of *D. subspicatus* and *Tetraselmis* sp.

| Microalgae | Factors | DF | MS | *F* | *P* | ECV (%) |
|---|---|---|---|---|---|---|
| *D. subspicatus* | Con | 3 | 0.3753 | 30.78 | **< 0.0001** | 27 |
| | Time | 2 | 1.0796 | 88.52 | **< 0.0001** | 40 |
| | Con:Time | 6 | 0.0699 | 5.73 | 0.000818 | 19 |
| | Residue | 24 | 0.0122 | – | – | 15 |
| | Total | 35 | 1.537 | – | – | 100 |
| *Tetraselmis* sp. | Con | 3 | 0.0051 | 18.348 | < 0.0001 | 19 |
| | Time | 2 | 0.0422 | 153.25 | < 0.0001 | 49 |
| | Con:Time | 6 | 0.0017 | 6.268 | 0.000457 | 18 |
| | Residue | 24 | 0.0003 | – | – | 14 |
| | Total | 35 | 0.0493 | – | – | 100 |

Significant values ($p < 0.05$) are identified in bold. DF = degrees of freedom, MS = average of the sum of the squares, F = calculated value of the ANOVA variance, p = significance value, and ECV (%) = components of variation in percentage.

and the toxicity mechanism on microalgae. ZnO NRs affected *Tetraselmis* sp. at lower concentrations and shorter periods of exposure (acute effect). However, the microalga *Tetraselmis* sp. recovered from the harmful effects of ZnO NRs after a certain period of exposure, demonstrated by the increase in cell density after 48 and 72 h (Fig. 2b).

In general, this recovery of cell density after a certain exposure time can be associated with the higher resilience of marine organisms (*Tetraselmis* sp.) than freshwater organisms (*D. subspicatus*). Similar studies have reported this effect of CuO NPs, where the recovery of cell density of microalgae was observed after exposure for 72 h [9,11,12,31]. However, an organism's resilience cannot be considered in isolation, because as previously discussed, physical-chemical mechanisms affect the toxicological behavior of ZnO NRs in the evaluated aquatic environments.

The mechanisms of ZnO NR agglomeration need to be considered since the tests were performed statically for 72 h (the medium was not renewed during this period). The agglomeration mechanisms may have affected the state of agglomeration of suspended ZnO NRs, reducing the NM surface area and causing sedimentation. The Pz data (Table 1) confirmed that both suspensions are unstable in the tested culture media. To minimize this effect, we kept all vessels under shaking during the tests. Additionally, the bioavailability of Zn ions seems not to be affected, since several studies have reported that Zn dissolution from ZnO NM was not statistically significant with time [12,47–50].

Aravantinou et al. [11] evaluated the long-term toxicity of ZnO NMs to *Scenedesmus rubescens* cultivated in different media. They reported that microalgal growth was affected by the exposure time and NM concentrations, but more so by the culture medium used. They reported also that the species of microalgae (freshwater or marine) will define the behavior of NPs and the mechanisms of the toxic effects, since NM dissolution depends on the aqueous matrix composition (pH, ionic strength, organic matter content, etc.) [11].

### 3.3. Evaluation of oxidative stress

ZnO NRs are known for their potential to induce oxidative stress in microalgal cells [47,48,50]. To evaluate oxidative stress effect, samples of microalgae exposed to ZnO NRs for 72 h were prepared to evaluate the

**Table 3**
Chronic toxicity results of ZnO NRs according to LOEC and NOEC (mg/L) of the microalgae *D. subspicatus* and *Tetraselmis* sp.

| NM | Microalgae | Exposure time (h) | Chronic toxicity data (mg/L) |
|---|---|---|---|
| ZnO NR | *D. subspicatus* | 48 | LOEC: 10<br>NOEC: 1 |
| | *Tetraselmis* sp. | 24 | LOEC: 0.1<br>NOEC: <0.1 |

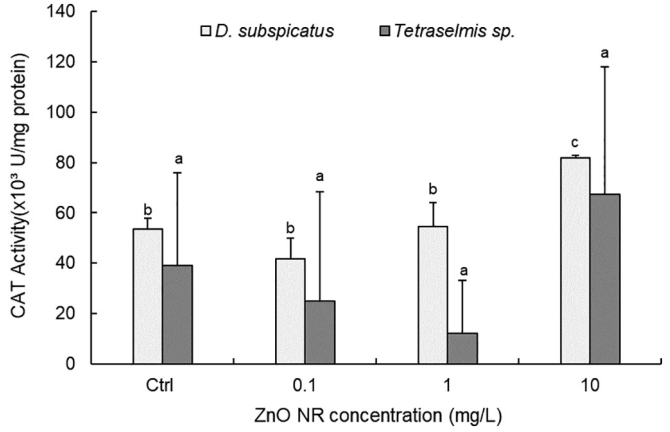


**Fig. 3.** Dose response of oxidative stress to ZnO NR exposure of *D. subspicatus* and *Tetraselmis* sp. microalgae, measured by CAT activity, at the exposure time of 72 h. Different letters indicate significantly different groups, according to the Tukey test ($p < 0.05$).

CAT activity. This activity was different between the two microalgae tested, as evidenced in Fig. 3.

For *D. subspicatus,* only at a concentration of 10 mg/L of ZnO NRs was different behavior than the other concentrations observed (one-way ANOVA, $p < 0.05$). This result evidenced that at this concentration, ZnO NRs induced an increase of CAT activity, and consequently induced oxidative stress in *D. subspicatus* when exposed to this concentration.

For *Tetraselmis* sp., there was no alteration of CAT activity at all tested concentrations of ZnO NRs after 72 h. The results of this assay indicate that ZnO NRs induced distinct oxidative stress effects on the microalgae of freshwater and marine ecosystems. The results of CAT activity corroborated the chronic toxicity results for both microalgae at 72 h.

The main toxicity mechanism of ZnO NMs is described in the literature as the release of Zn ions in the medium, causing the formation of ROS, which in turn induces oxidative stress [8,51]. When some metallic ions are released in the environment, the excess electrons in the valence layer cause the formation of highly reactive species that can promote the formation of ROS [51]. Although no method to quantify Zn ions was carried out in our study, a previous study with these ZnO NRs reported that the Zn ion release occurs to this NM when in suspension [8]. The main mechanism for inducing oxidative stress in microalgae is by releasing Zn ions into the medium.

Since CAT is an enzyme that catalyzes hydrogen peroxide ($H_2O_2$) molecules produced in the medium, the increase of the CAT activity of microalgae indicates that the microorganism has been exposed to some level of oxidative stress. Thus, *D. subspicatus* presented CAT activity values significantly different from the other treatments, at a concentration of 10 mg/L of ZnO NRs. *Tetraselmis* sp. showed no significant differences in CAT activity of ZnO NRs and the negative control treatments. This result showed that the antioxidant defense mechanisms of this marine microalga act differently from those of the freshwater microalga tested. This suggests that freshwater ecosystem organisms are more susceptible to the effects of oxidative stress by ZnO NRs.

## 4. Conclusions

We conducted a toxicological evaluation of the effects of ZnO NRs through chronic toxicity assays with two green microalgae species, *D. subspicatus* and *Tetraselmis* sp. We also evaluated the effects of oxidative stress induction through CAT activity in both organisms. Our results showed that ZnO NRs in suspension have low stability in both media tested, and have a tendency to agglomerate in the F/2 medium. This behavior can affect the bioavailability of NMs to the organism, and consequently the toxicity of ZnO NRs. Chronic toxicity assays with *D. subspicatus* exposed to ZnO NRs presented LOEC 10 mg/L and NOEC of 1 mg/L at exposure times of 48 and 72 h. Chronic toxicity assays with *Tetraselmis* sp. exposed to ZnO NRs showed LOEC of 0.1 mg/L and NOEC < 0.1 mg/L after 24 h exposure time. At 48 and 72 h, this effect was not significant. CAT activity behaved differently for the tested algae and corroborated the chronic toxicity results. *D. subspicatus* had higher CAT activity at the ZnO NR concentration of 10 mg/L. For *Tetraselmis* sp., there was no change in the enzymatic activity of CAT. Our results showed that the microalga from the freshwater ecosystem is more susceptible to the effects of oxidative stress by ZnO NRs.

In conclusion, the results of this study showed that ZnO NRs can induce chronic oxidative effects in green microalgae. However, the nature of the organism and its ecosystem can affect the response of ZnO toxicity, since distinct environments affected the behavior of NMs due to the chemical composition of the culture media.

## CRediT authorship contribution statement

**Ana Claudia O. de Almeida**: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing - original draft. **Lais F. dos Santos**: Methodology, Formal analysis. **Denice S. Vicentini**: Methodology, Formal analysis, Resources, Writing - review & editing. **William G. Matias**: Writing - review & editing, Resources, Funding acquisition. **Silvia P. Melegari**: Conceptualization, Resources, Writing - review & editing, Visualization, Supervision, Funding acquisition.

## Acknowledgments

We would like to acknowledge to Environmental Laboratory of Joinville Regional University (UNIVILLE, Joinville, Santa Catarina, Brazil) and Engineering and Malacoculture Laboratory (LEMAQUI – UFPR, Pontal do Paraná, Paraná, Brazil) to kindly provided the microalgae strains. We gratefully acknowledge the Central Electron Microscopy Laboratory (LCME/UFSC) for TEM images. This work was supported in part by the CNPq [Procs. no.552112/2011-9, no.473046/2013-0 and no.441265/2017-0] and CAPES/PNPD [Procs. no.2982/10 and no.2590/11].

## References

[1] S. Vijayakumar, B. Vaseeharan, B. Malaikozhundan, M. Shobiya, Laurus nobilis leaf extract mediated green synthesis of ZnO nanoparticles: characterization and biomedical applications, Biomed. Pharmacother. 84 (2016) 1213–1222, https://doi.org/10.1016/j.biopha.2016.10.038.

[2] R. Kumar, A. Umar, G. Kumar, H.S. Nalwa, Antimicrobial properties of ZnO nanomaterials: a review, Ceram. Int. 43 (2017) 3940–3961, https://doi.org/10.1016/j.ceramint.2016.12.062.

[3] H. Ma, P.L. Williams, S.A. Diamond, Ecotoxicity of manufactured ZnO nanoparticles—a review, Environ. Pollut. 172 (2013) 76–85, https://doi.org/10.1016/j.envpol.2012.08.011.

[4] I. Blinova, A. Ivask, M. Heinlaan, M. Mortimer, A. Kahru, Ecotoxicity of nanoparticles of CuO and ZnO in natural water, Environ. Pollut. 158 (2010) 41–47, https://doi.org/10.1016/j.envpol.2009.08.017.

[5] M.V.D.Z. Park, E.A.J. Bleeker, W. Brand, F.R. Cassee, M. Van Elk, I. Gosens, W.H. De Jong, J.A.J. Meesters, W.J.G.M. Peijnenburg, J.T.K. Quik, et al., Considerations for safe innovation: the case of graphene, ACS Nano 11 (2017) 9574–9593, https://doi.org/10.1021/acsnano.7b04120.

[6] J.M. Neff, Zinc in the ocean, Bioaccumulation Mar. Org, Elsevier 2002, pp. 175–189, https://doi.org/10.1016/b978-008043716-3/50011-7.

[7] World Health Organization, Zinc in drinking-water, in: WHO (Ed.), WHO Guidel. Drink. Qual, 3nd ed. 2003, pp. 1–10.

[8] S.P. Melegari, C.F. Fuzinatto, R.A. Gonçalves, B.V. Oscar, D.S. Vicentini, W.G. Matias, Can the surface modification and/or morphology affect the ecotoxicity of zinc oxide nanomaterials? Chemosphere 224 (2019) 237–246, https://doi.org/10.1016/j.chemosphere.2019.02.093.

[9] A.F. Aravantinou, V. Tsarpali, S. Dailianis, I.D. Manariotis, Effect of cultivation media on the toxicity of ZnO nanoparticles to freshwater and marine microalgae, Ecotoxicol. Environ. Saf. 114 (2015) 109–116, https://doi.org/10.1016/j.ecoenv.2015.01.016.

[10] M. Samei, M.H. Sarrafzadeh, M.A. Faramarzi, The impact of morphology and size of zinc oxide nanoparticles on its toxicity to the freshwater microalga, *Raphidocelis subcapitata*, Environ. Sci. Pollut. Res. 26 (2019) 2409–2420, https://doi.org/10.1007/s11356-018-3787-z.

[11] A.F. Aravantinou, F. Andreou, I.D. Manariotis, Long-term toxicity of ZnO nanoparticles to *Scenedesmus rubescens* cultivated in different media, Sci. Rep. 7 (2017) 1–11, https://doi.org/10.1038/s41598-017-13517-7.

[12] C. Zhang, J. Wang, L. Tan, X. Chen, Toxic effects of nano-ZnO on marine microalgae *Skeletonema costatum*: attention to the accumulation of intracellular Zn, Aquat. Toxicol. 178 (2016) 158–164, https://doi.org/10.1016/j.aquatox.2016.07.020.
[13] N.M. Franklin, N.J. Rogers, S.C. Apte, G.E. Batley, G.E. Gadd, P.S. Casey, Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and $ZnCl_2$ to a freshwater microalga (*Pseudokirchneriella subcapitata*): the importance of particle solubility, Environ. Sci. Technol. 41 (2007) 8484–8490, https://doi.org/10.1021/es071445r.
[14] T.L. Rocha, T. Gomes, V.S. Sousa, N.C. Mestre, M.J. Bebianno, Ecotoxicological impact of engineered nanomaterials in bivalve molluscs: an overview, Mar. Environ. Res. (2015) https://doi.org/10.1016/j.marenvres.2015.06.013.
[15] R. Bacchetta, B. Maran, M. Marelli, N. Santo, P. Tremolada, Role of soluble zinc in ZnO nanoparticle cytotoxicity in *Daphnia magna*: a morphological approach, Environ. Res. 148 (2016) 376–385, https://doi.org/10.1016/j.envres.2016.04.028.
[16] H.C. Poynton, J.M. Lazorchak, C.A. Impellitteri, M.E. Smith, K. Rogers, M. Patra, K.A. Hammer, H.J. Allen, C.D. Vulpe, Differential gene expression in daphnia magna suggests distinct modes of action and bioavailability for Zno nanoparticles and Zn ions, Environ. Sci. Technol. 45 (2011) 762–768.
[17] R.A. Gonçalves, A.L. de Oliveira Franco Rossetto, D.J. Nogueira, D.S. Vicentini, W.G. Matias, Comparative assessment of toxicity of ZnO and amine-functionalized ZnO nanorods toward *Daphnia magna* in acute and chronic multigenerational tests, Aquat. Toxicol. 197 (2018) 32–40, https://doi.org/10.1016/J.AQUATOX.2018.02.002.
[18] R. Bacchetta, N. Santo, M. Marelli, G. Nosengo, P. Tremolada, Chronic toxicity effects of ZnSO4 and ZnO nanoparticles in *Daphnia magna*, Environ. Res. 152 (2017) 128–140, https://doi.org/10.1016/j.envres.2016.10.006.
[19] N. Adam, F. Leroux, D. Knapen, S. Bals, R. Blust, The uptake and elimination of ZnO and CuO nanoparticles in *Daphnia magna* under chronic exposure scenarios, Water Res. 68 (2015) 249–261, https://doi.org/10.1016/j.watres.2014.10.001.
[20] A. Ivask, K. Juganson, O. Bondarenko, M. Mortimer, V. Aruoja, K. Kasemets, I. Blinova, M. Heinlaan, V. Slaveykova, A. Kahru, Mechanisms of toxic action of Ag, ZnO and CuO nanoparticles to selected ecotoxicological test organisms and mammalian cells in vitro: a comparative review, Nanotoxicology 8 (Suppl. 1) (2014) 57–71, https://doi.org/10.3109/17435390.2013.855831.
[21] L.M. Skjolding, M. Winther-Nielsen, A. Baun, Trophic transfer of differently functionalized zinc oxide nanoparticles from crustaceans (*Daphnia magna*) to zebrafish (*Danio rerio*), Aquat. Toxicol. 157 (2014) 101–108, https://doi.org/10.1016/j.aquatox.2014.10.005.
[22] L. Hao, L. Chen, J. Hao, N. Zhong, Bioaccumulation and sub-acute toxicity of zinc oxide nanoparticles in juvenile carp (Cyprinus carpio): a comparative study with its bulk counterparts, Ecotoxicol. Environ. Saf. 91 (2013) 52–60, https://doi.org/10.1016/j.ecoenv.2013.01.007.
[23] D. Xiong, T. Fang, L. Yu, X. Sima, W. Zhu, Effects of nano-scale TiO2, ZnO and their bulk counterparts on zebrafish: acute toxicity, oxidative stress and oxidative damage, Sci. Total Environ. 409 (2011) 1444–1452, https://doi.org/10.1016/j.scitotenv.2011.01.015.
[24] H.M. Mayton, D. White, I.M. Marcus, S.L. Walker, Influence of nano-CuO and -$TiO_2$ on deposition and detachment of *Escherichia coli* in two model systems, Environ. Sci. Nano. (2019) https://doi.org/10.1039/C9EN00857H.
[25] N. Padmavathy, R. Vijayaraghavan, Enhanced bioactivity of ZnO nanoparticles—an antimicrobial study, Sci. Technol. Adv. Mater. 9 (2008), 035004, https://doi.org/10.1088/1468-6996/9/3/035004.
[26] M. Li, D. Lin, L. Zhu, Effects of water chemistry on the dissolution of ZnO nanoparticles and their toxicity to *Escherichia coli*, Environ. Pollut. 173 (2013) 97–102, https://doi.org/10.1016/j.envpol.2012.10.026.
[27] Y.-W. Baek, Y.-J. An, Microbial toxicity of metal oxide nanoparticles (CuO, NiO, ZnO, and Sb2O3) to *Escherichia coli*, *Bacillus subtilis*, and *Streptococcus aureus*, Sci. Total Environ. 409 (2011) 1603–1608, https://doi.org/10.1016/j.scitotenv.2011.01.014.
[28] A.K. Gupta, M. Gupta, Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications, Biomaterials 26 (2005) 3995–4021, https://doi.org/10.1016/j.biomaterials.2004.10.012.
[29] T.C. Long, N. Saleh, R.D. Tilton, G.V. Lowry, B. Veronesi, Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity, Environ. Sci. Technol. 40 (2006) 4346–4352, https://doi.org/10.1021/es060589n.
[30] T. Xia, M. Kovochich, J. Brant, M. Hotze, J. Sempf, T. Oberley, C. Sioutas, J.I. Yeh, M.R. Wiesner, A.E. Nel, Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm, Nano Lett. 6 (2006) 1794–1807, https://doi.org/10.1021/nl061025k.
[31] Ana Letícia O.F. de Rossetto, S.P. Melegari, L.C. Ouriques, W.G. Matias, Comparative evaluation of acute and chronic toxicities of CuO nanoparticles and bulk using *Daphnia magna* and *Vibrio fischeri*, Sci. Total Environ. 490 (2014) 807–814, https://doi.org/10.1016/j.scitotenv.2014.05.056.
[32] J. Stevenson, Ecological assessments with algae: a review and synthesis, J. Phycol. 50 (2014) 437–461, https://doi.org/10.1111/jpy.12189.
[33] B.W. Brooks, J.M. Lazorchak, M.D.A. Howard, M.V.V. Johnson, S.L. Morton, D.A.K. Perkins, E.D. Reavie, G.I. Scott, S.A. Smith, J.A. Steevens, Are harmful algal blooms becoming the greatest inland water quality threat to public health and aquatic ecosystems? Environ. Toxicol. Chem. 35 (2016) 6–13, https://doi.org/10.1002/etc.3220.
[34] J.J. Korhonen, P. Köngäs, J. Soininen, Temporal variation of diatom assemblages in oligotrophic and eutrophic streams, Eur. J. Phycol. 48 (2013) 141–151, https://doi.org/10.1080/09670262.2013.779390.
[35] F. Štork, M. Bačkor, B. Klejdus, J. Hedbavny, J. Kováčik, Changes of metal-induced toxicity by H2O2/NO modulators in *Scenedesmus quadricauda* (Chlorophyceae), Environ. Sci. Pollut. Res. 20 (2013) 5502–5511, https://doi.org/10.1007/s11356-013-1541-0.
[36] J.C. Prata, B.R.B.O. Lavorante, M.C. da Maria, L. Guilhermino, Influence of microplastics on the toxicity of the pharmaceuticals procainamide and doxycycline on the marine microalgae *Tetraselmis chuii*, Aquat. Toxicol. 197 (2018) 143–152, https://doi.org/10.1016/j.aquatox.2018.02.015.
[37] ABNT, ABNT NBR 12648:2018 - Aquatic Ecotoxicology - Chronic Toxicity - Test With Algae (Chlorophyceae), 2018.
[38] OECD, Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD, 2011.
[39] ABNT, ABNT NBR 16181:2013 Aquatic Ecotoxicology - Chronic Toxicity - Test With Marine Microalgae, 2013 27 http://www.abntcatalogo.com.br/norma.aspx?ID=088829 (accessed March 4, 2016).
[40] R.M. Valer, M. Glock, Ecotoxicologia aquática inibição do crescimento de microalgas marinha, Acta Limnol. Bras. 11 (1998) 149–156.
[41] EPA, EPA-821/R02-013 Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms, 4th ed., 2002 , Washington, DC www.epa.gov/waterscience/WET/disk3/.
[42] S.P. Melegari, F. Perreault, R.H.R. Costa, R. Popovic, W.G. Matias, Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga *Chlamydomonas reinhardtii*, Aquat. Toxicol. 142–143 (2013) 431–440, https://doi.org/10.1016/j.aquatox.2013.09.015.
[43] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254 http://www.ncbi.nlm.nih.gov/pubmed/942051 (accessed July 24, 2011).
[44] M. Baalousha, Y. Nur, I. Römer, M. Tejamaya, J.R. Lead, Effect of monovalent and divalent cations, anions and fulvic acid on aggregation of citrate-coated silver nanoparticles, Sci. Total Environ. 454–455 (2013) 119–131, https://doi.org/10.1016/j.scitotenv.2013.02.093.
[45] S. Kashiwada, Distribution of nanoparticles in the see-through medaka (*Oryzias latipes*), Environ. Health Perspect. 114 (2006) 1697–1702, https://doi.org/10.1289/ehp.9209.
[46] Stephen J. Klaine, Pedro J.J. Alvarez, Graeme E. Batley, Teresa Fernandes, Richard D. Handy, Delina Y. Lyon, Shaly Mahendra, Michael J. McLaughlin, J.R. Lead, Nanomaterials in the Environment: Behvior, Fate, Bioavailability, and Effects, 2008 1825–1851.
[47] S.W.Y. Wong, P.T.Y. Leung, A.B. Djurišić, K.M.Y. Leung, Toxicities of nano zinc oxide to five marine organisms: influences of aggregate size and ion solubility, Anal. Bioanal. Chem, Springer 2010, pp. 609–618, https://doi.org/10.1007/s00216-009-3249-z.
[48] R.J. Miller, H.S. Lenihan, E.B. Muller, N. Tseng, S.K. Hanna, A.A. Keller, Impacts of metal oxide nanoparticles on marine phytoplankton, Environ. Sci. Technol. 44 (2010) 7329–7334, https://doi.org/10.1021/es100247x.
[49] M. Samei, M.H. Sarrafzadeh, M.A. Faramarzi, The impact of morphology and size of zinc oxide nanoparticles on its toxicity to the freshwater microalga, *Raphidocelis subcapitata*, Environ. Sci. Pollut. Res. 26 (2019) 2409–2420, https://doi.org/10.1007/s11356-018-3787-z.
[50] T.Y. Suman, S.R. Radhika Rajasree, R. Kirubagaran, Evaluation of zinc oxide nanoparticles toxicity on marine algae *Chlorella vulgaris* through flow cytometric, cytotoxicity and oxidative stress analysis, Ecotoxicol. Environ. Saf. 113 (2015) 23–30, https://doi.org/10.1016/j.ecoenv.2014.11.015.
[51] M. Auffan, J. Rose, M.R. Wiesner, J.-Y. Bottero, Chemical stability of metallic nanoparticles: a parameter controlling their potential cellular toxicity in vitro, Environ. Pollut. 157 (2009) 1127–1133, https://doi.org/10.1016/j.envpol.2008.10.002.