# ZnO Nanoparticles Alter Redox Metabolism of *Limnoperna Fortunei*

**Francine Girardello** ( fgirardello@yahoo.com.br )
University of Caxias do Sul: Universidade de Caxias do Sul
**Camila Custódio Leite**
University of Caxias do Sul: Universidade de Caxias do Sul
**Luciana Bavaresco Touguinha**
University of Caxias do Sul: Universidade de Caxias do Sul
**Mariana Roesch-Ely**
University of Caxias do Sul: Universidade de Caxias do Sul
**Andreia Neves Fernandes**
UFRGS: Universidade Federal do Rio Grande do Sul
**Richard Macedo de Oliveira**
UFSC: Universidade Federal de Santa Catarina
**Daniel L. G. Borges**
UFSC: Universidade Federal de Santa Catarina
**Chrys Katielli Hoinacki da Silva**
UFRGS: Universidade Federal do Rio Grande do Sul
**Mirian Salvador**
University of Caxias do Sul: Universidade de Caxias do Sul
**Izabel Vianna Villela**
Inn Vitro
**João Antonio Pêgas Henriques**
University of Caxias do Sul: Universidade de Caxias do Sul

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# Abstract

Nanoparticles incorporated in consumer and industrial products cause concerns about their potential ecological impacts. Zinc oxide nanoparticles (ZnO-NP) have several applications**,** which increases their potential for release to the environment, causing ecotoxicological problems. Bivalve mollusks are susceptible targets for nanoparticles toxicity, since nanomaterials can enter the cells by endocytosis mechanisms. *Limnoperma fortunei* (golden mussel) are validated for biomonitoring purposes and have a wide distribution in the South of Brazil, where it can be collected throughout the year. The aim of this study was to evaluate the influence of ZnO-NP on the redox metabolism by enzymatic and non-enzymatic antioxidant defense assessment in addition to DNA damage by DNA fragmentation assay in *L. fortunei* after exposure to ZnO-NP. Adult bivalves were placed in contact with 1, 10, and 50 μg $mL^{-1}$ ZnO-NP during three incubation times: 2, 4 and 24 h. Ionic Zn release, enzymatic and non-enzymatic antioxidant activity, oxidative damage to lipids and proteins and DNA damage were evaluated. Oxidative damage to proteins and lipids were observed after 4 h exposure and returned to baseline levels after 24 h. Superoxide dismutase levels decreased after 4 h exposure and increased after 24 h. No significant alteration was observed in catalase activity or even DNA double strand cleavage. The dissociation of ZnO may occur after 24 h, releasing ionic zinc ($Zn^{2+}$) by hydrolysis, which was confirmed as the ionic Zn concentration increased following 24 h exposure. In conclusion, ZnO-NP were able to induce oxidative stress in exposed golden mussels. The golden mussel is capable to modulate its own antioxidant defences in response to oxidative stress and seems to be able to hydrolyse the nanoparticle and consequently release $Zn^{2+}$ into the cellular compartment.


# 1. Introduction

The increasing production and use of nanoparticles in consumer and industrial products and the large range of applications related to these nanomaterials raises some concerns about their potential ecological impacts in different ecosystems and their adverse effect on human health (Simonet and Valcárcel 2009; Salieri, et al. 2015; Girardello, et al. 2016a; Girardello, et al. 2016b). Nanoparticles have a high surface-to-volume ratio, which results in high reactivity potential and unique physical and chemical properties that differ from those of their respective bulk materials (Mallevre, Fernandes, and Aspray 2014; Girardello, et al. 2016a; Girardello, et al. 2016b). The nanoparticle composition and solubility, and interaction modes with biological systems are highlighted as main factors for risk assessment of metal oxide nanoparticles (Wang, et al. 2010; Wu, et al. 2010).

Zinc oxide nanoparticles (ZnO-NP) have a large application field, including wastewater treatment (Anjum, et al. 2016), molecular biology (Navaei-Nigjeh, et al. 2018), cosmetics (Katz, Dewan, and Bronaugh 2015; Lu, et al. 2015; Khezri, Saeedi, and Maleki Dizaj 2018), sunscreen lotions (Lu, et al. 2018), as an additive (Nanthagopal, et al. 2017), in food industry (Venkatasubbu, et al. 2016), paints (Shi, et al. 2013) and construction materials (Hossain, et al. 2014; Schaumann, et al. 2015). Such widespread and expanding production and use of ZnO-NP increases the potential for their release to the environment, causing ecotoxicological problems (Jovanović and Palić 2012). The exposure of aquatic animals to nanoparticles

and their aggregates is a major concern, due to the potential harmful effects that may occur. Moreover, the nanoparticles bioavailability and uptake into cells and organisms may be affected by their association with naturally occurring colloids in aquatic systems, altering the nanoparticles behavior in this environmental compartment (Moore 2006).

The aquatic system has a variety of organisms and, once nanoparticles are into the organisms compartment, it may result in additional toxic effects, deregulate cell metabolism and generate reactive oxygen species (ROS) with effects that are related to concentration and period of exposure (Azqueta and Dusinska 2015; Marisa, et al. 2015). ROS can reason serious damage to biomolecules as lipids and proteins and deplete enzymatic defenses such as superoxide dismutase (Sod) and catalase (Cat). Non-enzymatic protein-bound sulfhydryl groups are also affected by ROS exposition (Katsumiti, et al. 2014). Nanoparticles can cause alteration in redox metabolism, modifying markers related to biomolecules oxidation (Iummato, et al. 2013; Girardello, et al. 2016a; Girardello, et al. 2016b). Furthermore, enzymatic antioxidant defenses are shown to change in different aquatic organisms (Hao, Wang, and Xing 2009; Canesi, et al. 2010; Zhu, Zhou, and Cai 2011; Faria, et al. 2014).

Ecotoxicity of ZnO-NP is related to their physical-chemical characteristics such as solubilization and photoreactivity, as well as the tested species. ZnO has photocatalytic properties and contribute to ROS generation. It is believed that solubilized $Zn^{2+}$ may contribute to cytotoxicity of these nanoparticles (Ma, Williams, and Diamond 2013). In the study of exposure of the freshwater mussel *Dreissena polymorpha* to ZnO-NP, the oxidative stress of these bivalves showed to be increased after exposition to the nanomaterial. Zn toxicity could be influenced by both solubilized or non-solubilized Zn complexes in freshwater mussels (Gagné, et al. 2019). However, toxicity studies on aquatic invertebrates exposed to ZnO-NP are very limited and its molecular mechanisms related to nanomaterial exposition should be further investigated.

Bivalve mollusks are sensitive to nanoparticles toxicity and may be internalized by endocytosis mechanisms (Canesi, et al. 2012; Barmo, et al. 2013; Canesi, et al. 2014; Girardello, et al. 2016a; Girardello, et al. 2016b). Mussels are filter feeders and stationary organisms and, for this reason, are largely used as biomonitors for environmental perturbations (Villela, et al. 2007; Villela, et al. 2013) and nanoparticles toxicity assessment (Girardello, et al. 2016a; Girardello, et al. 2016b). Furthermore, the ability of bivalves to bioaccumulate toxic compounds in their body determines its role in the transfer of environmental pollutants to higher trophic levels (Hunt, et al. 2003).

Golden mussel (*Limnoperna fortunei*) is an exotic organism from Asia, that lives in freshwater compartments and has been used for biomonitoring of environmental conditions (Mariano, et al. 2006; Iummato, et al. 2013; Villela, et al. 2013; Girardello, et al. 2016a; Girardello, et al. 2016b). *L. fortunei* are widely distributed in Rio Grande do Sul, the southernmost state of Brazil, and can be collected during the entire year, which makes this organism an adequate tool for biomonitoring nanoparticles and an excellent candidate to a sentinel organism (Mariano, et al. 2006; Villela, et al. 2006; Villela, et al. 2007; Villela, et al. 2013; Girardello, et al. 2016a; Girardello, et al. 2016b). Ecotoxicological effect models using *L. fortunei*

exposed to nanoparticles were validated in previous studies from our group (Girardello, et al. 2016a; Girardello, et al. 2016b).

Although there has been an increasing number of studies on nanoparticles toxicity, comprehensive knowledge on the impact of ZnO-NP on aquatic organisms is still not clear. In this sense, this study aims to evaluate the oxidative effects and DNA damage and modulation of enzymatic and non-enzymatic defenses in *L. fortunei*.

## 2. Material And Methods

## 2.1. Test organisms

Collection, handling and maintenance of *Limnoperna fortunei* bivalves were based on the protocols established by Villela et al. (2006) and Girardello et al. (2016a). Detailed information is available as Supplementary Material.

## 2.2. Zinc Oxide Nanoparticles (ZnO-NP)

ZnO-NP (purity > 97%) was obtained from Sigma-Aldrich. Physicochemical characterization of ZnO-NP was carried out following the procedure described by Girardello et al. (2016a). Transmission electron microscopy (TEM), x-ray diffraction (XRD), Brunauer-Emmett-Teller method (BET), dynamic light scattering (DLS) and zeta potential (ZP) were used to characterize the nanomaterial. The ZnO-NP solutions were prepared according to the procedure described by Girardello et al. (2016a). Full description of the analytical conditions, instruments and sample preparation protocols are available as Supplementary Material.

## 2.3. Exposure to zinc oxide nanoparticles

The mussels were exposed to ZnO-NP solutions (1, 10 or 50 μg $mL^{-1}$) for periods of 2, 4 or 24 h. Each individual exposure condition was carried out using four adult mussel specimens (2.05 ± 0.17 cm in length). The control group was exposed to the same conditions, except for the presence of ZnO-NP in the solutions. The mussel specimens were not fed during the exposure assays.

## 2.4. Quantitative analysis of Zn by ICP-MS

Soft tissue samples of *L. fortunei* exposed and not exposed (control group) to ZnO-NP were lyophilized and subsequently weighed on perfluoroalcoxy (PFA) vessels. The samples (0.0350 g) were digested in the open PFA flasks for 24 hours using 1.25 mL of doubly-distilled 14 mol $L^{-1}$ $HNO_3$ (Merck, Darmstadt, Germany) and 0.125 mL 30% $H_2O_2$ (Synth, São Paulo, Brazil). The residual solution was evaporated to dryness at 70°C and the dried residue was solubilized with 2.5 mL of doubly-distilled 14 mol $L^{-1}$ $HNO_3$, followed by dilution to 50 mL using deionized water at 18.2 MΩ cm produced in a Milli-Q system (Millipore, Bedford, USA) Additional details on the procedure adopted to determine Zn in the samples are available as Supplementary Material. The detection limit for Zn was 0.3 mg $g^{-1}$. The method was proven

to be free from interferences. The ICP-MS operating conditions are summarized in Table, which is available as Supplementary Material.

## 2.5. Enzymatic and non-enzymatic antioxidant defenses

Sample preparation for enzymatic and non-enzymatic antioxidant activity determination and oxidative damage to lipids and proteins evaluation was adapted from Iummato et al. (2013).

The enzymatic activities of Sod and Cat were determined following the protocols established by Bannister and Calabrese (1987) and Aebi (1984), respectively. Absorbance measurements were obtained using a Victor-X3 multilabel counter microplate reader (Perkin Elmer, Finland).

The protein sulfhydryl content assay was performed according to the protocol described by Aksenov and Markesbery (2001).

The screening method to determine the total antioxidant capacity followed the procedure described by Re et al. (1999).

Lipid peroxidation was induced following the procedure described by Wills (1966), with minor modifications as described by Girardello *et al.* (2006a). The oxidative damage to proteins was assessed following the method established by Levine et al. (1990) and modified by Girardello *et al.* (2006a).

Detailed description of all the procedures mentioned in this section are available in the Supplementary Material.

## 2.6. DNA fragmentation assay

The DNA fragmentation assay was carried out following the procedure described by Giradello *et al.* (2016a). Detailed description of the procedure is available in the Supplementary Material.

## 2.7. Statistical analysis

All experiments were performed with biological and technical triplicates and the results are expressed as mean ± standard deviation (SD). Statistical analysis using the SPSS 1.0 software was carried out to establish a statistical comparison between the exposed groups and the control group. ANOVA and Dunnett's multiple comparison test were applied to the results, as well as Tukey's post hoc test. Statistical significance was considered in cases where $*p \leq 0.05$ and $\#p \leq 0.001$.

# 3. Results

## 3.1. Characterization of nanoparticles

The results from the characterization of ZnO nanoparticle are summarized in Table 1. ZnO-NP were analyzed by TEM, which showed an average diameter of approximately 50 nm (Fig. 1). This result is in accordance with the diameter defined by the producer. The X-ray diffraction (XRD) patterns revealed the

presence of wurtzite phase (Fig. 2), with high diffraction peak with 2$\theta$ values of 32.41°, 34.24°, 36.14°, 47.35°, 56.39°, 62.56° and 66.94°, typical of ZnO hexagonal crystals (Sai Saraswathi, et al. 2017). The surface area of ZnO-NP was 26.86 $m^2\ g^{-1}$.

**Table 1.** Summary of ZnO-NP characterization by Trasmission Electron Microscopy, X-Ray Diffraction and Brunauer-Emmitt-Teller analysis.

| | ZnO-NP |
|---|---|
| TEM | ~ 50 nm |
| XDR | wurtzite phase |
| BET | 26.86 $m^2\ g^{-1}$ |

The scattering experiments using samples containing ZnO-NP revealed the presence of particle sizes larger than 500 nm in all samples, which extrapolated the maximum detection limit of the DLS technique (set as 500 nm) (data not shown). For all experiments using water collected in the State Park of Itapuã, the formation of aggregates with diameters larger than 500 nm was observed (Girardello, et al. 2016a). The stability of nanoparticles in aqueous solutions was evaluated by the zeta potential (ζ) parameter, which was employed to estimate the particle surface potential related to the nanoparticles aggregation rate. The zeta potential values ranged from − 13.58 ± 1.44 to − 17.98 ± 1.55 mV for the ZnO-NP solutions and − 20.46 ± 1.98 mV for the control (Itapuã water) (Table 2).

Table 2
Zeta potential of solutions in different ZnO-NP concentrations.

| **ZnO-NP Concentration** | **Average zeta potential (mV)*** |
|---|---|
| 1 μg $mL^{-1}$ | − 13,58 ± 1,44 |
| 10 μg $mL^{-1}$ | − 17,98 ± 1,55 |
| 50 μg $mL^{-1}$ | − 17,69 ± 0,98 |
| Itapuã water** | − 20,46 ± 1,98 |
| *Data are presented as the mean ± standard deviation for 10 measurements; **control. | |

# 3.2. Analysis by ICP-MS

The results from the determination of Zn by ICP-MS are shown in Fig. 3. As shown in this Figure, Zn was detected in the soft body portions of *L. fortunei* after exposure to different concentrations of ZnO-NP (Fig. 3). Zn concentrations associated to all treatments decreased after 2 h exposure, when compared to the control group (Fig. 3). After 4 h exposure, the Zn concentration decreased in the specimens exposed

to 1 and 50 μg $mL^{-1}$ ZnO-NP. On the other hand, the Zn concentration increased significantly after exposure for 24 h to 50 μg $mL^{-1}$ ZnO-NP.

## 3.3. Enzymatic and non-enzymatic antioxidant defenses

The results from Sod and Cat activities, protein sulfhydryl content and the TEAC are shown in Fig. 4. With increasing exposure time of mussels to ZnO-NP, the Sod antioxidant activity decreased after 4 h exposure, and the activity increased again after 24 h exposure. The exposure of *Limnoperna fortunei* to increasing concentrations of ZnO-NP resulted in a decrescent tendency of Sod activity after 2 h exposure, without statistical difference. After exposure for 24 h, the Sod activity increased significantly under 10 and 50 μg $mL^{-1}$ ZnO-NP treatments, when compared to the control (Fig. 4A).

The activity of Cat enzyme was not statistically affected by the increase in exposure time. However, the Cat antioxidant activity increased significantly after 24 h exposure to 10 and 50 μg $mL^{-1}$ ZnO-NP, when compared to the control. After 4 h exposure to ZnO-NP, the Cat levels increased, although without statistical significance, when compared to control (Fig. 4B).

Protein sulfhydryl content decreased significantly after 24 h exposure to ZnO-NP for the tested concentrations. With the exposure of *Limnoperna fortunei* to increasing concentrations of ZnO-NP, the protein sulfhydryl content increased significantly after 2 h in 50 μg $mL^{-1}$ ZnO-NP and no statistically significant alterations were observed for the additional exposure conditions tested (Fig. 4C).

The TEAC levels remained essentially unchanged upon changes in the exposure time and NP concentration, as shown in Fig. 4D.

## 3.4. Oxidative damage to lipids and proteins

The oxidative damage to lipids increased in the 4 h exposure group and decreased after 24 h, considering the exposure of mussels to ZnO-NP. No oxidative damage to lipids was observed following a 2 h exposure period to ZnO-NP (Fig. 4F).

Oxidative protein damage increased after 4 h of exposure to NP, although it decreased after being exposed to the NP for 24 h. No significant alterations, compared to the control, were detected in the group that was exposed to ZnO-NP for 2 h with increasing ZnO-NP concentration. Oxidative protein damage decreased for 1, 10 and 50 μg $mL^{-1}$ ZnO-NP after 4 h exposure when compared to the control group. In the 24 h exposure group, the oxidative protein damage decreased regardless of the ZnO-NP concentrations (Fig. 4E).

## 3.5. DNA fragmentation assay

The results obtained from DNA fragmentation assays have shown that the DNA double strand remained intact in the specimens that were exposed to ZnO-NP, as shown in Figure available as Supplementary Material.

# 4. Discussion

Physical and chemical properties of nanoparticles may interfere with ecosystems, since the release of NP to the environment is inevitable and generates different levels of ecotoxicity (Ma, Williams, and Diamond 2013; Châtel and Mouneyrac 2017). Shape, size, surface area, crystalline phase and particle surface potential are considered important factors in reactive oxidative species (ROS) generation, as demonstrated for $TiO_2$ nanoparticles ($TiO_2$-NP) in previous studies from our group (Girardello, et al. 2016a; Girardello, et al. 2016b). The results here presented indicate that ZnO-NP have larger average sizes when compared to $TiO_2$-NP (Girardello, et al. 2016a) and, consequently, lower surface area, which may imply in lower particle reactivity potential. These differences could be related to the lower incidence of negative effects on the biomolecules of *L. fortunei* cells exposed to ZnO-NP, when compared to other nanoparticles (Girardello, et al. 2016a; Girardello, et al. 2016b). High cytotoxic and phototoxic effects are related to larger surface area of nanoparticles (Xiong, et al. 2013). Smaller molecules present a larger fraction of the atoms on the surface causing high mobility and reactivity (Tarrahi, et al. 2018). In addition to physical and chemical characteristics of metal oxide nanoparticles, another factor to be considered for risk assessment is the mechanism which is involved in the interaction of this nanomaterial with biological systems (Wang, et al. 2010; Wu, et al. 2010). The toxic effect of metal oxide nanoparticles may involve distinct mechanisms of ion release from their complexes, producing chemical radicals or interacting with biological targets (Ma, Williams, and Diamond 2013).

The production of reactive oxygen and nitrogen species by nanoparticles may occur directly or indirectly and act as a key pathway in toxicity induction (Tarrahi, et al. 2018). This reactive species generation may be related to the modulation of antioxidant defenses (Azqueta and Dusinska 2015; Girardello, et al. 2016a; Girardello, et al. 2016b). ZnO-NP may induce toxic effects by their ability to disturb electron transfer processes into cells, increasing ROS generation and creating alternative routes of oxidative stress that are able to cause different damages to the cell (Ma, Williams, and Diamond 2013). Our results show that golden mussel exposure to nanoparticles induces a redox imbalance in mussel's cells, in both enzymatic and non-enzymatic antioxidant defenses. Oxidative damage to proteins and lipids were observed after 4 h exposure and the levels were stabilized after 24 h (Fig. 4E and **F**). On the other hand, Sod decreased after 4 h exposure and increased after 24 h (Fig. 4A). As a means to remove the produced ROS, the Sod and Cat enzymes are activated, decreasing the oxidative stress in the cell. Sod acts as a first antioxidant defensive system reducing the superoxide ion radical ($O^{2\bullet-}$) to hydrogen peroxide ($H_2O_2$), therefore suggesting that the radical $O^{2\bullet-}$ is produced upon exposure to nanoparticles (Halliwell and Gutteridge 2015). Catalase reduces $H_2O_2$, generating $H_2O$ and $O_2$ (Halliwell and Gutteridge 2015) in order to reduce the deleterious effect of $H_2O_2$. Sod enzyme is important to avoid the increase in $O^{2\bullet-}$ concentration, which may lead to oxidative damage. The $O^{2\bullet-}$ is an important reducing agent that is possibly responsible for generating carbonylated proteins and oxidized lipids at higher exposure concentrations. The imbalance between ROS and cellular antioxidants, with excessive production of ROS, allows specific species to attack the cellular macromolecules causing peroxidation of lipids in the cell

and mitochondrial membranes, mitochondrial dysfunction, inhibition of the enzymes activity and DNA damage, which ultimately results in cell death (Abdel-Daim, et al. 2019).

After 24 h exposure to ZnO-NP, Sod levels increased and lipid and protein damage decreased, which is possibly related to restoration of the redox metabolism. This modulation of Sod activity in organisms exposed to ZnO-NP suggest that the mussel *Limnoperna fortunei* tried to stimulate its detoxification system (Fig. 4). Indeed, this observation corroborate with data obtained by Huang and coauthors, who assessed the impact of ZnO-NP and ocean acidification on the antioxidant responses of *Mytilus coruscus* (Huang, et al. 2018). A study with blue mussels (*Mytilus edulis*) evaluated the effects of ZnO structures exposure on these bivalves and showed that ZnO-NP caused accumulation of oxidative lesions in proteins and lipids in the intracellular compartment (Falfushynska, et al. 2019).

One explanation to these changes after 24 h is that the dissociation of ZnO may occur, releasing zinc ions ($Zn^{2+}$) by hydrolysis (Fig. 5). Probably, after 24 h of ZnO-NP exposure, the cells had already encapsulated the nanoparticles in their cytosol by phagocytosis. This process may occur by the invagination of plasmatic membrane to form vesicles that enclose the nanoparticles and transport them into the cell (Châtel and Mouneyrac 2017). After phagocytosis of ZnO-NP, the fusion of lysosome with the phagosome occurs in order to degrade the particle. The hydrolysis of ZnO-NP starts in the phagolysosomes in the presence of water, and $Zn^{2+}$ release may occur, as a result of the reaction $ZnO + H_2O \rightleftharpoons Zn^{2+} + 2OH^-$ (Taccola, et al. 2011). The pH between 4.5 and 5 favors dissociation and subsequent ion release, corroborating to the results obtained by ICP-MS analysis (Fig. 3), which have shown high concentrations of $Zn^{2+}$ in *Limnoperna fortunei* after 24 h of exposure at high assay concentrations. This phagocytosis process is probably a defense mechanism of the cell when it perceives changes in its biomolecules caused by ZnO-NP exposure at higher concentrations and longer exposition time, corroborating to the model of ZnO-NP cytotoxicity on proliferating cells (Taccola, et al. 2011). This autophagosome formation was also observed in previous studies from our group after exposure to *Limnoperna fortunei*, with the golden mussel's cells encompassing $TiO_2$-NP (Girardello, et al. 2016b). The proposed mechanism of action of ZnO-NP in golden mussel cells and the defense mechanisms of these cells may be observed in the illustrative diagram of Fig. 5.

Swiatek and Bednarska *et al.* 2019 discussed the idea that the detoxification process may be energetically costly. The authors could not observe a clear relation between Zn exposure and *Eisena Andrei* energy reserves as carbohydrates were reduced after treatment. Another ecotoxicological study showed that the clearance rate is the component of the energy budget most affected by toxic compounds (Swiatek and Bednarska 2019). As an example, in mussels, the potential toxic elements may be associated to suspended particulate matter in the water column and the energy gain from its feed may be compromised, since there is an energy deviation for contaminant metabolism (Toro, Navarro, and Palma-Fleming 2003). Following this idea, the high energy cost to detoxicate ZnO-NP would also be related to the increase in ROS. This oxidative stress caused by the detoxification process could be increasing the amount of ROS in a synergistic way (Swiatek and Bednarska 2019).

It is interesting to note that the ZnO-NP exposure or the ROS increase did not lead to DNA fragmentation. In addition, the oxidative stress observed in the *Limnoperna fortunei* cells did not result in double strand cleavage in DNA, once the redox metabolism was restored after 24 h. The fragmentation assay employed identified solely double strand cleavage in DNA, whereas other forms of DNA damage, such as single strand cleavage and alkali labile site, were not detected. As evaluated by Girardello and co-authors in 2016 using the comet assay, exposure to $TiO_2$-NP may cause DNA damage to the haemocytes of *Limnoperna fortunei* (Girardello, et al. 2016a). This assay may detect primary DNA damage and the types of lesions detected by the comet assay are generally recent and subject to DNA repair (Villela, et al. 2006; Girardello, et al. 2016a).

# 5. Conclusions

In conclusion, ZnO-NP induces oxidative stress in exposed golden mussels by physical and chemical properties and detoxification processes. The golden mussel is capable to modulate its own antioxidant defences in response to oxidative stress and seems to be able to hydrolyse the nanoparticle complex, therefore releasing Zn ions. This study gives a perspective of a mechanism that relates to the internalization of ZnO-NP through phagocytosis and its oxidative effects; however, further studies are required to understand the molecular mechanism of oxidative stress induced by ZnO-NP.

# Declarations

### Ethical Approval

Not applicable

### Consent to Participate

Not applicable

### Consent to Publish

Not applicable

### Authors Contributions

Francine Girardello: Investigation, Conceptualization, Software, Formal analysis, Investigation, Methodology, Writing - original draft.

Camila Custódio Leite: Formal analysis.

Luciana Bavaresco Touguinha: Methodology.

Mariana Roesch-Ely: Formal analysis, Investigation, Methodology, Writing - review & editing.

Andreia Neves Fernandes: Investigation, Formal analysis, Investigation, Methodology, Writing - review & editing, Supervision.

Richard Macedo de Oliveira: Methodology.

Daniel L. G. Borges: Methodology, Writing - review & editing.

Chrys Katielli Hoinacki da Silva: Methodology, Writing - review.

Mirian Salvador: Investigation, Conceptualization, Formal analysis, Investigation, Methodology, Writing - review & editing, Supervision.

Izabel Vianna Villela: Investigation, Writing - review & editing.

João Antonio Pêgas Henriques: Investigation, Conceptualization, Formal analysis, Investigation, Methodology, Writing - review & editing, Supervision.

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### Competing Interests

The authors declare that they have no competing interests linked to this article.

### Availability of data and materials

All data generated or analysed during this study are included in this published article.

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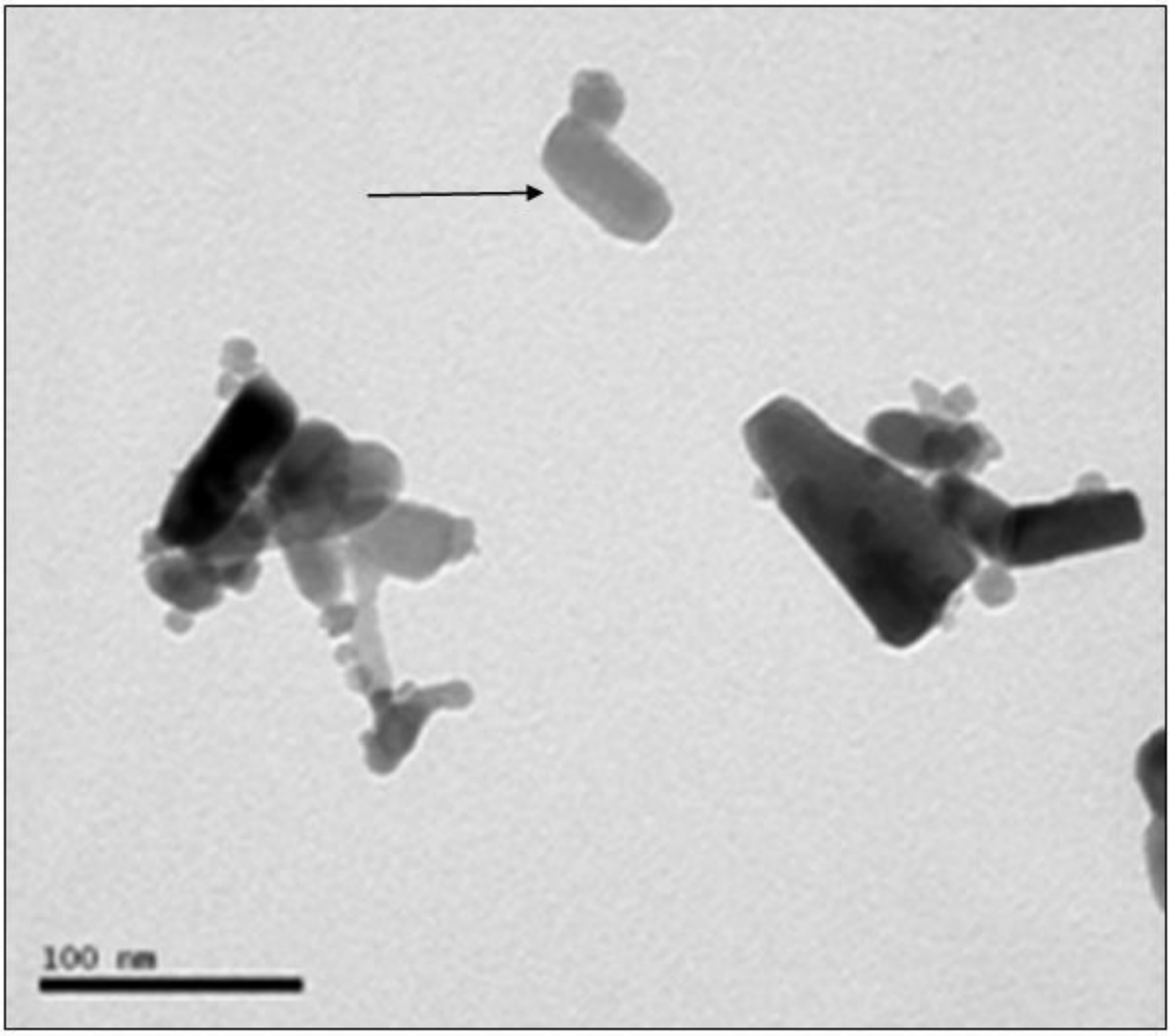
# Figures




## Figure 1

Transmission electron microscopic (TEM) of ZnO-NP powder. Arrow points to a ZnO-NP, presenting media dimension of nanoparticles of about 50 nm.

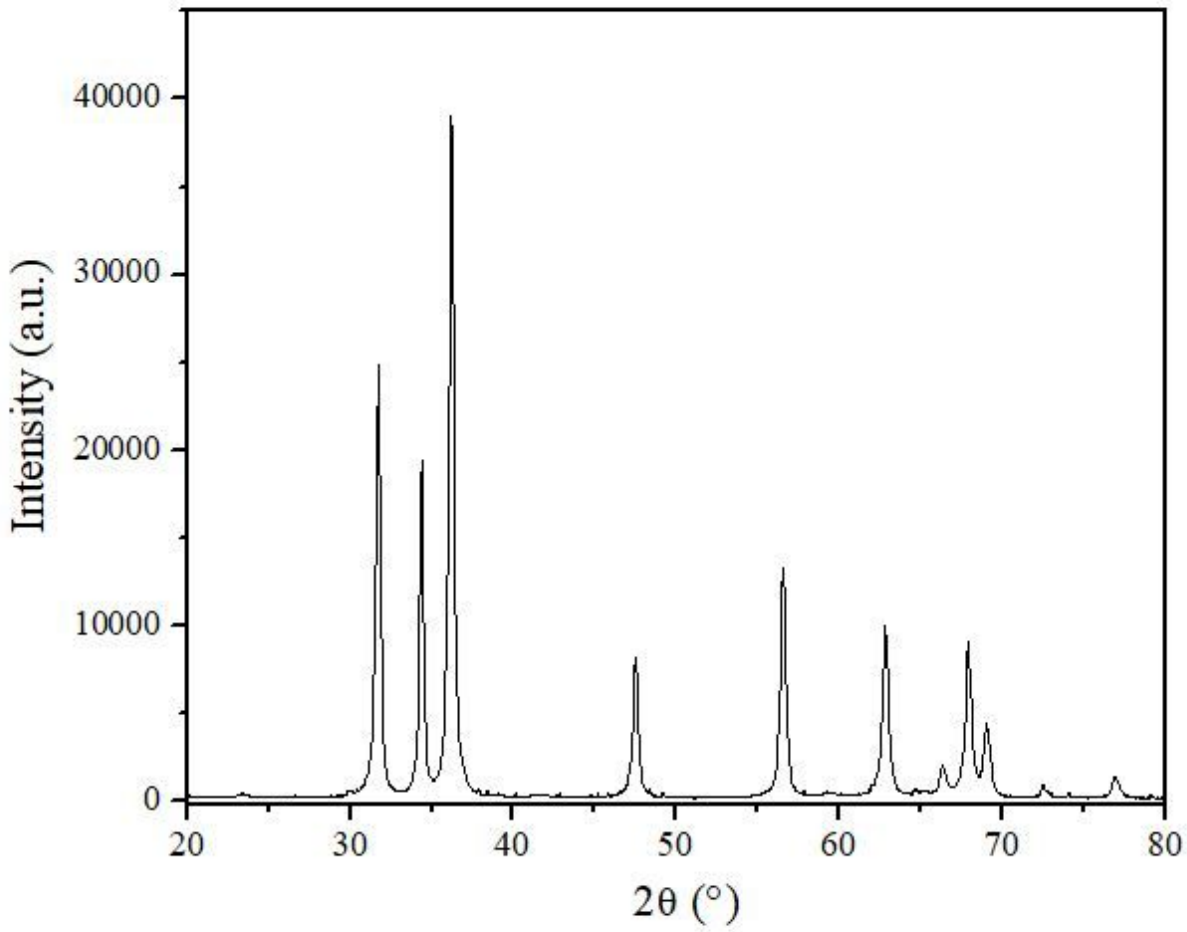
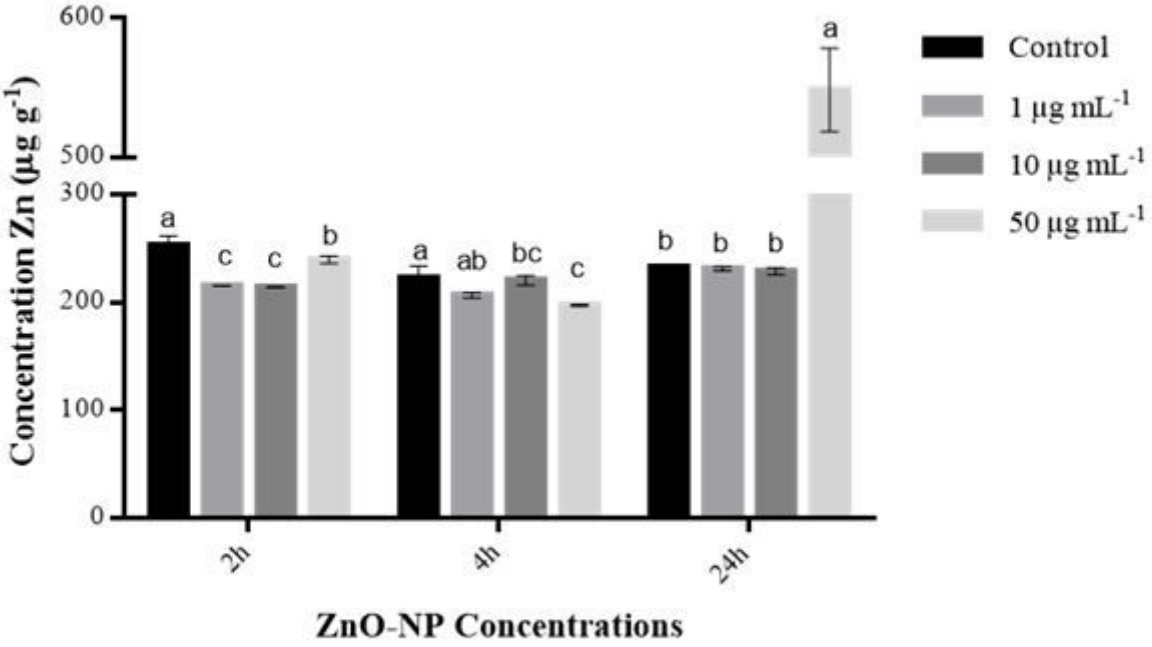


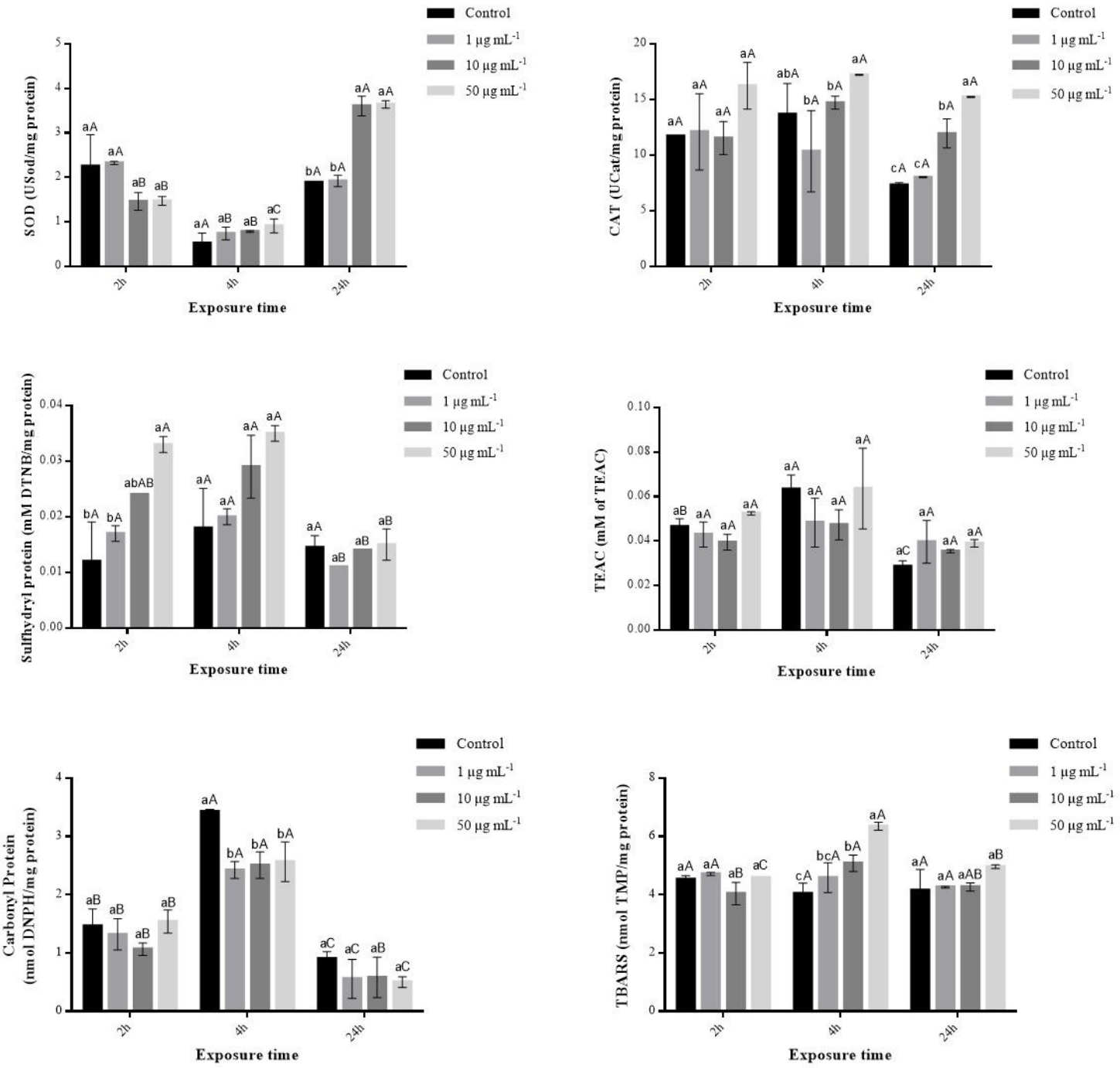

**Figure 2**

X-Ray diffraction (XDR) pattern of ZnO -NP; the crystalline phases are wurtzite.




## Figure 3

Results of ICP-MS analysis of L. fortunei after ZnO-NP exposure.




## Figure 4

Enzymatic and non-enzymatic antioxidant defenses of golden mussel's soft body in different ZnO-NP concentrations after 2, 4 and 24h exposure times: A) Sod; B) Cat; C) Protein sulfhydryl; D) TEAC; E) Carbonyl protein and F) TBARS.

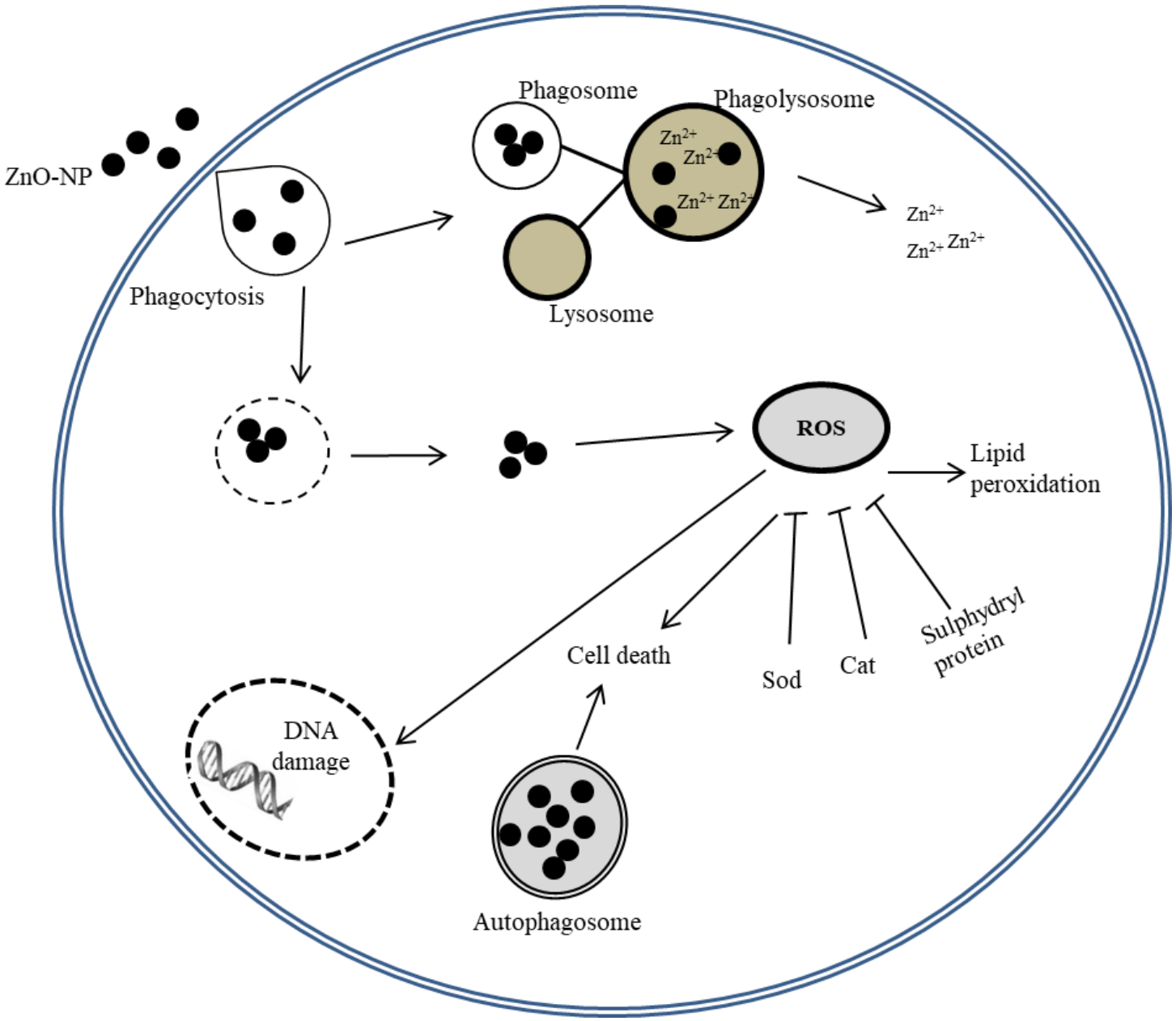



**Figure 5**

Schematic plot highlighting possible mechanism of phagocytosis of ZnO-NP. ZnO-NPs can be internalized through phagocytosis process, followed by the fusion of the phagosome with the lysosome, forming the phagolysosome. Inside of phagolysosome can occur the hydrolysis of ZnO-NP and the release of Zn2+ into the cytosol. In the cytosol, ZnO-NP can cause oxidative stress by generating ROS, damaging cellular organelles, as protein carbonylation and lipid peroxidation. ZnO-NP can also be internalized in autophagosomes and induce cell death. High ROS production and DNA damage can lead to cell death (Girardello, et al. 2016b; Taccola, et al. 2011; Mihai, et al. 2015; Sabella, et al. 2014).

# Supplementary Files

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