*Article*

Toxicology and Industrial Health

Toxicology and Industrial Health
1–12

DOI: 10.1177/0748233716687927
journals.sagepub.com/home/tih
SAGE

# Toxicological perturbations of zinc oxide nanoparticles in the *Coelatura aegyptiaca* mussel

**Sohair R Fahmy and Dawlat A Sayed**

**Abstract**

More research is needed to understand the interactions of nanoparticles (NPs) with aquatic organisms and their mechanism of toxic action. Zinc oxide nanoparticles (ZnONPs) are the most used engineered metal oxide NPs in consumer products. The present study was designed to evaluate the cytotoxicity, genotoxicity and digestive gland (DG) as well as gill histopathology of the freshwater molluscan bivalve *Coelatura aegyptiaca* following exposure to ZnONPs (2, 10 and 50 mg/L) for 6 consecutive days. Exposure to ZnONPs (10 and 50 mg/L) induced a significant increase in malondialdehyde, superoxide dismutase and nitric oxide with a concomitant decrease in reduced glutathione, glutathione-S-transferase and catalase levels in the haemolymph, DG and gills of the treated mussels. Following exposure to ZnONPs (50 mg/L), the DG exhibited gradual changes in glandular activity showing hypertrophy and hyperplasia in the glandular cells and irregularity of lamellae and swelling of filaments in the gills. The present investigation revealed that oxidative stress induction, genotoxicity in the haemocytes and histological alterations in the DG and gills of *C. aegyptiaca* could be the main mechanisms involved in ZnONPs toxicity in aquatic organisms. Thereby, it is suggested that ZnONPs should be applied with more precautions in relevant industries, and occupational health surveillance should be necessarily considered.

**Keywords**

Zinc oxide nanoparticles (ZnONPs), *Coelatura aegyptiaca*, oxidative stress, genotoxicity, gill, digestive gland, haemolymph, histopathology

Received 22 July 2016; Revised 3 December 2016; Accepted 9 December 2016

## Introduction

*Bivalve molluscs* are an ecologically important target group for nanoparticles (NPs) toxicity (Canesi et al., 2012). Freshwater bivalves are used as unique biomarkers of aquatic ecosystem pollution (Jagtap et al., 2011). Bivalves are stationary filter-feeding organisms that have long been recognized as valuable pollution bioindicators (Niyogi et al., 2001). The use of filter-feeding bivalves as valuable models for NPs toxicity studies is based on the extensive background of information that rely on pollutants and methodologies for identifying impaired biological responses (Ringwood et al., 2009). Egyptian freshwater mussel, *Coelatura aegyptiaca* is a molluscan bivalve belonging to *Unionoida*e, common in the Egyptian River Nile. *C. aegyptiaca* is widely distributed along the River Nile from Upper to Lower Egypt (Moloukhia and Sleem, 2011).

Nanotechnology is the most innovative field of the 21st century (Sabir et al., 2014; Yang et al., 2015). It is used in association with optics, electronics, biomedical and materials sciences (Klaine et al., 2008). In this regard, metal oxide NPs are highly suitable and desirable for many consumer products and industrial technologies because of their exceptional physicochemical properties (Montes et al., 2012). Zinc oxide nanoparticles (ZnONPs) are widely used in consumer products that have attracted

Zoology Department, Faculty of Science, Cairo University, Giza, Egypt

**Corresponding author:**
Sohair R Fahmy, Zoology Department, Faculty of Science, Cairo University, Giza 12613, Egypt.
Email: sohairfahmy@gmail.com

intensive research due to their unique properties (Kumar et al., 2014; Nakada et al., 2004). Since the aquatic environment receives run-off and waste water from domestic and industrial sources, it is being targeted with nanoscale environmental remediation techniques. Consequently, the potential impacts of ZnONPs on aquatic ecosystems have attracted special attention (Ali et al., 2012; Fahmy et al., 2014; Sales, 2013). ZnONPs were estimated as one of the most harmful NPs in the aquatic environment (Kahru and Dubourguier, 2010).

Oxidative stress (OXS) in terms of reactive oxygen species (ROS) generation is a convenient parameter to measure the ecotoxicity of ZnONPs in mollusca (Ali et al., 2012; Fahmy et al., 2014; Trevisan et al., 2014). One of the most important mechanisms of NP toxicity is OXS (Mocan et al., 2010). Once the NPs are exposed to the acidic environment of lysosomes, they induce ROS (Chang et al., 2012) or interact with oxidative organelles, such as mitochondria (Zhang and Gutterman, 2007). Canesi et al. (2012) demonstrated that the response of mussels to NP suspensions exposure involves changes in lysosomal and OXS biomarkers in the digestive gland (DG). Moreover, OXS may manifest as damage to tissue macromolecules, including proteins and DNA (Di Giulio et al., 1989). In addition, *in vitro* studies have shown that ZnONPs are capable of inducing OXS, which plays a crucial role in ZnONP-mediated apoptosis (Wang et al., 2014).

The bivalve *C. aegyptiaca* was chosen to determine its suitability as a bioindicator for ZnONPs in the aquatic environment and to show the extent of its tolerance towards NPs in freshwater ecosystems. This study provides basic information for evaluating the current status of NPs pollution in freshwater ecosystems in Egypt.

## Materials and methods

### Experimental animals

Freshwater mussels, *C. aegyptiaca* (8–12 cm shell length), were collected by local fishermen from the River Nile near Abu Rawash, Giza Governorate, Egypt, immediately transported to the lab and acclimatized to room temperature in fiberglass-reinforced plastic (FRP) tanks for 7 days. Animals were not fed during the experiment. The active acclimatized bivalves of approximately same size were selected for experiment.

### Chemicals

ZnONPs with average particle size of < 35 nm and 50 wt% in $H_2O$ (product no. 721077) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). A colloid solution with a concentration of 1000 mg $L^{-1}$ in distilled water was then prepared. The required doses of ZnO were then prepared with the following concentrations: 2, 10, 50 and 100 mg $L^{-1}$ in distilled water.

### Characterization of ZnONPs

The size and shape of the NPs were examined by transmission electron microscopy (TEM) using a JEM-2100F (JEOL Ltd, Tokyo, Japan) at an accelerating voltage of 200 kV.

### Zinc ion analysis in tested water

Zinc ion ($Zn^{2+}$) was determined in the test solution of ZnONPs with a flame atomic absorption spectrophotometer (ICP-MS; ELAN DRC II, PerkinElmer, Waltham, MA, USA) according to the method reported by Richardson (2003).

### Acute toxicity test

A preliminary experiment was conducted to give an indication of the mussels' response to a high dose of ZnONPs (Busk, 2011). The exposure solution was prepared by dispersing ZnONPs in dechlorinated freshwater to a concentration level of 100 mg $L^{-1}$, followed by incubation in an ultrasonic bath for 90 min. Freshwater mussels, *C. aegyptiaca*, were transferred into a FRP tank containing 3 L *dechlorinated* freshwater for acclimation (3 h) prior to the experiment. The dechlorinated freshwater was gently removed and mussels (five mussels per treatment) were exposed to the ZnONPs dechlorinated freshwater solution for 24 h. A parallel control group of mussels was kept in clean dechlorinated freshwater for 24 h. Animals were not fed during the experiment.

### Experimental design

The results of the preliminary experiment revealed that none of the five mussels died at the limit dose of 100 mg $L^{-1}$ for 24 h. The $LC_{50}$ was therefore taken as above 100 mg $L^{-1}$. Three different doses (2, 10 or 50 mg $L^{-1}$) were selected based on the proposed $LC_{50}$ obtained from the acute toxicity study. A stock suspension of 1000 mg $L^{-1}$ ZnONPs was prepared by dispersing the ZnONPs in dechlorinated freshwater and then incubated for 90 min in an ultrasonic bath; a further 20 min of sonication was conducted immediately before water replacement each day. The exposure solutions were prepared immediately prior to use

by diluting the stock solution in dechlorinated freshwater to the concentration levels of 2, 10 or 50 mg $L^{-1}$. Mussels (five mussels per each concentration level) were exposed to ZnONPs for 6 days. A parallel control group of mussels was kept in clean dechlorinated freshwater. ZnONPs dechlorinated freshwater solutions were changed daily, and the turbidity of the ZnONPs solutions was measured right before and after each water replacement in order to monitor the exposure concentrations. Animals were not fed during the experiment.

### Haemolymph sampling

Haemolymph was collected by gently prying the shell open approximately 2–3 mm with a thin knife. The shell was held open with tissue forceps. Under full-spectrum lighting, the anterior adductor muscle was visible between slightly gaping valves as a highly reflective glistening white muscle surface. This muscle mass was gently penetrated with a 25 gauge 5/8 inch needle. Having obtained the haemolymph sample, the needle was discarded to reduce shearing forces that can harm the cells during the subsequent expulsion of the haemolymph into an Eppendorf tube.

### Tissue preparation

Soft tissues were removed from the shell using forceps. DGs and gills of each control and exposed snail were quickly removed, washed with ice-cold saline (0.9%) and cleaned from accessory connective adipose tissues. DGs and gills were homogenized (10% w/v) in ice-cold 0.1 M Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 3000 r/min for 15 min at 4°C, and the resultant supernatant was stored at −80°C for biochemical analysis.

### Assessment of OXS markers

*Lipid peroxide level.* The concentration of malondialdehyde (MDA) as a marker of lipid peroxide (LPO) was determined according to the method of Ohkawa et al. (1979). Glutathione (GSH) content as a non-enzymatic antioxidant was measured according to the method of Beutler et al. (1963) at 412 nm. Superoxide dismutase (SOD) was measured according to the method of Nishikimi et al. (1972). Glutathione-S-transferase (GST) activity was measured using the method of Habig et al. (1974). Catalase (CAT) activity was measured according to the method of Aebi (1984). Nitric oxide (NO) activity was measured according to the method of Montgomery and Dymock (1961).

### Determination of DNA strand breakage

*Comet assay.* Comet assay was performed for measuring DNA damage in haemolymph after 6 days of ZnONPs exposure by single cell gel assay, which permits the detection of single-stranded DNA breaks in one cell according to the methods of Singh et al. (1988) and Grazeffe et al. (2008).

*Histological studies.* Tissue samples of DG and gills were dissected and fixed in 10% buffered formalin and then dehydrated in a graded series of ethanol, and finally immersed in xylene and embedded in paraffin wax (58–60°C) using an automatic processor. Paraffin blocks were trimmed to suitable size and sections of tissues were cut using a microtome at 5–6 μm thickness. After deparaffinization, sections were rehydrated, stained with haematoxylin and eosin, mounted with Cristal/Mount, and subsequently subjected to pathological assessment (Kim et al., 2006).

*Statistical analysis.* The data of the present study were analysed statistically according to Shapiro–Wilk and Kolmogorov analysis. All the raw data were normally distributed, and consequently, all the statistical analyses were performed on the basis of parametric analysis. Reported values were represented as means $\pm$ standard error (SE). Statistical analysis was evaluated by one-way analysis of variance. Statistical Processor System Support (SPSS version 20.0) for Windows software. A value of $p < 0.05$ was considered significant.

## Results

### Characterization studies

Figure 1 shows a TEM image of the ZnONPs, which indicates that most NPs were polygonal in shape with smooth surfaces, and the average diameter of ZnONPs was found to be around 20 $\pm$ 0.56 nm (Figure 2).

### Release of $Zn^{2+}$ concentration

The recorded results revealed that $Zn^{2+}$ concentration increased along with increase in ZnONPs dose (Table 1). $Zn^{2+}$ concentrations in test water were found to be 0.102, 0.512 and 2.56 mg/L at 2, 10 and 50 mg/L of ZnONPs exposure solution, respectively.

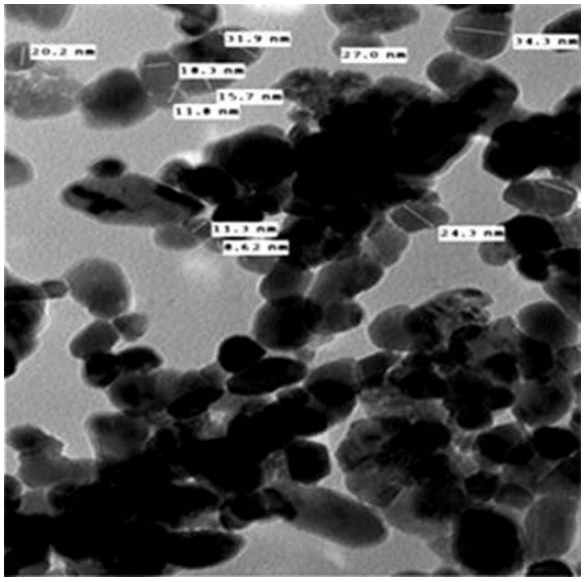

**Figure 1.** High-resolution TEM images of ZnONPs. TEM: transmission electron microscopic; ZnONPs: zinc oxide nanoparticle.

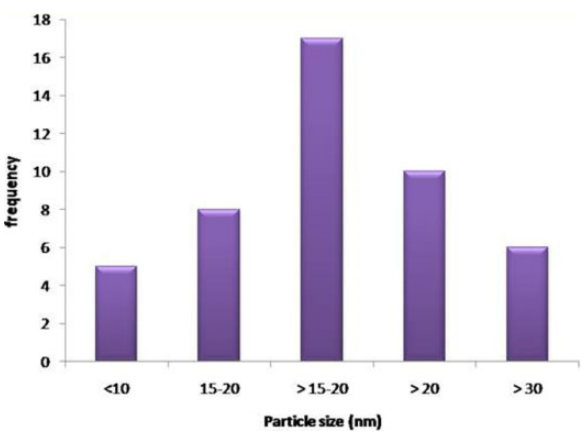


**Figure 2.** Number frequency histograms showing particle size distribution of ZnONPs in linear scale. The particle size data is based on the image analysis of more than 1000 particles. ZnONPs: zinc oxide nanoparticle.

**Table 1.** Concentration of $Zn^{2+}$ in ZnONPs tested solutions.

| ZnONPs (mg/L) | $Zn^{2+}$ (mg/L) |
|---|---|
| Control | 0.012 |
| 2 | 0.102 |
| 10 | 0.512 |
| 50 | 2.56 |

ZnONPs: zinc oxide nanoparticle.

## *Effects of ZnONPs on haemolymph, DG and gills LPO*

MDA levels were assessed as an indicator of LPO. Sublethal *in vivo* exposure of different concentrations of ZnONPs to the bivalve exhibited a significant elevation ($p < 0.05$) in MDA following 10 and 50 mg/L in the haemolymph, DGs and gills (Tables 2 to 4) while only DGs showed significant increase ($p < 0.05$) in MDA following exposure to ZnONPs at concentration of 2 mg/L. It was observed that the MDA level was elevated as exposure concentration and time increased (Tables 2 to 4).

## *Effects of ZnONPs on haemolymph, DG and gills reduced GSH levels*

As shown in Tables 2 to 4, GSH content of haemolymph, DG and gills was significantly ($p < 0.05$) decreased due to ZnONPs exposure in a concentration – dependent manner. The highest reduction in GSH level was observed in the mussels exposed to 50 mg/L of ZnONPs following 6 days of exposure (Tables 2 to 4).

## *Effects of ZnONPs on haemolymph, DG and gills GST*

Treatment with different concentrations of ZnONPs caused a significant ($p < 0.05$) reduction in GST activities in the haemolymph and DG of treated *C. aegyptiaca* mussels following exposure to 10 and 50 mg/L of ZnONPs (Tables 2 to 4). However, GST activities of gills tissues recorded a significant ($p < 0.05$) decrease only following exposure to 50 mg/L of ZnONPs.

## *Effects of ZnONPs on haemolymph, DG and gills CAT activity*

CAT activity in the haemolymph of *C. aegyptiaca* mussels was found to be decreased significantly ($p < 0.05$) at 10 and 50 mg/L of ZnONPs when compared with control group (Table 2). While in the DG and gills, CAT activities decreased significantly ($p < 0.05$) only following exposure to 50 mg/L ZnONPs for consecutive 6 days (Tables 3 and 4).

## *Effects of ZnONPs on haemolymphh, DG and gills SOD activity*

Concerning the effect of ZnONPs on SOD activity, a significant increase ($p < 0.05$) was recorded in haemolymph and DG tissues of the treated mussels with 10 and 50 mg/L (Tables 2 to 4). However, gills SOD

**Table 2.** Effect of different sublethal concentrations of ZnONPs for 6 days on some OXS markers in haemolymph of *C. aegyptiaca*.[a]

| Concentration | Control | 2 μg/ml | 10 μg/ml | 50 μg/ml |
|---|---|---|---|---|
| MDA (mmol/ml) | 2.48 ± 0.20 | 2.57 ± 0.32 | 4.27 ± 0.85[b] | 5.14 ± 0.39[b] |
| GSH (mmol/l) | 0.74 ± 0.11 | 0.65 ± 0.14 | 0.38 ± 0.02[b] | 0.36 ± 0.02[b] |
| GST (U/ml) | 0.72 ± 0.1 | 0.66 ± 0.14 | 0.41 ± 0.02[b] | 0.34 ± 0.04[b] |
| CAT (U/ml) | 0.31 ± 0.06 | 0.21 ± 0.49 | 0.127 ± 0.46[b] | 0.128 ± 0.62[b] |
| SOD (U/ml) | 201.92 ± 9.71 | 222.45 ± 7.5 | 256.73 ± 15.7[b] | 242.64 ± 10.7[b] |
| NO (μmol/l) | 23.37 ± 1.14 | 31.74 ± 1.7 | 35.15 ± 4.7[b] | 40.60 ± 3.3[b] |

ZnONPs: zinc oxide nanoparticle; OXS: oxidative stress; MDA: malondialdehyde; GSH: glutathione; GST: glutathione-S-transferase; CAT: catalase; SOD: superoxide dismutase; NO: nitric oxide; SE: standard error.
[a]Values are mean ± SE; $n = 5$ in each group.
[b]$p < 0.05$: significant as compared with the corresponding control.

**Table 3.** Effect of different sublethal concentrations of ZnONPs for 6 days on some OXS markers in gills of *C. aegyptiaca*.[a]

| Concentration | Control | 2 μg/ml | 10 μg/ml | 50 μg/ml |
|---|---|---|---|---|
| MDA (mmol/ml) | 6.66 ± 0.53 | 8.67 ± 0.62 | 10.18 ± 1.14[b] | 14.16 ± 1.33[b] |
| GSH (mmol/l) | 14.54 ± 1.18 | 13.52 ± 0.51 | 12.03 ± 0.53[b] | 9.95 ± 0.41[b] |
| GST (U/ml) | 18.28 ± 1.6 | 17.23 ± 1.25 | 15.50 ± 0.78 | 12.64 ± 0.65[b] |
| CAT (U/ml) | 81.58 ± 6.89 | 87.64 ± 3.34 | 90.95 ± 7.30 | 58.31 ± 9.77[b] |
| SOD (U/ml) | 14.72 ± 0.47 | 8.27 ± 0.32[b] | 7.61 ± 0.86[b] | 6.64 ± 1.43[b] |
| NO (μmol/l) | 1.35 ± 0.08 | 1.40 ± 0.5 | 1.61 ± 0.12 | 3.09 ± 0.26[b] |

ZnONPs: zinc oxide nanoparticle; OXS: oxidative stress; MDA: malondialdehyde; GSH: glutathione; GST: glutathione-S-transferase; CAT: catalase; SOD: superoxide dismutase; NO: nitric oxide; SE: standard error.
[a]Values are mean ± SE; $n = 5$ in each group.
[b]$p < 0.05$: significant as compared with the corresponding control.

**Table 4.** Effect of different sublethal concentrations of ZnONPs for 6 days on some OXS markers in the DG of *C. aegyptiaca*.[a]

| Concentration | Control | 2 μg/ml | 10 μg/ml | 50 μg/ml |
|---|---|---|---|---|
| MDA (mmol/ml) | 5.98 ± 0.40 | 9.72 ± 0.42[b] | 12.39 ± 0.36[b] | 15.19 ± 0.57[b] |
| GSH (mmol/l) | 18.72 ± 0.83 | 16.59 ± 0.91 | 13.96 ± 0.16[b] | 10.93 ± 1.06[b] |
| GST (U/ml) | 23.71 ± 1.1 | 21.68 ± 0.58 | 17.68 ± 1.44[b] | 14.02 ± 1.34[b] |
| CAT (U/ml) | 109.26 ± 10.30 | 127.57 ± 5.44 | 90.67 ± 9.05 | 68.09 ± 5.23[b] |
| SOD (U/ml) | 13.41 ± 0.58 | 14.52 ± 0.49 | 15.47 ± 0.19[b] | 19.15 ± 0.70[b] |
| NO (μmol/l) | 1.15 ± 0.09 | 1.54 ± 0.09[b] | 1.64 ± 0.04[b] | 1.71 ± 0.1[b] |

ZnONPs: zinc oxide nanoparticle; OXS: oxidative stress; DG: digestive gland; MDA: malondialdehyde; GSH: glutathione; GST: glutathione-S-transferase; CAT: catalase; SOD: superoxide dismutase; NO: nitric oxide; SE: standard error.
[a]Values are mean ± SE; $n = 5$ in each group.
[b]$p < 0.05$: significant as compared with the corresponding control.

activity increased significantly ($p < 0.05$) after exposure to all concentrations of ZnONPs (Table 3).

## *Effects of ZnONPs on haemolymphh, DG and gills NO activity*

Treatment with ZnONPs (10 and 50 mg/L) induced a significant increment ($p < 0.05$) in NO activity in haemolymph and DG of treated mussels (Tables 2 to 4) compared with controls. However, NO activity increased significantly ($p < 0.05$) following exposure to low concentration of ZnONPs (2 mg/L) in DG only (Table 4). While in gill tissues, NO activity increased significantly ($p < 0.05$) only after exposure to high concentration of ZnONPs (50 mg/L) (Table 3).

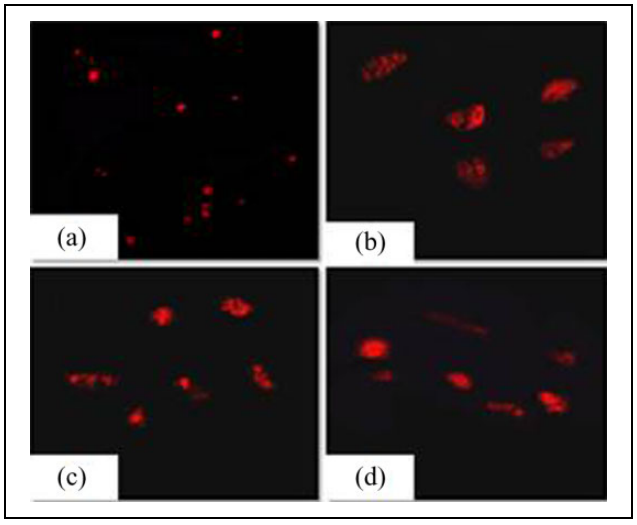


**Figure 3.** DNA damage in the haemocytes of *Caelatura aegyptiaca* after exposure to different concentrations of ZnONPs. (a) Control, (b) cells exposed to 2 mg/L, (c) cells exposed to mg/L and (d) cells exposed to 50 mg/L. ZnONPs: zinc oxide nanoparticle.

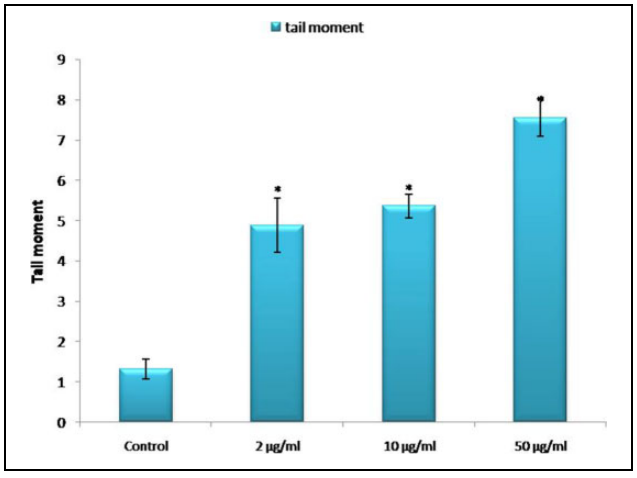


**Figure 4.** Effect of different concentrations of ZnONPs (2, 10 and 50 mg/L) on % tail DNA damage in the haemocytes of *C. aegyptiaca*. ZnONPs: zinc oxide nanoparticle. *$p$\0.05 = significant difference.

## *Effects of ZnONPs on DNA damage in the haemocytes*

DNA damage was measured as tail DNA (%) and olive tail moment in the control and ZnONPs exposed haemocytes (Figures 3 to 5). During electrophoresis, the cell DNA migrates more rapidly towards the anode at the highest concentration than at the lowest concentration of the NPs. Cells exposed to different concentrations of ZnONPs showed significantly ($p < 0.05$) more DNA damage than did the control cells. A gradual nonlinear increase in cell DNA damage was

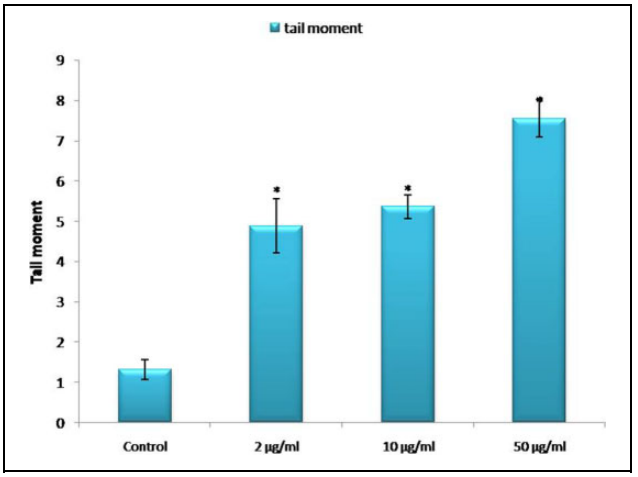


**Figure 5.** Effect of different concentrations of ZnONPs (2, 10 and 50 mg/L) on tail moment of DNA damage in the haemocytes of *C. aegyptiaca*. ZnONPs: zinc oxide nanoparticle. *$p$\0.05 = significant difference.

observed as the concentration of ZnONPs exposure increased. The most DNA damage was recorded at, the highest concentration, 50 mg/L (Figures 3 to 5).

## *Histopathological alterations in the DG ZnONPs exposure*

Representative photomicrograph of *C. aegyptiaca* mussels' DG sections is shown in Figure 6(a). DG of control *C. aegyptiaca* mussels consists of a great number of digestive tubules. The tubules are separated by a thin layer of loose connective tissue. Each digestive tubule is lined by columnar epithelial cells and secretory cells resting on a basement membrane. In the core of each digestive tubule, there is a narrow lumen. The obtained results showed more or less normal architecture of the DG following 2 and 10 mg/L ZnONPs exposure for 6 consecutive days (Figure 6(b) and (c)).

DG of bivalves exposed to chronic 50 mg/L ZnONPs for 6 days showed histopathological changes in their structure as observed in Figure 6(d). There was an increase in lumen size in the core of the lobule (arrow). Necrosis and degeneration of the cells were observed. Connective tissue became very thin. The overall damage of the hepatic follicles was observed.

## *Histopathological alterations in the gills following ZnONPs exposure*

Gills of *C. aegyptiaca* are composed of lamellae, filaments and water channels. Each gill filament consisted of two chitinous rods, an epithelium layer and

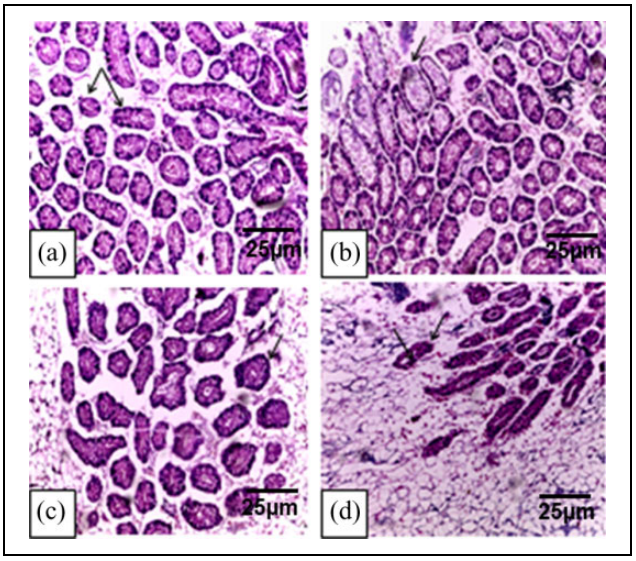


**Figure 6.** Photomicrograph of the *C. aegyptiaca* DG following 6 days of chronic exposure to ZnONPs. (a) Control mussels showing the tubular nature of the DG (arrows); (b) 2 mg/L ZnONPs treated mussels; (c) 10 mg/L ZnONPs treated mussels and (d) 50 mg/L ZnONPs treated mussels. ZnONPs: zinc oxide nanoparticle; DG: digestive gland.

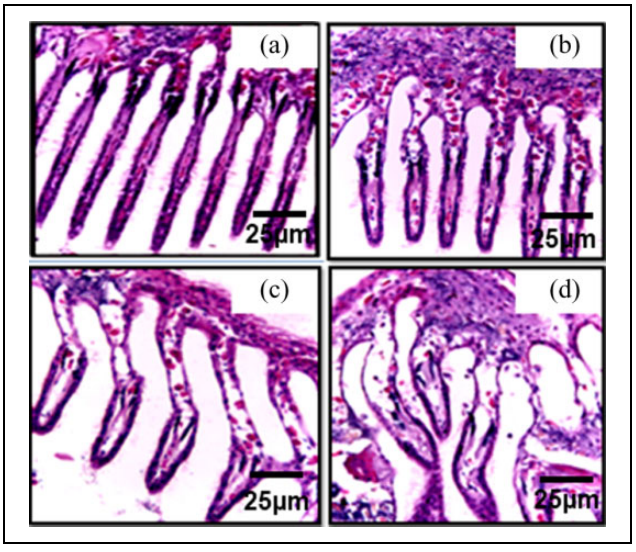


**Figure 7.** Photomicrograph of the *C. aegyptiaca* gills following 6 days of chronic exposure to ZnONPs. (a) Control mussels showing normal demibranchs; (b) 2 mg/L ZnONPs treated mussels; (c) 10 mg/L ZnONPs treated mussels and (d) 50 mg/L ZnONPs treated mussels. ZnONPs: zinc oxide nanoparticle.

vascular system covered with cilia. Histological observations showed normal structure of gill in the control mussel throughout the duration of the experiment (Figure 7(a)). Pathological lesions after 6 days exposure of three different sublethal concentrations are shown in Figure 6. In the 2 mg/L exposure group, gill filaments showed marginal channel dilation (Figure 7(b)). In the 10 mg/L exposure group, blood congestion, epithelial lifting, desquamation and necrosis were observed. Alteration in filament architecture and the loss of cilia were of serious concern (Figure 7(c)). Also, hyperplasia of epithelial cells, lamellar fusion, aneurism, filament disorganization and curling were recorded for the 50 mg/L exposure group (Figure 7(d)).

## Discussion

Water pollution is considered to be one of the most dangerous hazards affecting Egypt. The present investigation was designed to evaluate the possible mechanisms of ecotoxicological effects of ZnONPs on the freshwater mussel *C. aegyptiaca*, which are important aquatic organisms along the Nile River from Upper Egypt to Lower Egypt (Moloukhia and Sleem, 2011).

OXS is a convenient parameter that has been suggested as one of several possible mechanisms of ZnONPs toxicity in mollusca (Ali et al., 2012; Fahmy et al., 2014). However, when organisms are subjected to xenobiotic compounds, the rate of production of ROS exceeds their scavenging capacity (Halliwell and Gutteridge, 2007). The suspension-feeding bivalves are considered efficient toxin agent with a relative insensitivity to toxicants compared to other aquatic organisms (Martins et al., 2014). DG is the site of multiple oxidative reactions and may, therefore, be a site of substantial free radical generation. In addition, the gill is the main respiratory organ, which is concerned with filtration of particles and remaining suspended material from the water, so it is directly susceptible to xenobiotics and contaminants present in water (McDonagh and Sheehan, 2008).

LPO occurs as a result of the oxidative deterioration of polyunsaturated fatty acids, that is, those that contain two or more carbon–carbon double bonds (Reiter et al., 2014). MDA, considered as a byproduct of LPO, has been widely used to assess the effects of many pollutants on aquatic ecosystems (Wu et al., 2011). In conjunction with the reports of Suman et al. (2014) and Fahmy et al. (2014), data from the present investigation reflects that OXS is a common feature of ZnONPs toxicity in mussel *C. aegyptiaca*. In the present study, the intensity of OXS is measured as an increase in the levels of LPO end product, MDA, in the haemolymph, DG and gills of *C. aegyptiaca* following ZnONPs exposure. The present study also

showed a dose–response relationship between exposures to ZnONPs and MDA level in the studied tissues. The data demonstrated the high sensitivity of DG to very low concentrations of ZnONPs (2 mg/L) compared to other studied tissues that did not showed any significant change in their MDA levels at this concentration. In accord with the report of Borković-Mitić et al. (2011), the present study confirmed that DG of the bivalve *C. aegyptiaca* is of particular interest during ZnONPs toxicity because it is involved in most biotransformation processes and redox-cycling.

GSH is the most abundant non-protein thiol compound that serves many important physiological roles, particularly as a cellular antioxidant in peripheral tissue (Liu and Pravia, 2010). GSH plays a major role in catalysis, metabolism and transport. It also protects cells against free radicals, peroxides and other toxic compounds (Hiraishi et al., 1994). Indeed, GSH depletion increases the sensitivity of cells to various toxic insults GSH, a ubiquitous and abundant antioxidant cellular tripeptide, was found to be strongly depleted after exposure to ZnONPs (Alarifi et al., 2013). Because of their exposed sulphydryl groups, non-protein sulphydryls bind a variety of electrophilic radicals and metabolites that may be damaging to the cells (Szabo et al., 1992). Insufficiency in non-enzymatic antioxidant GSH following ZnONPs could be the consequence of increased utilization for trapping free radicals. The present study confirmed the finding of Ali et al. (2012) and Fahmy et al. (2014), who suggested that the decrease in GSH content in the haemolymph, DG and gills of *C. aegyptiaca* appears to be a common response of molluscs to metal exposure, partly explained by the high affinity of zinc metal for the GSH molecule.

GST is an enzyme that participates in the detoxification process due to conjugation reaction between GSH and xenobiotics (Adang et al., 1990). The present study showed significant decrease in GST activities in the haemolymph, DG and gills of ZnONPs-exposed mussels, *C. aegyptiaca*, as compared to the control group. The recorded results also showed that 10 and 50 mg/L of ZnONPs induced the highest significant reduction in GST activity. It has been reported that the enhanced free radical concentration resulting from the OXS conditions can cause loss of enzymatic activity (Koul et al., 2014; Lakshmi and Subramanian, 2014).

SOD and CAT act as mutually supportive antioxidative enzymes, which provide defence against ROS (Cerutti et al., 1994). The present study revealed a significant increase in SOD activity in all studied tissues following exposure to different concentrations of ZnONPs. The recorded increment in the SOD activity following exposure of ZnONPs could be attributed to their increased synthesis resulting from induction, since antioxidant enzymes are induced in response to OXS. Thus, this increase in the activities of SOD could be a compensation for GSH depletion. Viewed in conjunction with the report of Fahmy et al. (2014), the inhibition of CAT activities following ZnONPs (10 and 50 mg/L) toxicity in the present study may be due to the enhancement of the peroxidation end product MDA, which is known to inhibit protein synthesis and the activities of certain enzymes. Moreover, decreased CAT activity in *C. aegyptiaca* may be related to a reduced capability to neutralize ROS and an increased susceptibility to OXS (Pampanin et al., 2005).

NO is an important signalling molecule that plays an indispensable role in immunity of all vertebrates and invertebrates (Jiang et al., 2013). The NO system has been reported to regulate the metabolism, growth, cell proliferation, development and neurogenesis in vertebrates, insects and mollusks (Cáceres et al., 2011). The present investigation confirmed the finding of Fahmy et al. (2014), who reported a significant increase in NO activity following exposure to ZnONPs. The direct toxicity of NO is enhanced by reacting with superoxide radical forming peroxynitrite, which is capable of oxidizing cellular structures causing LPO (Weinstein et al., 2000).

Studies conducted to increase information on genotoxic risks related to exposure to emerging nanomaterials are of increasing interest (Landsiedel et al., 2009), mainly those regarding compounds with limited information regarding potential genotoxic risks. Genotoxicity is considered one of the most important parameters that have been suggested as possible mechanisms of ZnONPs toxicity testing and risk assessment (Ali et al., 2012; Heim et al., 2015). The results reported in the current study add new and interesting information on the genotoxic effects of ZnONPs in the bivalve *C. aegyptiaca*. Haemocytes are circulating cells that act as sentry cells, scanning the immediate environment to detect foreign material (Donaghy et al., 2009). In invertebrates, blood cells and haemocytes have traditionally been considered the primary vehicles for transporting metals (Anderson and Anderson, 1991). Thereby, the *C. aegyptiaca* haemocytes were used in the present study to

determine the genotoxical potential of ZnONPs. In conjunction with the finding of Gomes et al. (2013) and Ali et al. (2012), the present study revealed a genotoxic effect of ZnONPs in haemocytes of *C. aegyptiaca*, which may be mediated through OXS. The results from this investigation will act as a base line data for evaluation of the state of pollution by genotoxic pollutants like ZnONPs along the freshwater drains in Egypt using freshwater bivalve like *C. aegyptiaca* as the sentinel organisms.

The histological details of DG and gills of *C. aegyptiaca* are very similar to earlier reports on different bivalve species (Agwuocha et al., 2011; El-Shenawy et al., 2009; Kumar et al., 2011). Moreover, the DG is the site of multiple oxidative reactions and may, therefore, be a site of substantial free radical generation. In addition, pollutant accumulation through different routes is transported by haemocytes to the DG (Regoli et al., 2005). Histopathological alterations of gills of bivalve tissues have been shown to be sensitive to a wide range of contaminants because they play an important role in respiration and food collection (Abdel Nabi et al., 2007). So, DG and gills were used in the present investigation to investigate the pathological responses of *C. aegyptiaca* to ZnONPs. Johnson et al. (1996) reported active involvement of digestive tubule cells in intracellular digestion, absorption and toxicant detoxification as they are rich in acid phosphatase and specialized in structure. Following 6 days of ZnONPs (50 mg/L) exposure, the DG of the mussel exhibited gradual changes in glandular activity by showing hypertrophy and hyperplasia in the glandular cells, and gills showed irregularity of lamellae and swelling of filaments. This suggests the high sensitivity of the *C. aegyptiaca* DG and gills to ZnONPs, and the key role of these species in freshwater monitoring programmes. Similar histopathological changes in DG and gills were recorded by many workers on bivalves (Cid et al., 2015; Hu et al., 2014; Koehler et al., 2008; Vale et al., 2014) exposed to different NPs.

In conclusion, the present investigation has indicated that ZnONPs contamination in the aquatic system leads to disturbances in the metabolically active haemolymph as well as DG and gills of the freshwater mussel *C. aegyptiaca*. Determination of the OXS markers such as MDA, GSH, CAT and SOD beside the histological alterations in the DG and gills could be used as biomarkers for the assessment of ZnONPs toxicity in aquatic organisms. So, it is suggested that ZnONPs should be used with more precautions in relevant industries, and also occupational health surveillance should be necessarily considered.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

## References

Abdel Nabi IM, ElShenawy NS, Taha IA, et al. (2007) Oxidative stress biomarkers and bioconcentration of reldan and roundup in the edible clam *Ruditapes decussatus*. *Acta Zoologica Sinica* 53: 910–920.

Adang AE, Brussee J, Van Der Gen A, et al. (1990) The glutathione-binding site in glutathione S-transferases. Investigation of the cysteinyl, glycyl and gamma-glutamyl domains. *Biochemistry Journal* 269: 47–54.

Aebi H (1984) Catalase *in vitro*. *Methods in Enzymology* 105: 121–126.

Agwuocha S, Kulkarni BG and Pandey AK (2011) Histopathological alterations in hepatopancreas of *Gafrarium divaricatum* exposed to xylene, benzene and gear oil-WSF. *Journal Environmental Bioliogy* 32(1): 35–38.

Alarifi S, Ali D, Alkahtani S, et al. (2013) Induction of oxidative stress, DNA damage, and apoptosis in a malignant human skin melanoma cell line after exposure to zinc oxide nanoparticles. *International Journal Nanomedicine* 8: 983–993.

Ali D, Alarifi S, Kumar S, et al. (2012) Oxidative stress and genotoxic effect of zinc oxide nanoparticles in freshwater snail Lymnaea luteola L. *Aquatic Toxicolo*gy 124–125: 83–90.

Anderson DH and Anderson JHS (1991) The distribution of vanadium and sulfur in the blood cells and the nature of vanadium in the blood cells and plasma of the ascidian, *Ascidia ceratodes*. *Comparative Biochemistry and Physiology* 99A: 585–588.

Beutler E, Duron O and Kelly M B (1963) Improved method for the determination of blood glutathione. *Journal of Laboratory and Clinical Medicine* 61: 882–888.

Borković-Mitić SS, Kovačević TB, Perendija BR, et al. (2011) Superoxide dismutase and catalase activities in the digestive gland of the freshwater bivalve *Unio pictorum* from the Sava River. *Archive Biological Science, Belgrade* 63(1), 185–192.

Busk SA (2011) *Nanostructured titanium dioxide: fate in the aquatic environment and effects on the marine mussel Mytilus edulis*. Master's Thesis, University of Stavanger, Norway, p. 72.

Cáceres L, Necakov AS, Schwartz C, et al. (2011) Nitric oxide coordinates metabolism, growth, and development via the nuclear receptor E75. *Genes & Development* 25: 1476–1485.

Canesi L, Ciacci C, Fabbri R, et al. (2012) *Bivalve molluscs* as a unique target group for nanoparticle toxicity. *Marine Environmental Research* 76: 16–21.

Cerutti P, Ghosh R, Oya Y, et al. (1994) The role of the cellular antioxidant defense in oxidant carcinogenesis. *Environmental Health Perspectives* 102(10): 123–129.

Chang YN, Zhang MY, Xia L, et al. (2012) The toxic effects and mechanisms of CuO and ZnO nanoparticles. *Materials* 5(12): 2850e71

Cid A, Picado A, Correia JB, et al. (2015) Oxidative stress and histological changes following exposure to diamond nanoparticles in the freshwater Asian clam *Corbicula fluminea* (Müller, 1774). *Journal of Hazardous Materials* 284: 27–34.

Di Giulio RT, Washburn PC, Wenning RJ, et al. (1989) Biochemical responses in aquatic animals: a review of determinants of oxidative stress. *Environmental Toxicology and Chemistry* 8: 1103–1123.

Donaghy L, Lambert C, Choi K, et al. (2009) Hemocytes of the carpet shell clam (*Ruditapes decussatus*) and the Manila clam (*Ruditapes philippinarum*): current knowledge and future prospects. *Aquaculture* 297: 10–24.

El-Shenawy NS, Moawad TIS, Mohallal ME, et al. (2009) Histopathologic biomarker response of clam *Ruditapes decussates* to organophosphorus pesticide reldan and roundup: a laboratory study. *Ocean Science Journal* 44(1): 27–34.

Fahmy SR, Abdel-Ghaffar F, Bakry FA, et al. (2014) Ecotoxicological effect of sublethal exposure to zinc oxide nanoparticles on freshwater snail *Biomphalaria alexandrina*. *Archives of Environmental Contamination and Toxicology* 67(2): 192–202.

Gomes T, Araújo O, Pereira R, et al. (2013) Genotoxicity of copper oxide and silver nanoparticles in the mussel *Mytilus galloprovincialis*. *Marine Environmental Research* 84: 51–59.

Grazeffe VS, Tallarico LF, Pinheiroa AS, et al. (2008) Establishment of the comet assay in the freshwater snail Biomphalaria glabrata (Say, 1818). *Mutation Research* 654: 58–63.

Habig WH, Pabst MJ and Jakoby WB (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry* 249(22): 7130–7139.

Halliwell B and Gutteridge JMC (2007) *Free Radicals in Biology and Medicine*. 4th ed. Oxford and New York: Oxford University Press, pp. 1–851.

Heim J, Felder E and Tahir MN, et al. (2015) Genotoxic effects of zinc oxide nanoparticles. *Nanoscale* 7(19): 8931–8938.

Hiraishi H, Terano A, Ota S, et al. (1994) Protection of cultured rat gastric cells against oxidant-induced damage by exogenous glutathione. *Gastroenterol* 106(5): 1199–1207.

Hu W, Culloty S, Darmody G, et al. (2014) Toxicity of copper oxide nanoparticles in the blue mussel, *Mytilus edulis*: a redox proteomic investigation. *Chemosphere* 108: 289–299.

Jagtap JT, Shejule KB and Jaiswal DP (2011) Acute toxicity study of tributyltin chloride on the freshwater bivalve. *Lamellidens marginalis. World Journal of Fish and Marine Sciences* 3(2): 100–103.

Jiang Q, Zhou Z, Wang L, et al. (2013) The immunomodulation of inducible nitric oxide in scallop *Chlamys farreri*. *Fish Shellfish Immunology* 34(1): 100–108.

Johnson MA, Paulet YM, Donval A, et al. (1996) Histology, histo-chemistry, and enzyme biochemistry in the digestive system of the endosymbiont bearing bivalve (*Loripes lucinalis* Lamarck). *Journal of Experimental Biological and Ecology* 197(1): 15–38.

Kahru A and Dubourguier H C (2010) From ecotoxicology to nanoecotoxicology. *Toxicology* 269(2): 105–119.

Kim Y, Ashton-Alcox KA and Powell EN (2006) *Histological Techniques for Marine Bivalve molluscs: Update*. Silver Spring: NOAA Technical Memorandum NOS NCCOS 27.

Klaine SJ, Alvarez PJJ, Batley GE, et al. (2008) Nanomaterials in the environment: behavior, fate, bioavailability, and effects. *Environmental Toxicology and Chemistry* 27: 1825–1851.

Koehler A, Marx U, Broeg K, et al. (2008) Effects of nanoparticles in *Mytilus edulis* gills and hepatopancreas – a new threat to marine life. *Marine Environmental Research* 66(1): 12–14.

Koul A, Mohan V and Bharati S (2014) *Azadirachta indica* mitigates DMBA-induced hepatotoxicity: a biochemical and radiometric study. *Indian Journal of Biochemistry and Biophysics* 51(1): 37–45.

Kumar A, Najafzadeh M, Jacob BK, et al. (2014) Zinc oxide nanoparticles affect the expression of p53, Ras p21 and JNKs: an *ex vivo/in vitro* exposure study in respiratory disease patients. *Mutagenesis* 30(2): 237–245.

Kumar S, Pandey RK, Das S, et al. (2011) Pathological changes in hepatopancreas of freshwater mussel (*Lamellidens marginalis* Lamarck) exposed to sub-lethal concentration of dimethoate GERF. *Bulletin of Biosciences* 2(2): 18–23.

Lakshmi A and Subramanian S (2014) Chemotherapeutic effect of tangeretin, a polymethoxylated flavone studied in 7, 12-dimethylbenz(a)anthracene induced mammary carcinoma in experimental rats. *Biochimie* 99(1): 96–109.

Landsiedel R, Kapp MD, Schulz M, et al. (2009). Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations-many questions, some answers. *Mutation Research* 681: 241–258.

Liu RM and Pravia KAG (2010) Oxidative stress and glutathione in TGF-beta-mediated fibrogenesis. *Free Radical Biology and Medicine* 48: 1–15.

Martins JC, Campos A, Osório H, et al. (2014) Proteomic profiling of cytosolic glutathione transferases from three bivalve species: *Corbicula fluminea*, *Mytilus galloprovincialis* and *Anodonta cygnea*. *The International Journal of Molecular Science* 15(2): 1887–1900.

McDonagh B and Sheehan D (2008) Effects of oxidative stress on protein thiols and disulphides in *Mytilus edulis* revealed by proteomics: actin and protein disulphide isomerase are redox targets. *Marine Environmental Research* 66(1): 193–195.

Mocan T, Clichici S, Agoşton-Coldea L, et al. (2010) Implications of oxidative stress mechanisms in toxicity of nanoparticles (review). *Acta Physiologica Hungarica* 97(3): 247–255.

Moloukhia H and Sleem S (2011) Bioaccumulation, fate and toxicity of two heavy metals common in industrial wastes in two aquatic molluscs. *Journal of American Science* 7(8): 459–464.

Montes MO, Hanna SK, Lenihan HS, et al. (2012) Uptake, accumulation and biotransformation of metal oxide nanoparticles by a marine suspension-feeder. *Journal of Hazardous Materials* 225–226: 139–145.

Montgomery HAC and Dymock JF (1961) The determination of nitrite in water. *Analyst* 86: 414–416.

Nakada T, Hirabayashi Y, Tokado T, et al. (2004) Novel device structure for Cu(In,Ga)$Se_2$ thin film solar cells using transparent conducting oxide back and front contacts. *Solar Energy* 77(6): 739–747.

Nishikimi M, Appaji N and Yagi K (1972) The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochemical and Biophysical Research Communications* 46: 849–854.

Niyogi S, Biswas S, Sarkers M, et al. (2001) Antioxidant enzymes in brackishwater oyster, *Saccostrea cucullata* as potential biomarkers of polyaromatic hydrocarbon pollution in Hooghly Estuary (India): seasonality and its consequences. *Science of the Total Environment* 281: 237–246.

Ohkawa H, Ohishi N and Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry* 95: 351–358.

Pampanin DM, Volpato E, Marangon I, et al. (2005) Physiological measurements from native and transplanted mussel (*Mytilus galloprovincialis*) in the canals of Venice. Survival in air and condition index. *Comparative Biochemistry & Physiology A* 140: 41–52.

Regoli F, Gorbi S, Machella N, et al. (2005) Pro-oxidant effects of extremely low frequency electromagnetic fields (ELFEM) in the land snail *Helix aspersa*. *Free Radical Biology & Medicine* 39: 1620–1628.

Reiter RJ, Tan DX and Galano A (2014) Melatonin reduces lipid peroxidation and membrane viscosity. *Frontiers in Physiology* 5: 377.

Richardson SD (2003) Water analysis: emerging contaminants and current issues. *Anal Chem* 75(12): 2831–2857.

Ringwood A, Polyachenko NL and Carroll D (2009) Fullerene exposures with oysters: embryonic, adult, and cellular responses. *Environmental Science & Technology* 43: 7136–7141.

Sabir S, Arshad M and Chaudhari S K (2014) Zinc oxide nanoparticles for revolutionizing agriculture: synthesis and application. *Scientific World Journal* 2014: 925494.

Sales J (2013) Effects of pharmacological concentrations of dietary zinc oxide on growth of post-weaning pigs: a meta-analysis. *Biological Trace Element Research* 152(3): 343–349.

Singh NP, McCoy MT, Tice RR, et al. (1988) A simple technique for quantification of low levels of DNA damage in individual cells. *Exp. Cell Res*. 175: 184–191.

Suman TY, Radhika Rajasree SR and Kirubagaran R (2014) Evaluation of zinc oxide nanoparticles toxicity on marine algae chlorella vulgaris through flow cytometric, cytotoxicity and oxidative stress analysis. *Ecotoxicology and Environmental Safety* 113C: 23–30.

Szabo S, Nagy L and Plebani M (1992) Glutathione, protein sulfhydryls and cysteine proteases in gastric mucosal injury and protection. *Clinica Chimica Acta* 206: 95–105.

Trevisan R, Delapedra G, Mello DF, et al. (2014) Gills are an initial target of zinc oxide nanoparticles in oysters *Crassostrea gigas*, leading to mitochondrial disruption and oxidative stress. *Aquatic Toxicology* 153: 27–38.

Vale G, Franco C, Diniz MS, et al. (2014) Bioavailability of cadmium and biochemical responses on the

freshwater bivalve Corbicula fluminea – the role of $TiO_2$ nanoparticles. *Ecotoxicology and Environmental Safety* 109: 161–168.

Wang J, Deng X, Zhang F, et al. (2014) ZnO nanoparticle-induced oxidative stress triggers apoptosis by activating JNK signaling pathway in cultured primary astrocytes. *Nanoscale Research Letters* 9(1): 117.

Weinstein DM, Mihm MJ and Bauer JA (2000) Cardiac peroxynitrite formation and left ventricle dysfunction following doxorubicin treatment in mice. *The Journal of Pharmacology and Experimental Therapeutics* 294: 396–401.

Wu S, Wu E, Qiu L, et al. (2011) Effects of phenanthrene on the mortality, growth, and anti-oxidant system of earthworms (*Eisenia fetida*) under laboratory conditions, *Chemosphere* 83(4): 429–434.

Yang X, Shao H, Liu W, et al. (2015) Endoplasmic reticulum stress and oxidative stress are involved in ZnO nanoparticle induced hepatotoxicity. *Toxicology Letters* 234(1): 40–49.

Zhang DX and Gutterman DD (2007) Mitochondrial reactive oxygen species-mediated signaling in endothelial cells. *American Journal of Physiology*. *Heart and Circulatory Physiology* 292: 2023–203110.