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Impacts of ZnO nanoparticles on growth and antioxidant enzymes of the green alga *Scenedesmus obliquus*

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Abstract

The growing passion for new industrial technology, using innovative materials such as nanoparticles (NPs) which reached water bodies as wastewater, heavily influenced aquatic organisms and consequently the human life. Physiological characteristics of the micro-green alga *Scenedesmus obliquus* can be used as biomarker to evaluate the effects of the newly invited zinc oxide nanoparticles (ZnO NPs). *S. obliquus* was exposed to different concentrations of ZnO NPs. The recorded results clarify not only an obvious inhibition in growth but also disturbance in the production of the tested antioxidant enzymes, where both Glutathione reductase (GR) and Glutathione peroxidase gradually increase with ZnO NPs concentration giving its maximum within 100 μg Zn NP.L^{-1} at the end of the experiment (562 and 0.42 μg.μg^{-1} respectively). However, in the case of Glutathione (GSH), Glutathione S-transferase (GST) and catalase (CAT) the gradual increase recorded only during the first 24 h, while Superoxide Dismutases (SOD) started to decrease within concentration of 50 μg L^{-1}ZnO NPs. Scanning electron microscope clarifies abnormalities in the cytomorphological characteristics of the treated cells where the cells tended to aggregate in a cluster and take elongated and spindle-shaped and/or wrapping. The various responses to ZnO NPs concentrations reflected, the disposal of ZnO nanoparticles in the environment affecting growth, morphological, and physiological characteristic of the cell.

Keywords: *ZnO nanoparticles, Scenedesmus obliquus, Antioxidant, Cytomorphology*

1. Introduction

The world as a whole innovates and develops technology to satisfy human needs, this development always accompanied by the presence of new materials. Technological attention focuses on the development of nanotechnology applications by the demonstration of different quantum size effects in nanoscale particles, where it expected that nanomaterials will be the key part in manufacturing most of the future devices (Barhoum and Makhlouf, 2018). Nanoparticles are picking up the global interest, due to their unique characteristics (Jeevanandam *et al*., 2018). A zinc oxide nanoparticles (ZnO NPs) is one of the most commonly utilized nanoparticles. Thus, ZnO NP was highlighted as a subject of many research papers during previous years and produced in large quantities everywhere in the range of 100 and 1,000 times more than other nanomaterials

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(Piccinno *et al*., 2012). Due to their large surface area, chemical stability, strong adsorption ability, long service life, and anti-friction that enhance its efficiency, ZnO NPs have been intensively used in many fields (Ovais *et al*., 2019). In addition, ZnO NPs have a broad range of applications in many industries such as the production of chemicals, rubber, ceramic, fibers, electronics and paint beside some medical uses (Hou *et al*., 2018; and Bhuvaneshwari *et al*., 2018). As regards, the inevitable fate of this mania in use, the ZnO NPs will certainly reach the aquatic environment through wastewater. Consequently, potential release and discharge of ZnO NPs represent a risk for living organisms in water, soil and sediments (Manzo *et al*., 2010) and subsequently on human health (Hou *et al*., 2018). Increased human blood viscosity, damage of liver, spleen, pancreas and even brain can be caused by exposure to ZnO NPs.

Biomarkers are biological parameters measuring, physiology, behavior, cell integrity, biochemistry and gene structure of organisms in response to ecological changes (Mofeed and Mosleh, 2013; Mofeed and Abdel-Aal, 2015; and Mofeed and El-Bilawy, 2020). Algae are the primary producers in the food chain, where phytoplankton providing food for diverse communities of invertebrates and fishes in the aquatic ecosystem. Moreover, microalgae are a source of various compounds that can be utilized in numerous fields (Abdel-Aal *et al*., 2015; Abdel-Aal and Mofeed, 2015; Mofeed, 2019; and Deyab *et al*., 2020). Freshwater algae, such as different species of *genus Scenedesmus*, are frequently used as helpful test organisms in ecotoxicological researches, due to their universal dissemination and their rapid physiological response to changes in aquatic ecosystem (Mosleh and Mofeed, 2014; and Mofeed, 2017). Hence, it often provides one of the first signals of environmental problems, clarifying the approximate effect of heavy metals contamination on aquatic species (Fodorpataki *et al*., 2009; and Mofeed, 2017).

During typical cell metabolic reactions, highly reactive compounds called free radicals are produced in the cell. These compounds are unstable as they have one pair of free electrons, consequently, become highly reactive. Where, they react with cellular molecules such as carbohydrates, lipids and proteins and then denature them. Therefore, this oxidative stress causes damage to vital cellular structures and functions. Antioxidant enzymes have the ability to deactivating the free radicals before they attack cellular components, to maintain cell stability. In general, most heavy metals can encourage the production of reactive oxygen free radicals in cells, and accordingly promote the production of antioxidative defenses enzymes such as catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR) (Mosleh and Mofeed, 2014) and Superoxide Dismutases (SOD) beside glutathione (GSH). Where, these enzymes act to minimize the damage of those harmful free radicals by giving up some of their electrons, or by interrupting with the oxidizing chain reaction.

It can be concluded that, physiological parameters are useful tools to evaluate the effects of nanoparticles, as a group of the newly invited pollutants, on cell viability and consequently its potential impact on the aquatic environment, aiming to minimize the possible injuries to the biota by establishing maximum acceptable levels of ZnO NPs. However, the effect of ZnO NPs on bioactivity, physiology and cytomorphology of organisms of most aquatic organisms is still in its nascent phase due to lack of sufficient information. So, the core intent of the present work was to study the sensitivity of the antioxidative defenses enzymes in *S. obliquus* that exposed to different concentrations of ZnO NPs.

2. Materials and methods

2.1. Nanoparticle

ZnO NPs produced by Nano Gate Company. The size of used nanoparticles was 20 ± 3 nm, with spherical like shape. A 250 $\mu g.L^{-1}$ stock solution was used for preparing appropriate concentrations.

2.1.1. Preparation of nanoparticle

Series of ZnO NP concentrations (10, 25, 50, 75, 100 µg.L^{-1}) was prepared by suspending ZnO NPs in bidistilled water.

2.2. Growth conditions and treatments

S. obliquus used in the present study was obtained from the algological collection of the National Institute of Oceanography and Fisheries. The algal strain was stored under the reference number NIOF-116. *S. obliquus* was cultivated in mineral growth medium (Couderchet and Böger, 1993) in temperature-controlled conditions at 25 ± 1 ºC and pH 6.3. The culture of *S. obliquus*, was with cell density of 10^6cells.ml^{-1}, was used during the exponential growth phase. Under sterile conditions, a total volume of 100 ml algal sample was inoculated in 250 ml volumetric flask. The tested algal species (*S. obliquus*) was exposed to different concentrations of Zn ONPs (control, 10, 25, 50, 75 and 100 $\mu g.L^{-1}$) for 24, 48 and 72 h. *S. obliquus* was placed on an orbital shaker at 130 rpm to avoid sticking; under continuous illumination of 65 μmol m^{-2} s^{-1}provided by white fluorescent

lamps (FORA 50 W) and continuously aerated with filtered air. Measurement of cell density for each sample was done in triplicate for each tested concentration of ZnO NPs in order to monitor the change of cell density Spectrophotometeric at 750 nm by using a Win Aspect plus (07745 Jena-Germany) Spectrophotometer, a standard curve was determinedto evaluate the algal growth.

2.2.1. Growth inhibition

The growth inhibitory effect was studied according to OECD (2011) method during the entire period of the experiment.

2.3. Estimation of total chlorophyll

Chlorophyll content of the tested alga was determined spectrophotometrically at optical densities (OD) at 663 and 645 nm according to Dere *et al*. (1998).

2.4. Enzyme assays

The algal cultures were incubated in medium supplied with the different ZnO NPs concentrations, under the conditions described above for the determination of enzyme activities. *S. obliquus* samples were collected, and the enzymes extracts were obtained after centrifugation (5 min, 4000 rpm; 8 ºC). The algal pellet was resuspended in 250 ml of sodium phosphate buffer (0.1 M, pH 7) and was ground with some fine glass beads in amalgamator for 2 min, and washed by potassium phosphate buffer (50 mM, pH 7.5). Enzyme extracts were then collected and centrifuged for 25 min at 1500 rpm (at 5 ºC). Total soluble protein was determined according to Bradford (1976).

The activity of GR was determined as a decrease in NADH concentration (Park *et al*., 2008) and expressed as micromoles per minute per milligram of protein. While, the CAT activity was determined spectrophotometrically by following the consumption of H_2O_2 (Lars *et al*., 1988). GSH was measured spectrophotometrically at 420 nm as described by Ren *et al*. (2003). SOD activity was determined by the photochemical method according to Bayer and Fridovich (1987). GST activity was assayed spectrophotometrically as the increase of absorbance at 340 nm due to the conjugation of GSH as described by Adachi *et al*. (1980). Glutathione peroxidase (GPx) activity was measured according to the method proposed by Paglia and Valentine (1967), where one GPx unit is defined as the amount of enzyme that produces 1 μmol min^{-1} oxidized guaiacol under the experimental conditions.

2.5. Scanning Electron Microscope (SEM)

The surface morphological characteristics of both the untreated (control) and treated *S. obliquus* cells that exposed to ZnO NPs (by concentration: 100 $\mu g.L^{-1}$) at the end of the exposure period were examined by using JEOL JSM 6510 SEM, according to Erdos (1986). Where, algal suspensions were centrifuged at 4000 rpm for 1 h at 25 ºC. After that, algal cells were chemically fixed for 2 h using 2.5% glutaraldehyde and then washed three times by PBS. Subsequently, the samples were dehydrated in a graded ethanol series (30, 50, 70, 90 and 100% twice), washed with isoamyl acetate and all of the samples after each step above were centrifuged at 8000 rpm for 5 min at 4 ºC. Finally, the samples dried under vacuum for 12 h. Images were obtained using the JEOL JSM 6510 SEM.

3. Statistical analysis

All experiments were performed in triplicates and repeated three times. Data presented in this study are the means ± standard deviation (SD). Significant differences between controls and contaminated samples were determined by the Mann and Whitney test and p-values < 0.05 were considered significant (*). All the statistical analysis was carried out using Sigma Stat 2.03 (SSCP Inc.) for Windows.

By using the MVSP program Cluster analysis was performed, where it is a multidimensional analysis clarifying the similarity between parameters (Legendre and Legendre, 1998).

4. Results

Anent, the recorded cell density of *S. obliquus* during the investigation period revealed that, the increase in the concentration of ZnO NPs was accompanied by a decrease in cell density. This growth inhibition was encouraged with time compared to the control, which acquired the maximum growth (Figure 1). Where, the maximum growth was recorded with the high ZnO NPs concentration (100 μg ZnO NP .L – 1). In more or less phenomena, total chlorophyll of *S. obliquus* was deeply affected by both the increase in ZnO NP

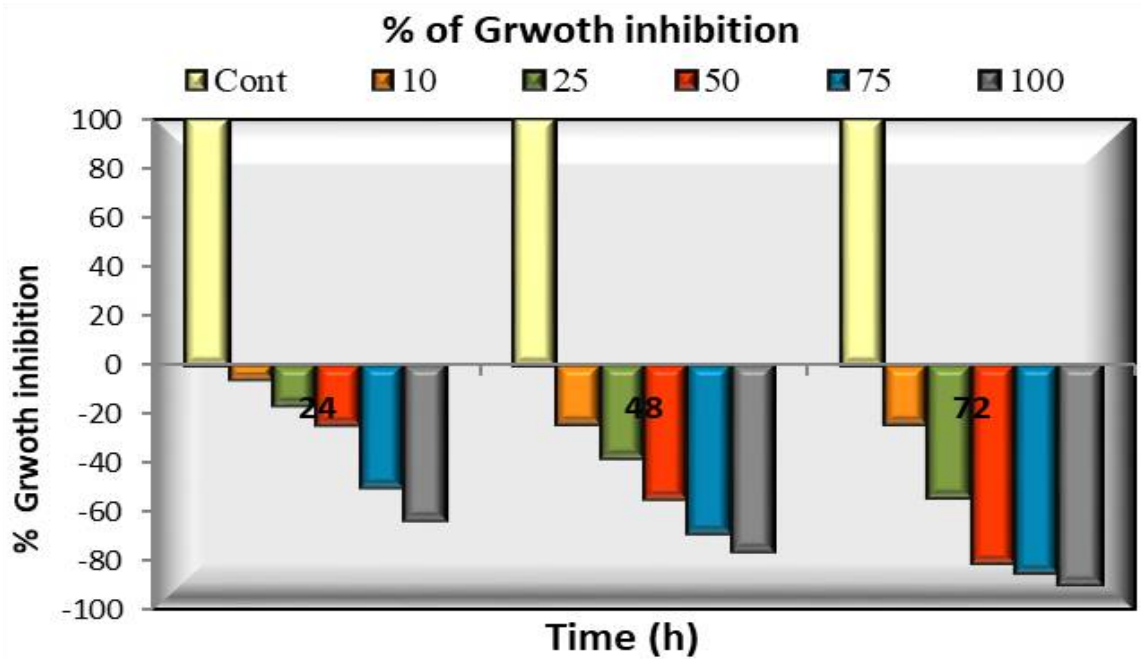


Figure 1: Inhibition in cell density of *S. obliquus* exposed to different concentrations of ZnO nanoparticles (*µ*g Zn NPs.L^{-1}) compared to control over time (*n* = 3)

concentrations and the exposure period, especially after 48 and 72 h. Inspection of figure 2 revealed that, the maximum productivity of total chlorophyll during the inter period of investigation obtained within the control (0.75 – 0.9 *µ*g.ml-1). In this context, any increase in ZnO NPs concentration was accompanied by a decrease in total chlorophyll content. Where, the minimum chlorophyll value (0.15 *µ*g.ml – 1) was recorded within the

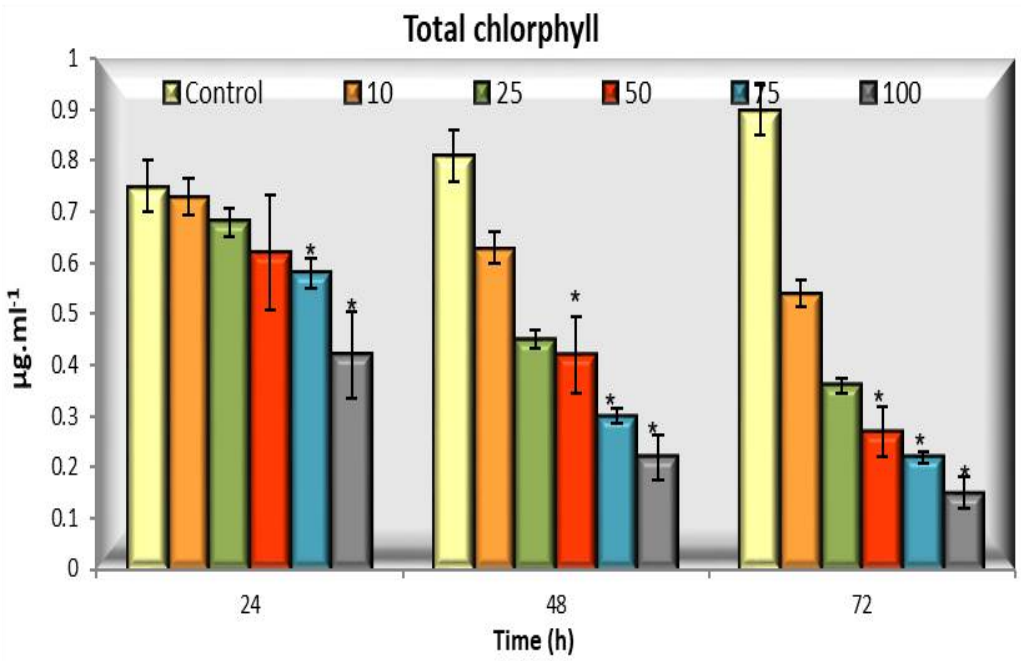


Figure 2: Total chlorophyll of *S. obliquus* exposed to different concentrations of ZnO nanoparticles (*µ*g Zn NPs.L^{-1})

Note: * Significantly different from control at $p < 0.05$ in a Student-Newman–Keuls test ($n = 3$).

maximum ZnO NPs concentration (100μg ZnONP.L – 1) after 72 h. Paradoxically, an inspection of figure (3-a) revealed, the gradual increase in GSH of *S. obliquus* with the tested ZnO NPs concentrations during the first 24 h, giving its maximum GSH value (3.8 μg.ml^{-1} protein) with 100 μg ZnO.L^{-1} concentration. The same trend continued after 48 and 72 h except within the maximum concentration where the GSH decrease (3.5 and 3.1 μg.mg^{-1} protein,respectively). While, the maximum values (4.4 and 4.1 μg.mg^{-1}protein after 48 and 72 h respectively) were recorded within concentration 75 μg ZnO NP L^{-1}. A glance on figure (3-b) revealed that, GST follow the same trend of GSH, giving its maximum (1152 μg.mg^{-1}protein) within 75μg ZnO NP L^{-1}concentration after 72 h. It is noticeable that, the high Zn concentrations reduce the production of both GSH and GST in the cell after 48 and 72 h.

In this context, CAT enzyme again gradual increase with concentrations gradient after 24 h, while both 50 and 75 μg ZnO NP.L^{-1} support the maximum CAT activity (113 mM H_2O_2.ml^{-1}) after 48 h figure (3-c). It is of interest to mention that, after 72 h the superiority in the motivation of CAT activity referred to 50 μg ZnO NPL^{-1}only, where it reached (118 mM H_2O_2.ml^{-1}). While, the high ZnO concentration suppressed CAT activity after 48 and 72 h. It is noticeable from figure (3-d & e) that, both GR and GPx in *S. obliquus* cell was gradually increased with concentrations gradient with a more or less the same pattern during the inter period of investigation, giving its maximum activity (562 and 0.42 μg.μg^{-1} protein respectively after 72 h of exposure) within the high ZnO NPs concentrations. As it was illustrated in figure (3-e), it is of interest to mention that, the increase in GPx concentration significantly induced with time during the entire period of investigation

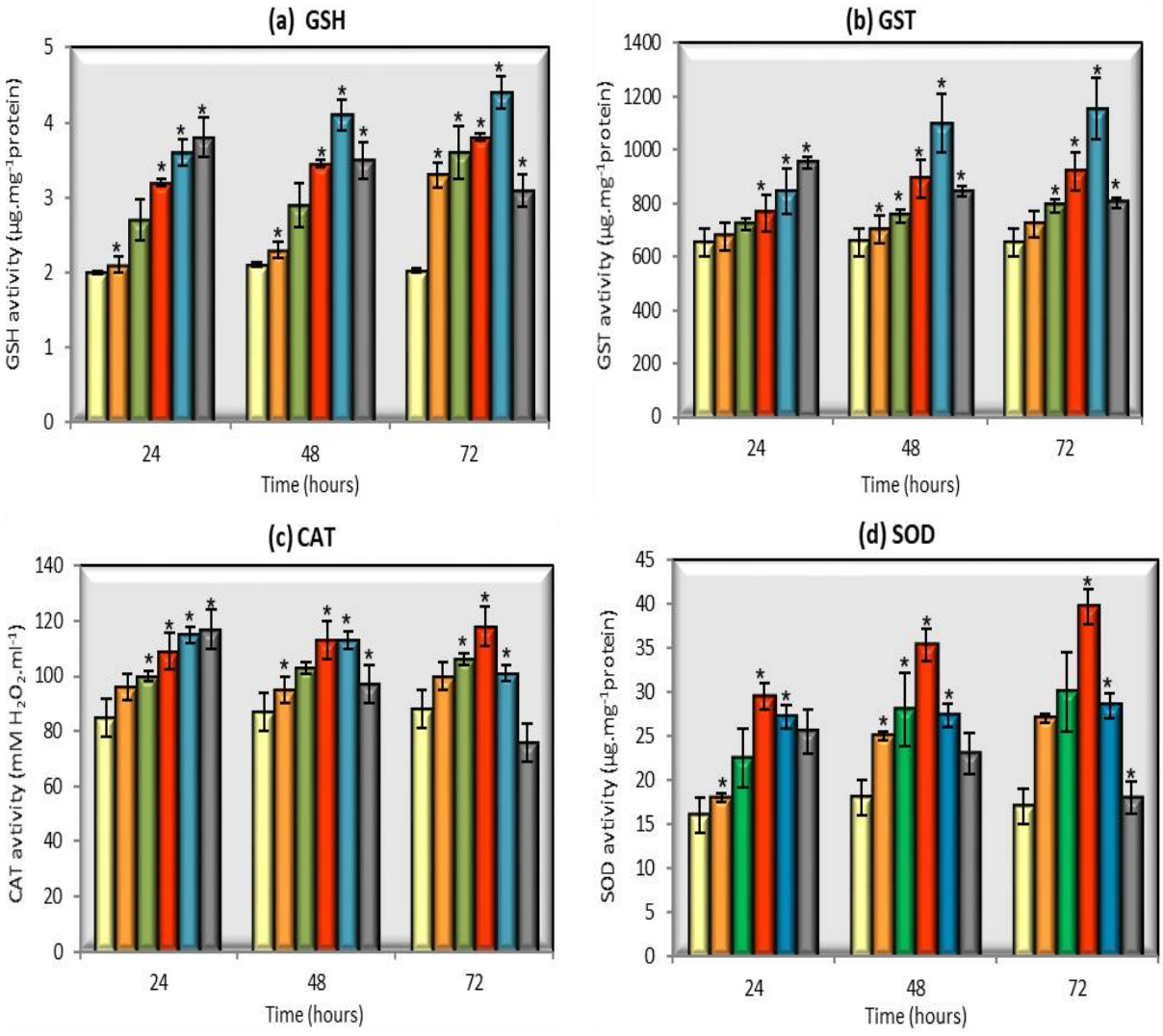
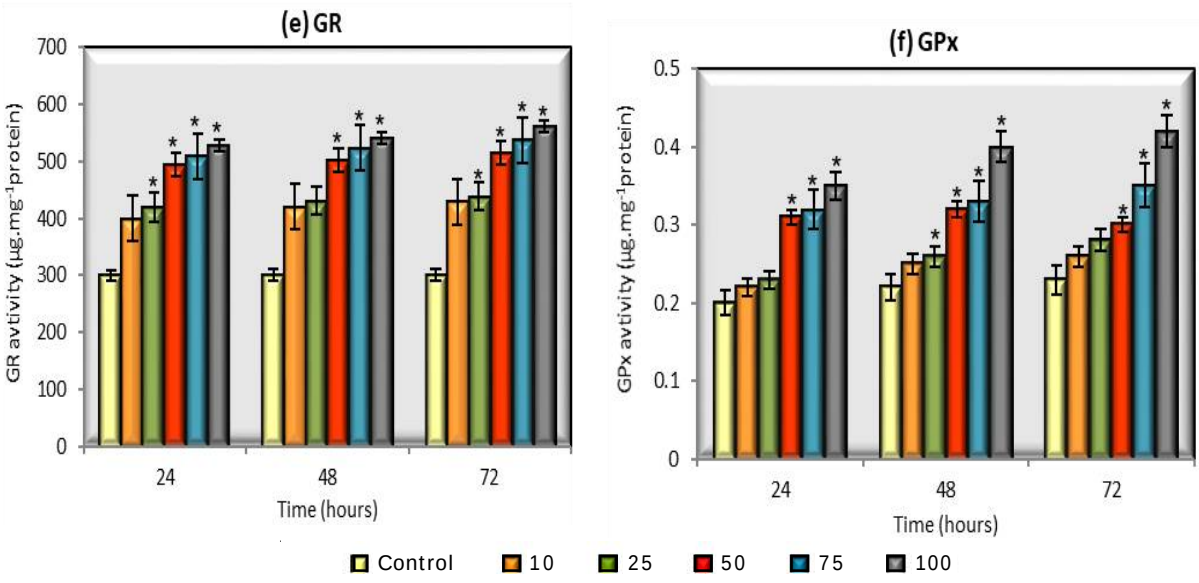


Figure 3: Changes in antioxidant enzymes. activities: (a) GSH; (b) GST; (c) CAT; (d) SOD; (e) GR and (f) GPx in *S. obliquus* exposed to different concentrations of ZnO nanoparticles (μg Zn NPs.L^{-1})



Note: * Significantly different from control at $p < 0.05$ in a Student-Newman–Keuls test ($n = 3$).

Figure 3 (Cont.)

especially, at high concentration. Both GR and GPx are the enzymes responsible for preventing cell damage due to the presence of free radicals like lipid peroxides and hydrogen.

In a different manner, the activity of SOD, reversely respond to the Zn NPs from the first 24 h, where the SOD increase with concentration gradient until 50 µg ZnO NP L^{-1} (29.5 µg.μg^{-1}protein) and then tended to decrease with the higher concentrations (27.1 and 25.5 µg.μg^{-1}protein within 75 and 100 µg Zn NP L^{-1} respectively) but not lower than the control (16 µg.mg^{-1}protein). In a significant variation, the concentration of 50 µg ZnO NP L^{-1}had the superiority in the stimulation of SOD activity after 48 and 72 h (35.3 and 39.7 µg.mg^{-1}protein respectively) over the higher concentrations. Generally, it is noticeable that the lower activities always recorded in the control.

The dendrogram produced by the Cluster analysis (Figure 4) summarized the similarity in response between the tested antioxidant enzymes in *S. obliquus* exposed to different concentrations of ZnO NPs, where it classified the enzymes into two sub-groups. Both GPx and GR were grouped together in minor sub-group with high

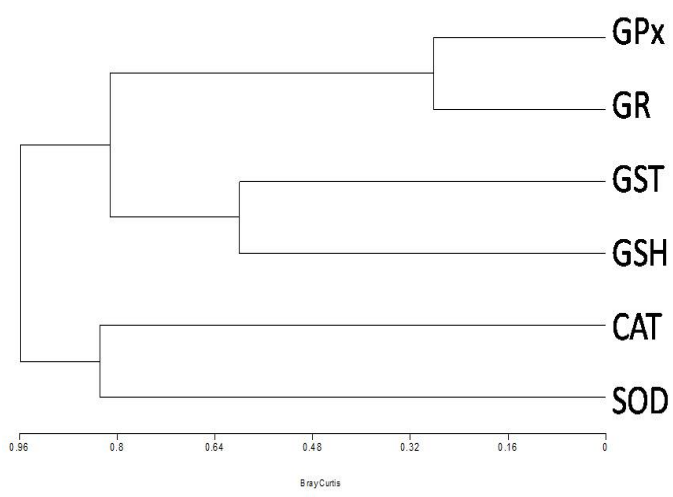


Figure 4: Dendrogram produced by the cluster analysis clarified the similarity between the tested antioxidant enzyme in *S. obliquus* exposed to different concentrations of ZnO nanoparticles (µg Zn NPs.L^{-1})

similarity, followed by that of GST and GSH. Meanwhile, CAT and SOD occupied the other sub-group with a high dissimilarity.

Taking into consideration the obtained result from SEM, in the present study, where both control and the treated *S. obliquus* cells (exposed to 100 μg.L^{-1} ZnO NPs) were examined by using SEM at the end of the exposure period. A glance on plate (1-a) reflected that, the algal cells kept intact and with the ideal cytomorphological characteristics of S. *obliquus* cell in the control with a normal range of length (12.01-12.3 μm) and width

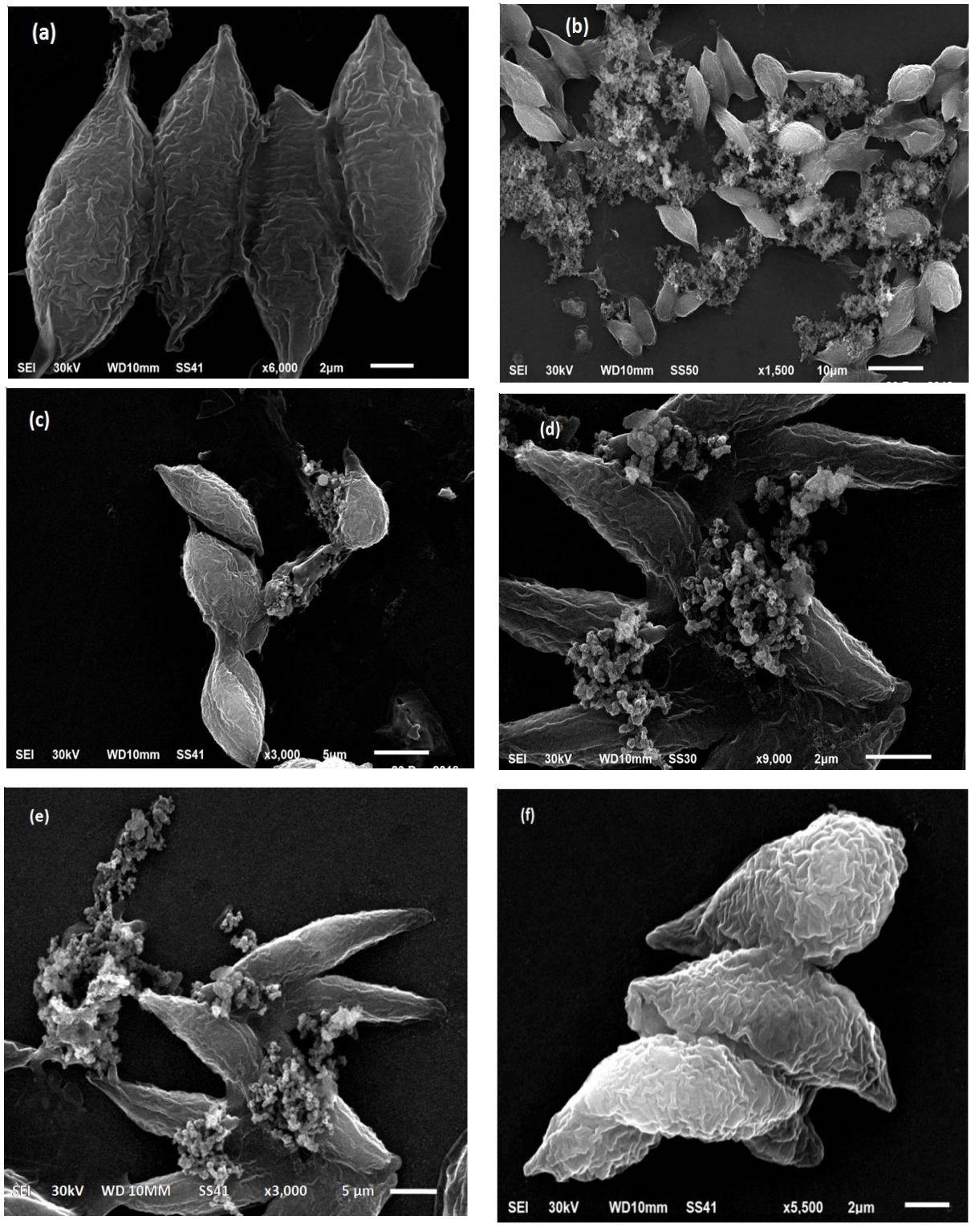


Plate 1: Scanning Electron Microscope (SEM) observation of the cytomorphology of *S. obliquus* of both (a) untreated (control) and (b, c, d, e and f) treated cells exposed to 100 mg.L^{-1} of ZnO NPs nanoparticles

(4.82-5.03 μm). However, as seen in plate (1-b) a number of ZnO NPs was adsorbed on the surface of *S. obliquus* cells and enhancing the cells to aggregate in a cluster. Meanwhile plate (1-c) clarifies the damage in treated algal cells and many free cells were present with abnormal cytomorphology. Focusing on the cell shape reveal that, the cells tended to take elongated and spindle-shaped with pointed ends (plate. 1-d and e) and/or wrapping the algal cells (plate 1-f) with length range from 19.31 to 22.1 μm and width from 4.01 to 4.53 μm.

5. Discussion

The increase in the concentration of ZnO NP was accompanied by a decrease in cell density of *S. obliquus*. The results, which in agreement with Tang *et al*. (2013), who indicated the growth of *Anabaena* sp. remarkably decreased with increasing Zn nanoparticle concentration. Moreover, Sibi *et al*. (2017) described that, the variation in microalgal growth in the presence of different metal nanoparticles depends on its concentration. Kumar *et al*. (2014) and Mosleh and Mofeed (2014) also investigated that, Zn concentrations already have observed negative an effect even with low concentrations on different microalgae. Meanwhile, Nguyen-Deroche *et al*. (2012) noted that, the effect of zinc supplementation on the algal cell varies from species to another, where cell density decreased in *Amphora* sp., and dramatically decreased in *Entomoneis paludosa*, while it increased in *Nitzschia palea*. In a comparative toxicity test between Nano-ZnO and bulk ZnO particles to the freshwater algae *Pseudokirchneriella subcapitata*, Aruoja *et al*. (2009) demonstrated that, Nano-ZnO had a higher level of toxicity than bulk ZnO. Bhuvaneshwari *et al*. (2018) in his review summarized that, acute toxicity of ZnO NP causes different responses with different spices.

Total chlorophyll of *S. obliquus* was deeply affected by both the increase in ZnO NP concentrations and the exposure period, where any increase in ZnO NPs concentration was accompanied by a decrease in total chlorophyll content, giving its minimum value after 72 h within the maximum ZnO NPs concentration. However, the presence of zinc in small amount may play an important role in the photosynthetic electron transport of oxygen in thylakoids within the cell, besides the participating in the enzymes synthesis, which eliminate the reactive oxygen species (ROS) such as, ascorbate peroxidase (APX) and SOD (Pinto *et al*., 2003). However, Mosleh and Mofeed (2014) described that, low concentrations of both zinc and copper act as micronutrients favoring some physiological activities and then supporting the algal growth. While, the higher concentration of Cu and Zn beside Cd reduced both carotenoids and chlorophyll. Miller *et al*. (2010) mentioned in his study about the toxicity of ZnO NPs on marine phytoplankton that, the uptake of zinc nanoparticles apparently inhibited the algal growth and chlorophyll content. High heavy metal concentrations can cause acute inhibition in photosynthesis, which both destroy the chloroplast of the cell and interrupt the physiological properties (Lamaia *et al*., 2005; and Mosleh *et al*., 2014). In this context, several studies on culture protocols were agreed that excess metallic nanoparticles, including zinc, reduce the chlorophyll production in marine diatoms (Nguyen-Deroche *et al*., 2012), *Chlorella vulgaris* (Kralova *et al*., 2004), *Synechococcus* (Chintamani and Mohanty, 1988) and *Pavlova viridis* (Li *et al*., 2007). Generally, high zinc nanoparticles concentration resulted in bleaching of photosynthetic pigments (Padmapriya and Anand, 2010; and Sibi *et al*., 2017) and finally degradation of cells (Mofeed, 2017). However, there is no specific reasonable hypothesis to suggest the way used by Zn to influence chlorophyll in the current state of our knowledge.

Beside the mentioned effects of ZnO NP on growth and chlorophyll content of the algal cell, the metal can enhance oxidative damage by increasing the ROS concentration in the cell (Winterbourn, 1982) by disturbing the antioxidant efficiency (Mofeed, 2015). SOD, GPx, CAT, lipid peroxidase, GST, GR peroxiredoxin, GPx and glutathione (GSH) considered as the main natural antioxidant enzymes (Mofeed and Mosleh, 2013). Glutathione (GSH) is responsible for protecting the important cellular components from damage by action of free radicals. The cited results showed a gradual increase in GSH and GST in *S. obliquus* with the tested ZnO NPs concentrations except within the maximum concentration after 48 and 72 h where the GSH decrease. Nagalakshmi and Prasad (2001) described that, the defense mechanisms, as antioxidant is responsible for the alteration between synthesis and utilization of GSH. It is worth to mention that, GSH is the most plentiful and widely distributed thiol-redox-derivative compound in the cell, which beside its important role as nonspecific reductant, it can act as a substrate for catalyzed enzymatic reactions, particularly in the highly oxidizing environment (Kishore and Mahajan, 2016). Where, GSH is powerful in the reduction of peroxides resulted during the partial reduction of oxygen. In a previous study on *Scenedesmus vacuolatus* exposed to $ZnCl_2$, the increase of free Zn^{+2} concentrations in growth media lead to a notable encouragement in GSH concentration in

comparison to control (Gaucher *et al*., 2018). In this connection, Kirubagaran *et al*. (2015), indicated that, the concentrations of GSH and GST of the algal cell usually increased significantly in response to metallic nanoparticle exposure to regulate protein synthesis and modulate the enzymatic activities.

As mentioned in the results, CAT again gradual increase with concentrations gradient after 24 h, giving its maximum activity within both 50 and 75 μg ZnO NP L^{-1} after 48 h, while the high concentration (100 μg ZnO NP L^{-1}) suppressed CAT activity after 48 and 72 h. Bhuvaneshwari *et al*. (2018) indicated that, CAT enzyme plays an important role as antioxidant especially at lower metallic nanoparticles concentrations on contrary to high concentrations. A more or less phenomena were reported by Srivastava *et al*. (2006) who added that, the activity of CAT enzyme depends on both concentration and exposure time.

In contradiction, the recorded results showed that, the heavy metals support the activities of both GR and GPx within all concentrations during the entire period of the experiment. Previous studies described that, in aquatic organisms, the oxidative stress and the production of ROS could be induced by heavy metals nanoparticles (Bhuvaneshwari*et al*., 2018).

One of the main defense lines against damage of free radicals is SOD, where approximately 70% of the measured total antioxidant activity attributed to the SOD activity (Hassan and Scandalios, 1990). In the recorded results, SOD increases with a concentration gradient until 50 μg ZnO NP L^{-1} and then tended to decrease with the higher concentrations. Interestingly, the SOD activity in the cell can serve as an indicator of pollution (Allen and Tresini, 2000; and Mofeed, 2015). Yilmaz and Sezgin (2014) described that; the cell can be protected by maintaining the steady-state of ROS by action of SOD and CAT. While Okamoto *et al*. (2001) mentioned that, in some marine dinoflagellate, zinc can induce SOD, APX and carotenoid levels only in low concentrations. In another study, *Anabaena variabilis*, Padmapriya and Anand (2010) reported that, a noticeable increase in SOD activity in cells exposed to low ZnO NPs concentrations, similar results were observed in *Chlorella vulgaris* (Kirubagaran *et al*., 2015).

Besides the physiological verifications, the SEM has been widely used to investigate any alter in morphological characteristics of algal cells due to exposure to NPs (Zheng *et al*., 2011). Chang *et al*. (2012) concluded that, ZnO NPs interrupt not only the growth but also the morphological characteristics, and the integrity of membrane in *Chlorella* sp, leading to mechanical cell damage. Concerning, the obtained result from SEM revealed many malformation and abnormalities in the cytomorphological characteristics of *S. obliquus* cells due to the exposure to ZnO NPs. Where the cells aggregate in a cluster and tended to take elongated and spindle-shaped with pointed ends and some cells were wrapping.

6. Conclusion

It is well known that, the global interest to use nanoparticles in many industries will expose the environment to high risk. The present study clarifies that, disposal of ZnO NPs in the environment poses danger influencing the growth, cytomorphological, and physiological characteristic of *S. obliquus* cell and consequently other aquatic organisms. Therefore, it needs more attention and precaution and more strict laws must be regulated and implemented for disposal of ZnO NPs in aquatic environments. Therefore, it is essential to improve a systematic design in order to restrict human exposure to lethal nanoparticles to safe levels. It is worthwhile that, antioxidant enzymes can be used as heuristic biomarkers to evaluate the ecological risk and toxicity effects of ZnO NPs to be used in the future as an efficient tool for risk assessment of ZnO NPs toxicity in real exposure scenarios.

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