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# Ecotoxicity of Ag-Nanoparticles to Microalgae

**Amal A. Hazani[1], Mohamed M. Ibrahim[2,3]*, Ibrahim A. Arif[3], Afaf I. Shehata[1], Gehan EL-Gaaly[1], Mohamed Daoud[4], Dalia Fouad[4], Humaira Rizwana[1] and Nadine Moubayed[1]**

[1]Botany and Microbiology Department, Science College, King Saud University, P.O. Box, 22452, Riyadh, 11495 Riyadh, Saudi Arabia.
[2]Alexandria University, Faculty of Science, Botany and Microbiology Department, P. O. Box 21511 Alexandria, Egypt.
[3]Prince Sultan Research Chair for Environment & WildlifeScience College, King Saud University, Riyadh, Botany and Microbiology Department, P.O. Box, 2455, Riyadh, 11451 Riyadh, Saudi Arabia.
[4]Department of Biochemistry, College of Science, king Saud University, P.O. Box 2455, Riyadh 11451 Riyadh, Saudi Arabia.

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**Increasing application of nanotechnology highlights the need to clarify and understand nanotoxicity. In this work, the sub-acute toxicity of silver nanoparticles (Ag-NPs) to fresh water microalga *Chlorella vulgaris* and marine microalga *Dunaliella tertiolecta* were assessed. To induce Ag-NPs effect we exposed both algae to various concentrations of Ag-NPs (0 - 200 mg/L). Cellular viability and reactive oxygen species (ROS) formation were determined to evaluate the toxic effect of Ag-NPs on algal growth. Superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities and lipid peroxidation (MDA) levels in algal cells were varied with concentration of Ag-NPs suspensions and exposure time (up to 8 d). As a result, 100 and 200 mg/L Ag-NPs caused statistically significant decrease in cell viability, as well as, SOD, CAT and POD activities and significant increase in ROS formation and MDA levels in tissues ($P < 0.05$), suggesting that the algal cells exposed to these two concentrations of Ag-NPs suffered from the oxidative stress. The extent of depletion of antioxidant enzymes activities and the elevation of MDA in the *D. tertiolecta* was the greatest, indicating that *D. tertiolecta* might be the most susceptible to Ag-NPs exposure. These results indicated a potential risk from Ag-NPs released into the aqueous environment.**

**Key words**: Antioxidant, Chlorella, *Dunaliella*, Nanoparticles, oxidative stress.

Nanotechnology manipulates matter at the nanoscale (1–100 nm) producing nanoproducts and nanomaterials (NMs) that can have novel and size-related physicochemical properties differing significantly from those from larger particles (Nel *et al*., 2006). The novel properties of NMs have been exploited widely for use in medicine (Barnett *et al*., 2007; Dong and Feng, 2007), cosmetics (Lens, 2009), renewable energies (Wei *et al*., 2008), environmental remediation (Tungittiplakorn *et al*., 2004), and electronic devices (Kachynski *et al*., 2008). Silver is considered relatively harmless to humans. Indeed, silver's bactericidal properties have been exploited by certain groups commercializing colloidal silver suspensions as 'health supplements'. The last decade is distinguished by the drastic growth of production and use of manufactured nanoparticles (NPs). NPs

* To whom all correspondence should be addressed.
E-mail: m_ibramim2004@yahoo.com; mibrahim@ksu.edu.sa

of metal oxides such as ZnO and $TiO_2$ are already widely used in personal care products (e.g., sunscreens), coatings and paints; CuO is used in gas sensors, photovoltaic cells, in catalyst applications and in heat transfer nano-fluids. Subsequently, the risk of natural water contamination by synthetic NPs continuously increases (Klaine *et al*., 2008). The environmental impacts of AgNPs are, as yet, unknown. However, previous knowledge on the environmental and physiological implications of exposure to dissolved silver ions and silver salts in freshwater and seawater organisms provides a baseline for assessment and a reason for concern; from this baseline the potential effects and impacts of AgNPs to organisms and to ecosystems can be developed. Prior to the interest in NPs, the silver ion ($Ag^+$ (aq)) was considered the most toxic form of silver in water (Ratte, 1999). As with all metals, the chemistry of the surrounding environment affects association of silver ions with various ligands, in turn influencing bioavailability and toxicity (Luoma *et al*., 2008; Adams and Kramer, 1998; Erickson *et al*., 1998). For instance, in freshwater systems organic matter and sulfide, with a high silver affinity, probably dominate Ag speciation and reduce silver bioavailability. In seawater systems the silver chloro complex is highly bioavailable and it is the primary form in waters of salinity greater than about 3 (Luoma, 2008; Luoma *et al*., 1995).

Algae species vary widely in their response to different toxic chemicals (Boyle, 1984). Park *et al*. (2010) reported that AgNPs have selective inhibitory effects on the harmful cyanobacterium *Microcystis aeruginosa* and this alga was more sensitive to AgNPs than green algae. Klaine *et al*. (2008) reported that major differences exist in the chemical behavior of nanoparticles in seawater compared to freshwater that will impact on the behavior of nanoparticles and therefore the habitats or organisms being exposed.

As the nanoparticles have a large surface area to volume ratio it is thought that AgNPs react strongly with compartments of the cell which may cause problems such as an increase in free radical production, causing oxidative stress, which may fatally damage the cells. Nanoparticle toxicity could be due to algae cell-wall. Perreault *et al*., 2011 compared aggregate formation in the wild type *Chlamydomonas reinhardtii* to its cell wall-deficient mutant exposed 48 h to glycodendrimer-coated gold NPs. They observed that the wild type *Chlamydomonas reinhardtii* formed large aggregates while no aggregates were observed when the cell-wall was lacking. It was reported that $SiO_2$ and $TiO_2$ NPs were able to interact directly with the algal cell surface through adsorption to the cell walls (Van Hoecke *et al*., 2008; Sadiq *et al*., 2011). Aggregate formation might reduce the light available to algal cells and thus inhibit their growth (Navarro *et al*., 2008; Perreault *et al*., 2011), or alter the cellular acquisition of essential nutrients by clogging to the walls (Wei *et al*., 2010). Nanoparticles affect different microorganisms and induce the generation of free radical which results in the deleterious effect on cellular functions. Oxidative stress is an important factor in nanoparticles-induced toxicity and parallel with induction of antioxidant defense system including non enzymatic compounds such as ascorbic acid, glutathione and Carotenoids. Antioxidant enzymes activities such as SOD, CAT and POD are very sensitive to the stress of pollutants and can be used as oxidative stressed signal for the early warning of environmental pollution (Nel *et al*., 2006).

Lipid peroxidation can be defined as the oxidative deterioration of cell membrane lipids and has been used extensively as a marker of oxidative stress (Sayeed *et al*., 2003).

In the present study, we aimed to make the toxicological assessment of Ag-NPs exposure to freshwater microalgae *Chlorella vulgaris* and marine microalga *Dunaliella tertiolecta*. The sub-acute toxicity effects on algae were examined including physiological responses by documenting the induction of reactive oxygen species formation and antioxidant enzymes in addition to the lipid peroxidation (MDA) level.

## Materials and Methods

### Algal culture

The fresh water microalgae *Chlorella vulgaris* and the marine green alga *Dunaliella tertiolecta* were obtained from the Culture collection for algae and protozoa (United Kingdom). The microalgae *Chlorella vulgaris* was grown in sterile BG-11 liquid medium and the stock culture was aerated with bubbling air (Rippka *et al*., 1979) and *Dunaliella tertiolecta* was cultivated in sea water growth medium according to McLachlan (1960).

The cells were grown under continuous and constant light intensity (100 mmol $m^{-2}$ $s^{-1}$, GRO-LUX Aquarium Wide Spectrum fluorescent lamps at 25 ºC). Aliquot of algal samples was used when cellular cultures were in their exponential growth phase. We note here that *Dunaliella tertiolecta* is a cell-wall lacking alga (Oliveira etal., 1980).

AgNPs characterization

Silver nanoparticles (Ag-NPs, particle size in 50 nm, a surface area of (30 ± 10) $m^2$/g, and crystal structure, with a purity > 97.0%) were purchased from Sigma-Aldrich Co., Ltd., USA. Ag-NPs suspensions were sonicated for 20 min in a bath type sonicator (100 W, 40 kHz) to disperse the particles. To investigate the suspension stability of Ag-NPs in the nutrient algal medium, different concentrations of Ag-NPs suspensions were prepared in triplicates. The Ag-NPs concentrations in aqueous solution were determined every day for 8 d according to the method introduced by Abdallah *et al*. (2012).

Oxidative stress parameters analysis

According to preliminary test results, the algae were exposed to 10, 50, 100 and 200 mg/L Ag-NPs for 8 d using a semi-static exposure test. The experiment was designed to allow for sub-lethal physiological effects over the exposure period. The exposure time of 8 d was chosen to enable some physiological or biochemical responses to the exposure. Algae from three flasks per treatment were randomly collected each at day 2, 4, 6 and 8, respectively for biochemical analysis. Algal cells were immediately snap-frozen in liquid nitrogen and stored at –20°C until needed. Viability of cells, as well as, the reactive oxygen species formation was measured and lipid peroxidation level was also measured for the content of malondialdehyde (MDA). All assays were preformed in triplicates.

The frozen cells were rinsed in 9-fold chilled 100 mmol/L, pH 7.8 sodium phosphate buffer solution and homogenized by a hand-driven glass homogenizer. The homogenates were centrifuged at 10000 ×*g* at 4°C for 20 min and the supernatant was stored in Eppendorf tubes at 4°C. The prepared supernatants were analyzed for antioxidant enzymes, i.e., superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities to determine possible effects on oxidative stress and antioxidant defense.

The SOD activity was estimated based on its ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated by riboflavin according to the method of Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the quantity of SOD required to produce a 50% inhibition of NBT reduction under the experimental conditions. The CAT activity was determined using the method of Beaumont *et al*.(1990) by measuring the initial rate of the decrease in absorbance at 240 nm as a consequence of $H_2O_2$ consumption over 1 min. Activity was expressed as a unit (one activity unit defined as absorbance at 240 nm changes 0.01 per min). The POD activity was assayed using guaiacol as a hydrogen donor by measuring the change at 470 nm over 1 min as reported previously by Chance and Maehly (1955). Enzyme activity is defined as unit (one activity unit defined as absorbance at 470 nm changes 0.01 per min) per gram fresh weight of tissue. Lipid peroxidation was measured using the thiobarbituric acid (TBA) assay by the method of Buege and Aust (1978). The level of lipid peroxidation was expressed as ¼mol MDA/ g fresh tissue.

**Determination of viable cells**

Fluorescein diacetate (FDA) is a non-polar ester that passes through cell membranes. Once inside the cell, FDA is hydrolyzed by esterase (an enzyme present in viable cells) to produce fluorescein, which accumulates inside viable cell walls and fluoresces under UV light (Regel *et al*, 2002). Viability of algal cells was estimated using the FDA method (Mayeretal, 1997). Each Ag NPs' treatment and control was treated with 5 mM of FDA in 1mL of solution. The fluorescence was measured using an excitation wavelength of 485 nm and an emission wavelength of 530nm.

**Determination of reactive oxygen species (ROS) formation**

22 ,72 -Dichlorodihydrofluorescein diacetate is a cell-permeable non-fluorescent probe. It is de-esterified intracellularly and turns to highly fluorescent 22 ,72 -dichlorofluorescein upon oxidation. 22 ,72 -Dichlorodihydrofluorescein diacetate is sensitive and rapid quantitation of oxygen-reactive species resulted from oxidative metabolism. ROS formation was measured qualitatively by using the cell permeable indicator 22 ,72 -Dichlorodihydrofluorescein diacetate (Gerber and Dubery,2003). Cellular esterases

hydrolyze the probe to the non-fluorescent 22 , 72 - dichlorodihydrofluorescein, which is better retained in the cells. In the presence of ROS and cellular peroxidases, 22 , 72 -Dichlorodihydro-fluorescein diacetate is transformed to the highly fluorescent 22 , 72 - dichlorofluorescein (DCF). Each AgNPs' treatment and control was treated with 5 mM of 22 , 72 -Dichlorodihydrofluorescein diacetate in 1mL of solution. The DCF fluorescence was measured using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All the fluorescence data were collected using a fluorescence plate reader.

**Statistical analysis**

Each treatment was replicated three times for statistical analysis. The results were expressed as mean ± standard deviation. The differences between the experimental and the control groups were tested for significance using one way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

## RESULTS

Several samples for different concentration of Ag-NPs were prepared to confirm the suspension stability of the nanoparticles in solution. According to our results, we can concluded that the most important factors affecting the suspension stability are the nanoparticle concentration, viscosity of base liquid and pH value (Data not shown).

**Reactive oxygen species production (ROS) after exposure to Ag NP**

Exposure of *Chlorella vulgaris* and *Dunaliella tertiolecta* culture to Ag-NPs for 8 days induced an increase in intracellular ROS concentrations throughout the experimental period. After 8 d exposure, ROS formation increased, respectively, in *Chlorella vulgaris* and *Dunaliella tertiolecta* by 68% and73% compared to control ($p \leq 0.05$) at 50 mg/L AgNPs (Fig.1). At 100 and 200 mg/L Ag NPs' ROS formation increased by 3.2 and 4.3 times for *Chlorella vulgaris* and by 4 and 6 times for *Dunaliella tertiolecta* compared to control at the end of experimental period ($p \leq 0.05$). Therefore, Ag NPs can lead to toxicological injury through the production of Cellular viability and reactive oxygen species (ROS).

**Viable cells**

Algal cells' viability for *Chlorella vulgaris* and *Dunaliella tertiolecta* was evaluated by fluorescein diacetate indicator (FDA), revealed that exposure of algae to 10–200 mg/L Ag NPs

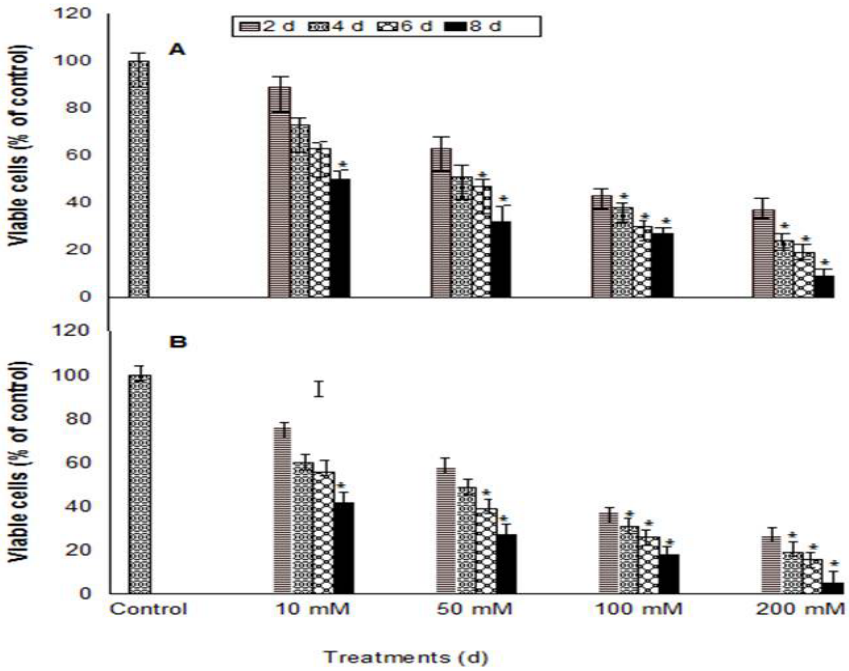


**Fig. 1.** Effect of various concentrations of AgNPs on reactive oxygen species level/ cell viability of *Chlorella vulgaris* (A) and *Dunaliella tertiolecta* (B) during 8 days of treatment

resulted in a highly significant reduction of viable cells compared to the control ($p<0.05$) (Fig.2 ). A great reduction in viable cell was observed throughout the experimental period especially with higher concentrations of Ag-NPs', 100 and 200 mg/L. After 2 days exposure, the frequency of oxidative stress indices in the exposed algae were elevated gradually with increasing Ag-NPs' concentration, and significant difference can be observed for the exposure to more than 100 mg/L Ag-NPs compare to the control (Fig. 2). Exposure of algae to 50 mg/L AgNPs' for 8 d induced a 68 % and 73% decrease of viable cells, respectively, for *Chlorella vulgaris* and *Dunaliella tertiolecta* compared to the control ($p<0.05$). Reduction of viable cells reached 91 % and 95 % at 200 mg/L Ag-NPs', respectively, for *Chlorella vulgaris* and *Dunaliella tertiolecta* after the same duration of AgNPs exposure.

**Oxidative stress and antioxidant defense**

In our study, there was obviously change in antioxidant enzymatic activities of *Chlorella vulgaris* and *Dunaliella tertiolecta* after algae exposed to the Ag-NPs concentration of 100 mg/L or higher. SOD activities in *Chlorella vulgaris* and *Dunaliella tertiolecta* treated with a various Ag-NPs' concentration and exposure time was expressed in Figure 3 A& B. Exposure to 10 mg/L Ag-NPs', SOD activities were stimulated and showed a significant increase, which might be due to the synthesis of new enzymes and/or the enhancement of pre-existing enzyme under lower concentrations. At 50 mg/L, the SOD activity initially increased and peaked at day 2 in both treated cells, and at day 6, then went down close to that of the control. This trend for the reduction of SOD activity might be an indication that the antioxidant defense systems of these algae were being stressed. However, at 100 and 200 mg/L Ag-NPs, there was a small rise at the beginning and then a sharp decrease in SOD activity, indicating that due to over-produced ROS and decreased defense capability, the SOD activity was inhibited. CAT and POD are also the key enzymes in antioxidant defense systems to convert the resulting free radicals $H_2O_2$ to water and oxygen. According to our results, CAT and POD activities in the two studied algal species fluctuated with a concentration and an exposure time, respectively, as shown in Figure 3 C-F. Exposure to 10 mg/L Ag-NPs, the CAT activity showed a slight decrease

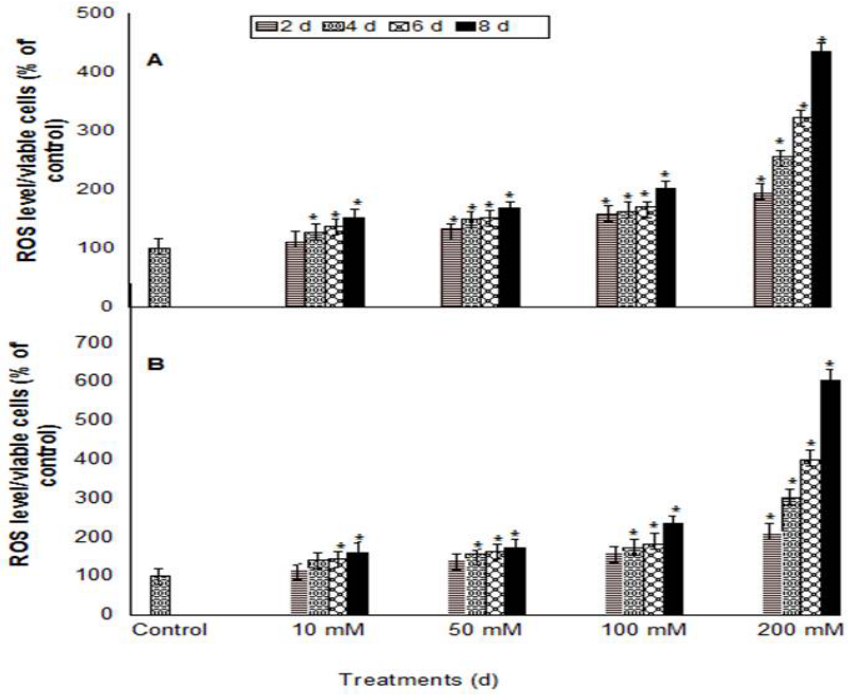


**Fig. 2:** Effect of various concentrations of AgNPs on cell viability of *Chlorella vulgaris* (A) and *Dunaliella tertiolecta* (B) during 8 days of treatment

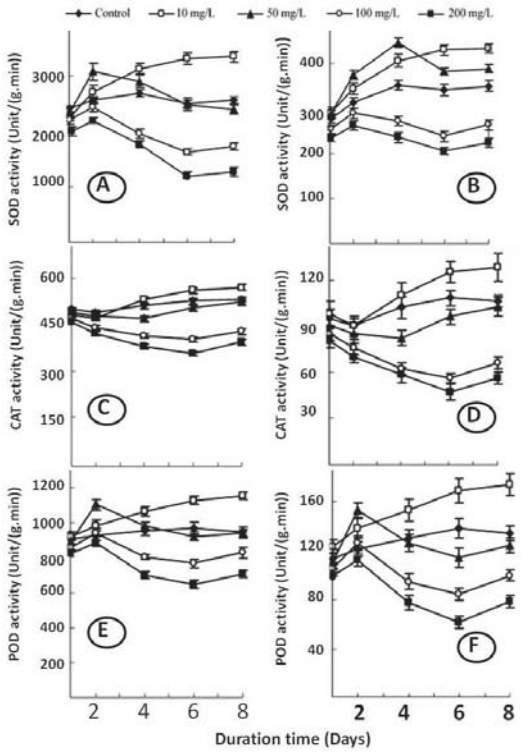


**Fig. 3:** Effect of various concentrations of AgNPs on antioxidant enzymes activities of *Chlorella vulgaris* and *Dunaliella tertiolecta*; SOD, A& B; CAT, C & D; POD, E & F during 8 days of treatment

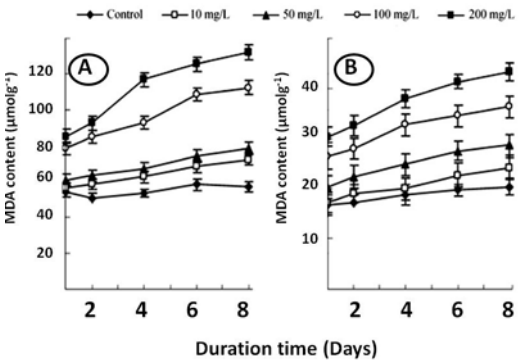


**Fig. 4:** Effect of various concentrations of AgNPs on malondialdehyde content (MAD) of *Chlorella vulgaris* (A) and *Dunaliella tertiolecta* (B) during 8 days of treatment

up to day 2 and then a remarkable increase. At 50 mg/L, the CAT activity slowed down until day 4, and then increased close to control level till the end of exposure time. However, 100 and 200 mg/L Ag-NPs caused a substantial decrease in CAT activity up to day 6 in both studied species. Results indicated that under the stress CAT activity was inhibited, and ROS scavenging weakened and accumulated gradually in the cells (Fig. 3 C &D). Similar to SOD, the POD activities followed a similar pattern in different tissues with a remarkable increase at lower concentrations of Ag-NPs and a considerable reduction at higher concentrations (Fig. 3 E &F). In addition, the CAT and POD activity in *Chlorella vulgaris* were 2–3 folds and 5–10 folds of that in *Dunaliella tertiolecta* at the same exposure concentration, respectively.

The lipid peroxidation indicated by MDA content in both studied species were not obviously different from those in control after exposed to 10 and 50 mg/L Ag-NPs, however, the significant increase in MDA level was found after 6 &8 d of exposure to 100 and 200 mg/L Ag-NPs (Fig. 4).

## DISCUSSION

Knowledge on nanoparticle physicochemistry, as well as biotic and abiotic factors affecting NPs behavior over time, is necessary for the fuller understanding of the mode of action of Ag NPs to cells. Indeed, initial results have shown a size and shape dependent interaction with prokaryotic organisms, with small (<10 nm) and truncated Ag NPs being the particles most likely to be taken up by microorganisms and affect cellular viability (Choi *et al*., 2010; Shrivastava *et al*., 2007). Other sizes and shapes appear to be less damaging to prokaryotes, suggesting that differences in shape and aggregate size could be responsible for some of the variability in observed toxicity, as metal composition remains the same. The toxicity of silver nanoparticles (AgNPs) to fresh microalgae *Chlorella vulgaris* and marine microalgae *Dunaliella tertiolecta* has been examined by measuring some oxidative stress indices such as reactive oxygen species level and lipid peroxidation indicated by malondialdehyde content, together with the measurement of cell viability. Reactive oxygen species (ROS) have been reported to affect the physiology, growth, and survival of algae (Di *et al*., 2013). Handy *et al*. (2008) reported that aggregation of nanoparticles in seawater is more likely than in freshwater and that the pH of the water may also influence the aggregation

rate depending on the surface charge of the particles involved.

The frequency of oxidative stress indices expressed by reactive oxygen species level as well as the cell viability in our studied algae were elevated gradually with increasing Ag-NPs' concentration, and significant difference can be observed for the exposure to more than 100 mg/L Ag-NPs compare to the control *Chlorella vulgaris* and *Dunaliella tertiolecta* cells exposed to the highest Ag-NPs concentration showed the indication of killing after short duration period. The phenomenon indicated that physiological changes of algal cells were affected by higher Ag-NPs' concentrations. Similarly, the abnormal behavioral changes with *Chattonella marina* exposed to Ag-NPs were reported previously (Di *et al*., 2012). Reduction of viable cells reached 91 % and 95 % at 200 mg/L Ag-NPs', respectively, for *Chlorella vulgaris* and *Dunaliella tertiolecta* after the same duration of AgNPs exposure. Other studies also showed the low toxicity of Ag-NPs' caused a highly significant reduction in the viability of algal cells (Abdallah *et al*., 2012).

Algae, like higher plants, possess well-developed antioxidant defense systems for neutralizing the toxic effects of ROS (Pandey *et al*., 2003). These defense systems include antioxidant enzymes (e.g., SOD, CAT and POD) and low-molecular weight, non enzymatic antioxidants (e.g., glutathione, GSH and ascorbic acid, ASA) (Vander *et al*., 2003; Ibrahim and Bafeel 2011).

The SOD-CAT-POD system provides the first defense against oxidative toxicity at a cellular level (Fang and Zheng, 2002). Super oxide dismutase (SOD) considered as the first enzyme to deal with oxyradicals and to catalyze the dismutation of superoxide radical $O_2^{\cdot}$ to $O_2$ and $H_2O_2$. The depletion of SOD activity is used as an indication of free radical scavenging ability, showing that the antioxidant defense system is overwhelmed by ROS , and oxidative stress occurred (Patra *et al*., 2009; Krishnaraj *et al*., 2012).

According to our results, the SOD activity in *Chlorella vulgaris* were 4–9 folds of that in *Dunaliella tertiolecta* at the same exposure concentration, showing that the *Dunaliella tertiolecta* might be the more sensitive algae to Ag-NPs exposure. Our results are consistent with the results obtained by Christian *et al*., 2013 who reported that the SOD activity was inhibited significantly in wheat plant after Ag-NPs' treatment and caused oxidative stress.

Variations of CAT and POD activity were not the same, but they were coordinated with each other and jointly played roles in antioxidant defense systems. Significant response of CAT and POD in *Dunaliella tertiolecta* again indicated that the *Dunaliella tertiolecta* might be the susceptible algae to Ag-NPs exposure. Similarly, Renault *et al*., 2008 showed that the CAT activity in two freshwater algal species was significantly induced after exposure to various concentrations of gold nanoparticles.

The oxidative deterioration of cell membrane lipids has been used extensively as a marker of oxidative stress and estimated by measuring the Malondialdehyde content. Our results indicated that *Chlorella vulgaris* and *Dunaliella tertiolecta* after exposure to different concentrations of Ag NPs' were undergoing oxidative stress, which was consistent with our results of higher concentration of AgNPs exhibiting more potent effects on disturbance to the antioxidant defense systems in algal cells. Similarly, Di *et al*., 2012 observed that AgNPs' increase reactive oxygen species and cause an orchestrated sequence of synergistic oxidative stress effect in algae.

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