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ECOTOXICITY OF AG-NANOPARTICLES ON TWO MICROALGAE, *CHLORELLA VULGARIS* AND *DUNALIELLA TERTIOLECTA*

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Abstract – The increasing application of nanotechnology highlights the need to clarify and understand it. In this work, the subacute toxicity of Ag-NPs to the fresh water microalga *Chlorella vulgaris* and marine microalga *Dunaliella tertiolecta* were assessed. The effect of Ag-NPs was induced by exposing both algae to increasing concentrations of Ag-NPs (0, 10, 50, 100 and 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 the algal cells varied with the concentration of Ag-NPs suspensions and exposure times (up to 8 d). As a result, 100 and 200 mg/L Ag-NPs caused a statistically significant decrease in cell viability, as well as SOD, CAT and POD activities, and a 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 oxidative stress. The extent of depletion of antioxidant enzyme activities and the elevation of MDA in *Dunaliella tertiolecta* was the greatest, indicating that *Dunaliella tertiolecta* might be the most susceptible to Ag-NP exposure. These results indicated a potential risk from Ag-NPs released into the aqueous environment.

Key words: Antioxidant, Chlorella, *Dunaliella*, nanoparticles, oxidative stress

INTRODUCTION

Nanotechnology manipulates matter at a nanoscale (1-100 nm), producing nanoproducts and nanomaterials (NMs) that can have novel and size-related physicochemical properties differing significantly from those of 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 dramatic growth in production and use of manufactured nanoparticles (NPs). NPs of metal oxides such as ZnO and TiO2 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 nanofluids. Subsequently, the risk of natural water con-

tamination by synthetic NPs continuously increases (Klaine et al., 2008).

The environmental impacts of Ag-NPs are as yet unknown. However, previous knowledge on the environmental and physiological implications of exposure to dissolved silver ions and silver salts in fresh- and seawater organisms provides a baseline for assessment and a reason for concern; from this baseline, the potential effects and impacts of Ag-NPs to organisms and 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 the 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 water with a salinity greater than about 3 (Luoma, 2008; Luoma et al., 1995).

Algae species vary widely in their responses to different toxic chemicals (Boyle, 1984). Park et al. (2010) reported that Ag-NPs have selective inhibitory effects on the harmful cyanobacterium *Microcystis aeruginosa*, and this alga was more sensitive to Ag-NPs 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 thereby the habitats or organisms being exposed.

As nanoparticles have a large surface area-to-volume ratio, it is thought that Ag-NPs react strongly with compartments within and outside of the cell, which may cause problems such as an increase in free radical production, causing oxidative stress that may fatally damage the cells. Nanoparticles' toxicity could be due to the algae cell wall. Perreault et al. (2011) compared aggregate formation in 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 the walls (Wei et al., 2010).

Nanoparticles affect different microorganisms and induce the generation of free radicals, which results in the deleterious effect on cellular functions. Oxidative stress is an important factor in nanoparticle-induced toxicity. Antioxidant enzyme activities such as SOD, CAT and POD, are very sensitive to the stress of pollutants and can be used as an oxidative-stress signal for an 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 assess the effects of exposure of freshwater microalgae *Chlorella vulgaris* and marine microalgae *Dunaliella tertiolecta* to Ag-NPs. The subacute toxicity effects on the algae were examined, including physiological responses, by documenting the induction of ROS formation and antioxidant enzymes in addition to the lipid peroxidation (MDA) level.

MATERIALS AND METHODS

Algal culture

The fresh water microalgae *Chlorella vulgaris* and marine green alga *Dunaliella tertiolecta* were obtained from the Culture Collection for Algae and Protozoa

(United Kingdom). The microalga *Chlorella vulgaris* was grown in sterile BG-11 liquid medium and the stock culture was aerated with bubbling air (Rippka et al., 1979); *Dunaliella tertiolecta* was cultivated in a seawater 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 °C). An 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 et al., 1980).

Ag-NP characterization

Silver nanoparticles (Ag-NPs, particle size 50 nm, surface area (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 water, different concentrations (5, 10, 25, 50, 100, 200 and 400 mg/l) of Ag-NP suspensions were prepared in triplicate. The Ag-NP concentrations in aqueous solution were determined every day for 8 days according to the method introduced by Zhang et al. (2006).

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 days using a semi-static exposure test. The experiment was designed to allow for sublethal physiological effects over the exposure period. The exposure time of 8 days was chosen to enable some physiological or biochemical responses to the exposure. Algae from three flasks per treatment were randomly collected 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. The viability of cells, as well as reactive oxygen species formation, was measured and the lipid peroxidation level was also measured for the content of malondialdehyde (MDA). All assays were preformed in triplicate.

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 enzyme, i.e., superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), activities to determine possible effects on oxidative stress and antioxidant defense.

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.

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 a 0.01 change in absorbance at 240 nm per min).

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 one unit (one unit of activity is defined as a 0.01 change in absorbance at 470 nm 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 μmol 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-NP treatment and the control was treated with 5 mM of FDA in 1 mL of solution. The fluorescence was measured using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Determination of reactive oxygen species (ROS) formation

2′,7′-Dichlorodihydrofluorescein diacetate is a cell-permeable non-fluorescent probe. It is de-esterified intracellularly and turns to highly fluorescent 2′,7′-dichlorofluorescein upon oxidation; 2′,7′-Dichlorodihydrofluorescein diacetate is used for the rapid quantitation of ROS in response to oxidative metabolism. ROS formation was measured using the cell permeable indicator 2′,7′-dichlorodihydrofluorescein diacetate (Gerber and Dubery, 2003). Cellular esterases hydrolyze the probe to the non-fluorescent 2′, 7′-dichlorodihydrofluorescein, which is better retained in the cells. In the presence of ROS and cellular peroxidases, 2′, 7′-dichlorodihydrofluorescein diacetate is transformed to the highly fluorescent 2′,7′- dichlorofluorescein (DCF). Each Ag-NP treatment and control was treated with 5 mM of 2′,7′-dichlorodihydrofluorescein diacetate in 1 mL 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 control groups were tested for significance using one way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

RESULTS

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 days of exposure, ROS formation increased in *Chlorella vulgaris* and *Dunaliella tertiolecta* by 68% and73%, respectively, compared to the control ($p \leq 0.05$) at 50 mM Ag-NPs (Fig.1). At 100 and 200 mg/L Ag-NPs, ROS formation increased 3.2- and 4.3-fold in *Chlorella vulgaris,* and 4- and 6-fold in *Dunaliella tertiolecta* compared to control at the end of experimental period ($p \leq 0.05$). Therefore, AgNPs can lead to toxicological injury through the production of cellular viability and reactive oxygen species (ROS).

Viable cells

Algal cell viability for *Chlorella vulgaris* and *Dunaliella tertiolecta* was evaluated by fluorescein diacetate (indicator FDA), revealing that exposure of the algae to 10-200 mg/L Ag-NPs resulted in a highly significant reduction of viable cells compared to the control ($p \leq 0.05$) (Fig. 2). A great reduction in viable cells was observed throughout the experimental period, especially with higher concentrations of Ag-NPs, 100 and 200 mg/L. After 2 days of exposure, the frequency of oxidative stress indices in the exposed algae was elevated gradually with increasing Ag-NP concentrations, and a significant difference can be observed for the exposure to more than 100 mg/L Ag-NPs compared to the control (Fig. 2). Exposure of algae to 50 mg/L Ag-NPs for 8 days induced a 68 % and 73% decrease of viable cells for *Chlorella vulgaris* and *Dunaliella tertiolecta*, respectively, compared to the control ($p<0.05$). Reduction of viable cells reached 91% and 95% at 200 mg/L Ag-NPs for *Chlorella vulgaris* and *Dunaliella tertiolecta*, respectively, after the same duration of Ag-NP exposure.

Oxidative stress and antioxidant defense

In our study, there was obvious change in the antioxidant enzymatic activities of *Chlorella vulgaris* and *Dunaliella tertiolecta* after their exposure to the Ag-NP concentration of 100 mg/L or higher. SOD activities in *Chlorella vulgaris* and *Dunaliella tertiolecta* treated with various Ag-NP concentrations

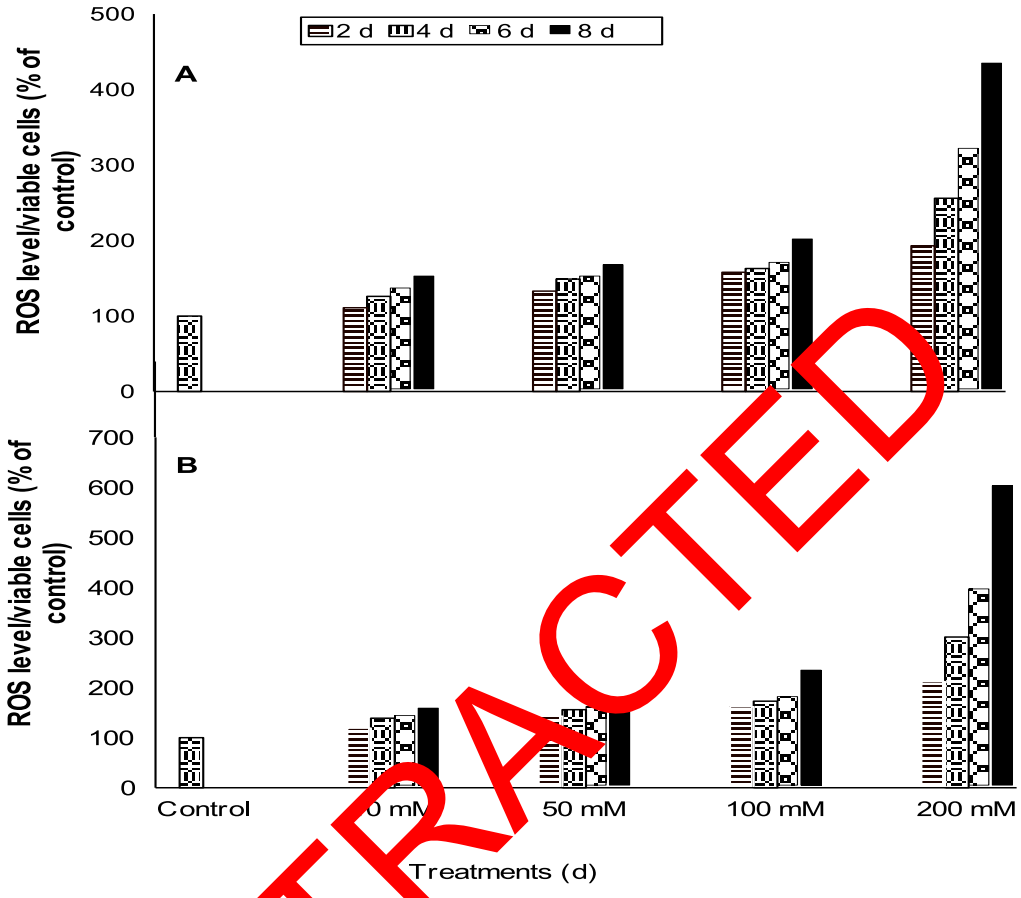

Fig. 1. Effect of various concentrations of Ag-NPs on reactive oxygen species level/cell viability of *Chlorella vulgaris* (A) and *Dunaliella tertiolecta* (B) during 8 days of treatment.

and exposure time are expressed in Figs. 3A and B. Exposed to 10 mg/L Ag-NP, SOD activities were stimulated and showed a significant increase, which might be due to the synthesis of new enzymes and/or the enhancement of a pre-existing enzyme under lower concentrations. At 50 mg/L, SOD activity initially increased and peaked on day 2 in both treated cells, and on day 6 decreased close to that of the control. This trend in the reduction of SOD activity might be an indication that the antioxidant defense systems of these algae were under stress. 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 an overproduction of ROS and decreased defense capability, the SOD activity was inhibited.

CAT and POD are also key enzymes in antioxidant defense systems for the conversion of 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 concentration and exposure time, respectively, as shown in Figs. 3C-F. After exposure to 10 mg/L Ag-NPs, CAT activity showed a slight decrease up to day 2 and then a remarkable increase. At 50 mg/L, CAT activity slowed down until day 4 and then increased close to control level until 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 gradu-

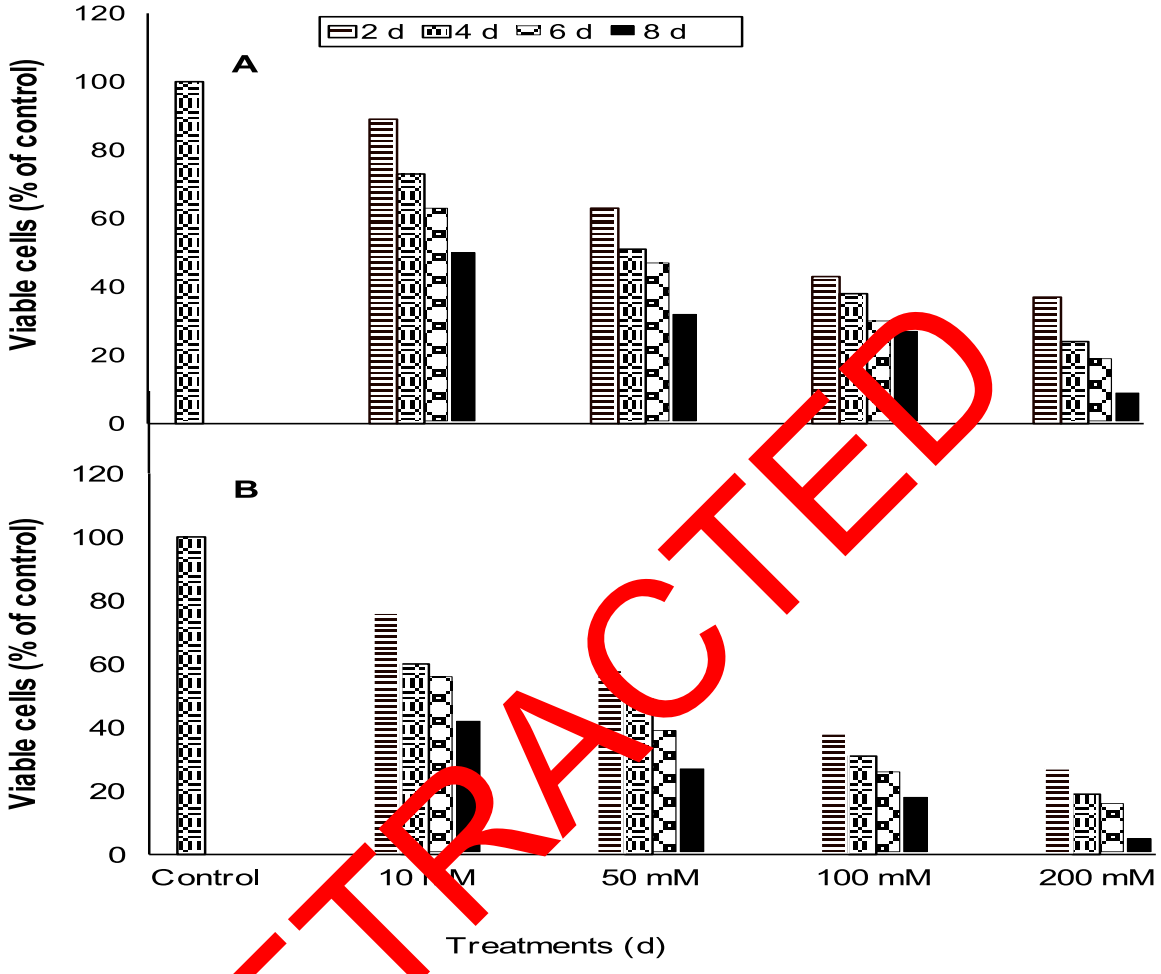

Fig. 2. Effect of various concentrations of Ag-NPs on cell viability of *Chlorella vulgaris* (A) and *Dunaliella tertiolecta* (B) during 8 days of treatment.

ally in the cells (Figs. 3 C & D). Like SOD, 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 (Figs. 3 E & F). In addition, the CAT and POD activities in *Chlorella vulgaris* were 2-3-fold and 5-10-fold higher than that in *Dunaliella tertiolecta* at the same exposure concentration, respectively.

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

DISCUSSION

Knowledge about nanoparticle physicochemistry, as well as biotic and abiotic factors affecting NP behavior over time, is necessary for a fuller understanding of the mode of action of Ag-NPs on 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

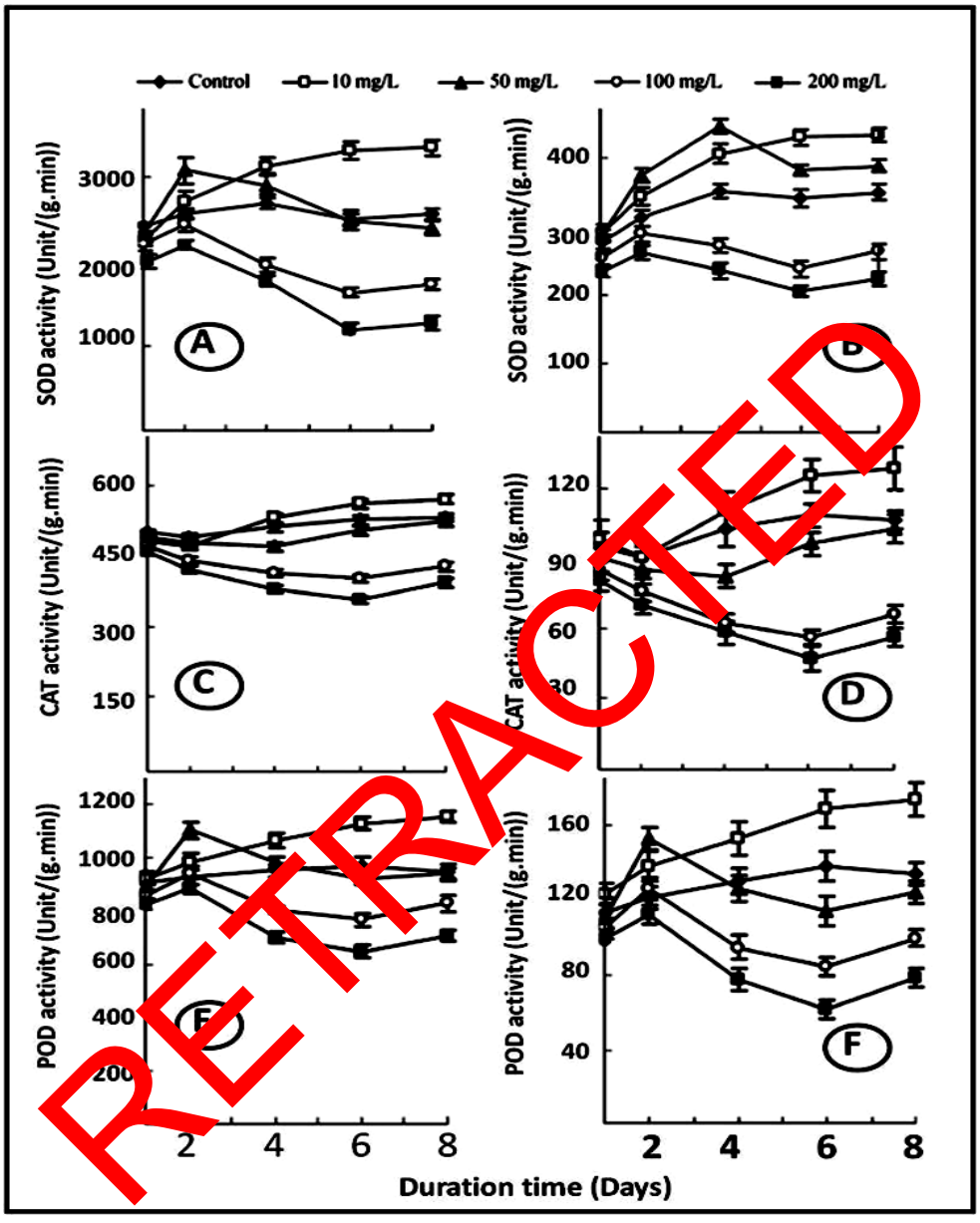

Fig. 3. The effect of various concentrations of Ag-NPs on antioxidant enzymes activities of *Chlorella vulgaris* and *Dunaliella tertiolecta*; SOD, A& B; CAT, C & D; POD, E & F during 8 days of treatment.

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 the metal composition remains the same.

The toxicity of silver nanoparticles (Ag-NPs) to fresh microalgae *Chlorella vulgaris* and marine microalgae *Dunaliella tertiolecta* has been examined by measuring some oxidative stress indices such as reactive oxygen species levels and lipid peroxida-

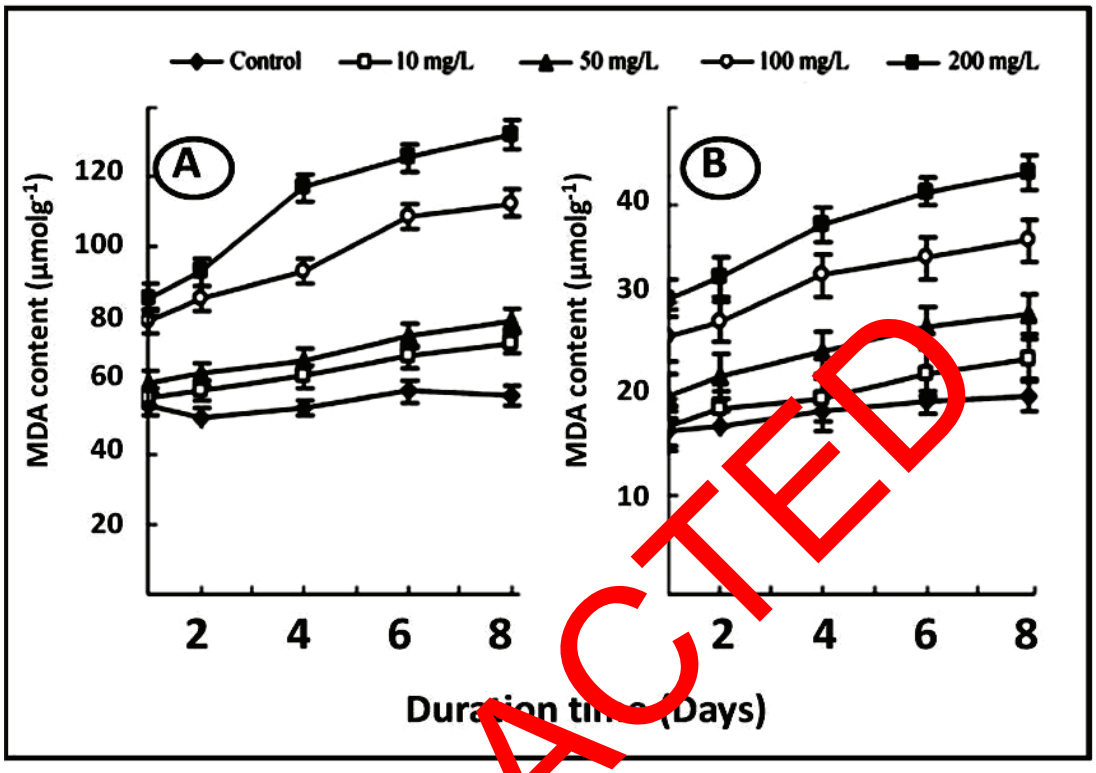

Fig. 4: The effects of various concentrations of Ag-NPs on malondialdehyde content (MAD) of *Chlorella vulgaris* (A) and *Dunaliella tertiolecta* (B) during 8 days of treatment.

tion 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 an 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-NP concentration, and a significant difference can be observed for the exposure to more than 100 mg/L Ag-NPs compared to the control. *Chlorella vulgaris* and *Dunaliella tertiolecta* cells exposed to the highest Ag-NP concentrations exhibited the signs of death after a short duration. The phenomenon indicated that physiological changes of algal cells were affected by higher Ag-NP concentrations. Similarly, the abnormal behavioral changes in *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 Ag-NP 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). SOD is considered the first enzyme to deal with oxyradicals and to catalyze the dismutation of superoxide radicals $O_2^{\cdot-}$ 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 that oxidative stress has occurred (Patra et al., 2009; Krishnaraj et al., 2012).

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

Variations of CAT and POD activities were not the same, but they were coordinated with each other and jointly played roles in the antioxidant defense systems. The significant response of CAT and POD in *Dunaliella tertiolecta* again indicated that *Dunaliella tertiolecta* might be the susceptible algae to Ag-NP exposure. Similarly, Renault et al., 2008 showed that 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, underwent oxidative stress, which was consistent with our results of higher concentration of Ag-NPs exhibiting more potent effects on the antioxidant defense systems in algal cells. Similarly, Di et al., 2012 observed that Ag-NPs increase reactive oxygen species and cause an orchestrated sequence of synergistic oxidative stress effect in algae.

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