

Growth, primary metabolites, and cell morphogenesis of *Scenedesmus opoliensis* in response to zinc oxide nanoparticles stress

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Abstract:

Zinc oxide nanoparticles (ZnO-NPs) are widely used in industrial and agricultural applications in addition to cosmetic products. However, the extensive use of ZnO-NPs can impose serious environmental problems on the aquatic ecosystem. So far, the hazardous impact of ZnO-NPs on phytoplankton is not well studied. Among myriad aquatic microorganisms, microalgae are sensitive indicators for water pollution. The current study investigated the toxic effects of different concentrations of ZnO-NPs (10-200 mg/L) on growth, metabolic profile, and morphology of the green microalga, *S. opoliensis*. The results revealed that ZnO-NPs significantly decreased cell growth, chlorophyll-a (Chl-a), and carbohydrate content with a concentration-dependent manner. In contrast, the protein and lipid contents were progressively increased after ZnO-NPs treatment compared to the control condition. The morphological examinations revealed obvious changes in the microalgal cell particularly at high ZnO-NPs concentrations. The present study provides a new report on the ecotoxicology of ZnO-NPs contamination on the aquatic ecosystem and highlights its toxic impact on *S. opoliensis*.

Keywords *S. opoliensis*; Toxicity; ZnO-NPs; growth; metabolites; morphological deformations.

Introduction

The global nanotechnology market is a fast-expanding field of science and technology, it is estimated at approximately US\$41.8 billion in 2020 (**BCC Research 2015**) and is expected to exceed to US\$ 125 Billion by 2024 (**Global Nanotechnology Market Report 2018**). Metal oxides such as ZnO-NPs are the

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third top annually manufactured nanoparticles (550 tons per year) after silica dioxide and titanium dioxide, respectively (**Piccinno *et al.*, 2012**). ZnO-NPs are widely used in various potential applications, the personal care products such as cosmetics and sunscreen (**Newman *et al.*, 2009**). In the agriculture field, ZnO-NPs are employed as Nanofertilizer (**Yusefi-Tanha *et al.*, 2020**), Nanofungicides (**Arciniegas-Grijalba *et al.*, 2019**), Nanopesticides (**Hamed *et al.*, 2019** and **Jameel *et al.*, 2020**).

Unfortunately, nanoparticles could be released into the aquatic ecosystem through industrial discharges, personal usage, and surface runoff from soils. The accumulation of these nanoparticles in the aquatic environment introduced new risks of toxicity to the ecosystem due to their small size and high reactivity (**Gupta and Xie 2018; Luo *et al.*, 2018** and **Nguyen *et al.*, 2020**). Nanoparticles can consequently be moved from the aquatic waterways and easily reach humans through the food chains by several processes (**Wang *et al.*, 2019**).

Microalgae are the primary producers of organic matter, which provide the food base for most marine and freshwater living organisms. Therefore, any alteration in microalgal growth, activity, and diversity induced by toxic contaminants led to consecutive disturbances in the complete food chain, and ultimately to the whole aquatic ecosystem (**Nowack and Bucheli 2007** and **Auffan *et al.*, 2011**). Thus, investigating the toxicity of nanoparticles on microalgae is an essential step to predict and develop a strategy for assessing their adverse impact on food webs and the whole ecosystem (**Manzo *et al.*, 2013**).

Microalgae and cyanobacteria are sensitive biological indicators for several xenobiotics and water pollutants (**Hamed *et al.*, 2020** and **2021**). They were demonstrated as ideal model organisms to study the metal oxide nanoparticles toxicity. Also, they can potentially be used as a biosensor to evaluate water quality and aquatic toxicity (**Cattaneo 2018; Espinasse *et al.*, 2018** and **Hamed *et al.*, 2020; 2021**). The green microalga, *Scenedesmus* is prevalent phytoplankton in the freshwater ecosystem. It has been used to study the effect of metal oxide nanoparticles on the aquatic system (**Vogs *et al.*, 2013**).

The present study aimed to investigate the effect of ZnO-NPs on growth profile, primary metabolites content, and morphological structure of the freshwater microalga *S. opoliensis*. The present investigation may also provide a

comprehensive overview of the adverse impacts of ZnO-NPs on food webs and water ecosystems.

Materials and Methods

1. Preparation of ZnO-nanoparticles

ZnO-NPs were phyco-synthesized using the aqueous extract of the brown macroalga *Cystoseira crinite* with ZnSO₄, 0.05 M (Elrefaey *et al.*, 2021). A stock solution of ZnO-NPs was prepared by dissolving 1 g of the bio-fabricated ZnO-NPs in 100 ml double-distilled water (DDW) (using ultrasonic (Emmi-12HC, Germany) for 15 min to get a homogenous solution.

Culturing of *Scenedesmus opoliensis* and toxicity assay test

The green microalga, *S. opoliensis* was obtained from the Algae Culture Collection at Al-Azhar University (ACCAZ), Cairo, Egypt. The microalgal strain was cultivated in sterilized Z-medium (Staub 1961) and was illuminated by the cool white fluorescent lamp (5000 Lux), under 12 Hrs.:12 Hrs. (light: dark) cycles and was incubated at 22–27 °C. Toxicity assay test was determined by testing five different concentrations of ZnO-NPs, (control (0), 10, 50, 100, and 200 mg/L) on microalgal growth. Briefly, 200 ml from the culture of *S. opoliensis* at the log phase (day 6- day 16) was inoculated in 500 mL Erlenmeyer flasks. Cultures were individually spiked with different ZnO-NPs doses, and cultures were incubated under the same growth conditions as aforementioned for 4 days. All treatments were conducted in three conducted in 3 replicates.

2. Measurement of algal growth

Cell growth (represented as cell count/ml) was determined using the Neubauer cell counting chamber after 96 Hrs. of incubation. The mean counts of three replicates were expressed as the number of coenobia/ml of algal suspension ± SD.

Measurement of Chlorophyll *a*

For determining the chlorophyll (Chl-*a*), 3 ml from each culture cell suspension was centrifuged at 3000 rpm for 15 mins., and the pellets were immersed in hot methanol at 65 °C for 60 minutes or until the cell pellets converted to almost colorless. The absorbance of the Chl-*a* extract was determined at 653 nm, 666 nm using UV-vis spectrum (UV-2100, UNICO, U. S. A.). Chl-*a* was calculated using the equations, $\text{Chl-}a = 15.65A_{666} - 7.34A_{653}$ (**Lichtenthaler and Wellburn 1983**).

3. Estimation of primary metabolic content

3.1. Carbohydrate content

The total carbohydrates were assayed by the anthrone sulphuric acid method (**Badour 1959**), after extraction of 10 ml of the microalgal suspension in 4 N HCL for 2 Hrs. at 100 °C. The carbohydrate content was calculated by referring to the glucose standard curve.

3.2. Protein content

Protein contents were extracted using the alkali method, where 10 ml of algal samples were centrifuged and 10 ml of 1N NaOH was added to the pellet with shaking for 30 mins. The protein content was estimated using **Lowry *et al.* (1951)**. A calibration curve of casein was used for the calculation of the protein content.

3.3. Lipid content

The assay of total lipid was achieved through the sulfo-phospho-vanillin method (SPV), colorimetric method (**Mishra *et al.*, 2014**). The standard lipid was prepared using commercial canola oil.

4. Microscopic examination

The phenotypic examination of *S. opoliensis*, both control and ZnO-treatment were done using the compound microscope (Zeiss, Jena, Germany). Where the morphology of cell surface and changes of microalga was checked, and micrographs were obtained using an Eyepiece camera (MDE4-500BC, China) adapted in the microscopes.

5. Statistical analysis

The mean and standard error values for all treatments were calculated ($n = 3$). To determine the significant differences between ZnO-NPs concentrations, The one-way ANOVA model was used, followed by Tukey's post hoc test for multiple pairwise comparisons, comparisons with $P \leq 0.05$ were considered statistically significant. Data were analyzed using the Minitab® 18.1 statistical software package.

Results and Discussion

1. ZnO-NPs inhibited microalgal growth

The toxic effect of ZnO-NPs on the growth of *S. opoliensis* was primarily assessed based on the changes in total cell count. Our results showed that the ZnO-NPs had a significant inhibitory effect on cell growth with a concentration dependent-response. Where concentrations above 50 mg/L were highly toxic, and 200 mg/L recorded the maximum growth reduction by 68.76 % compared to the control condition as revealed in Fig. 1. Previous reports attributed the cytotoxicity mechanisms of NPs on microalgae to adhering of these nano-sized materials to the cell surface of the algal cell and interaction with the cell wall that resulted in the formation of aggregates (**Oukarroum *et al.*, 2012; Handy *et al.*, 2012 and Hamed *et al.*, 2019**). These aggregates could inhibit the uptake of nutrients by microalgae (**da Costa *et al.*, 2015**) and induce cellular oxidative stress due to the production of the reactive oxygen species (ROS), leading to damages of cellular components (**Wang *et al.*, 2011 and Manke *et al.*, 2013**) (e.g., DNA damage and prevention of cell division (**Aruoja *et al.*, 2009; Ji *et al.*, 2011 and Chen *et al.*, 2019**)).

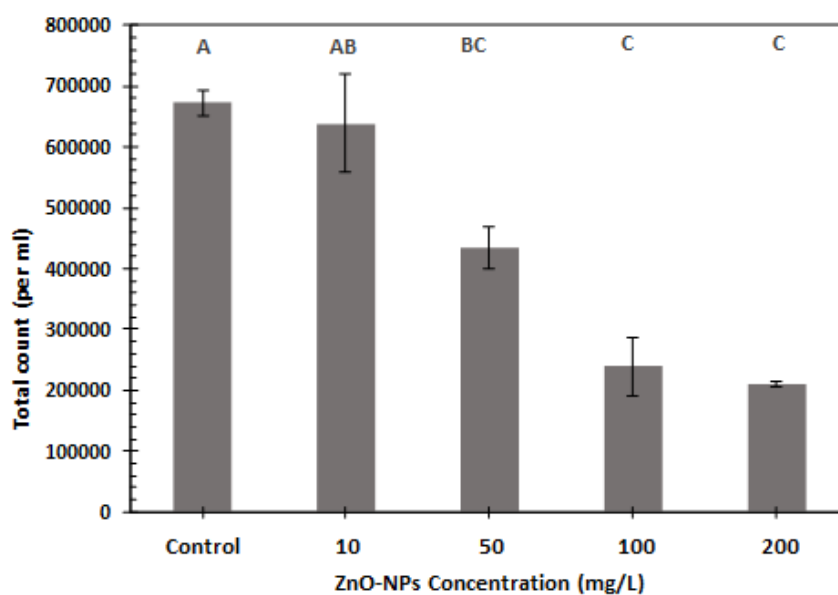


Fig. 1. Cell growth (represented as cell count/ ml) of *S. opoliensis* under different concentrations of ZnO-NPs (control (0), 10, 50, 100, and 200 mg/L). Different letters represent significant differences at $P \leq 0.05$ among ZnO-NPs treatments compared to the control condition. Error bars represent the standard error (\pm SE).

2. ZnO-NPs decreased photosynthetic pigment content

Data in Fig.2 showed that exposure of *S. opoliensis* to ZnO-NPs significantly decreased Chl-*a* content with a concentration-based response. The lowest inhibitory effect was observed at 10 mg/L by 2.84 %. Meanwhile, 50 mg/L and 100mg/L decreased Chl-*a* by 25.85%, 39.83%, respectively. While the highest reduction was detected at 200 mg/L by 55.64%. Similar observations were reported by (Aravantinou *et al.*, 2017 and Djearmane *et al.*, 2019) in *Scenedesmus* sp. and *Chlorella* sp. under ZnO-NPs.

Chl- *a* is the main photosynthetic pigment, the growth profile of algal cells can be evaluated by monitoring the Chl-*a* content (Movafeghi *et al.*, 2018). Thereby, the decrease in Chl-*a* content could be explained by inhibiting the

photosynthesis process (Perreault *et al.*, 2012; Li *et al.*, 2015 and Chen *et al.*, 2018). The nanoparticles were also demonstrated to create impairment of the cell structure, disrupting the function of photosynthetic apparatus and inhibiting respiration through damaging the mitochondrial membrane of microalgae which led to growth inhibition (Chen *et al.*, 2018 and Fazelian *et al.*, 2019).

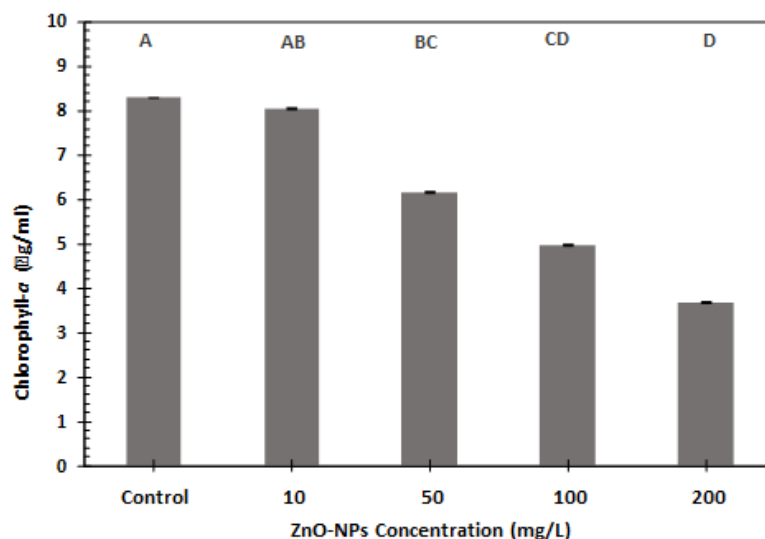


Fig. 2. Effect of different concentrations of ZnO-NPs on the chlorophyll-*a* content of the *S. opoliensis*. Different letters denote significant differences among ZnO-NPs concentrations compared to control conditions using pairwise comparison. Error bars represent the standard error (\pm SE).

3. ZnO-NPs induced considerable alteration in primary metabolic profile

To get a better insight into the physiological and biochemical responses of *S. opoliensis* following ZnO-NPs exposure, we analyzed the primary metabolic content. Results in Fig. 3 showed that ZnO-NPs significantly decreased the carbohydrate content, and this reduction was concentration-dependent.

Interestingly, 10 mg/L had no negative effect on carbohydrate content, where it showed comparable content to control condition by 324 $\mu\text{g/ml}$. Whilst the high ZnO-NPs concentration 50 and 100 mg/L showed a significant reduction in carbohydrate content by 29.64% and 43.56 % respectively.

The highest content of carbohydrate (50 %) was achieved by 200 mg/L compared to the control sample ($P \leq 0.05$). Carbohydrates are the major photosynthetic products of microalgae, and they are stored as starch grains in the chloroplasts (Huang *et al.*, 2016). Hu *et al.* (2014) attributed the reduction in carbohydrate content to the inhibitory effects of NPs on the photosynthetic and respiration processes resulting in a metabolic disturbance that affected the carbohydrate synthesis. A similar observation by Anusha *et al.* (2017), was reported a significant decrease in carbohydrates production in microalgae *Microcystis* sp. and *Oscillatoria* sp. when exposed to cobalt nanoparticles.

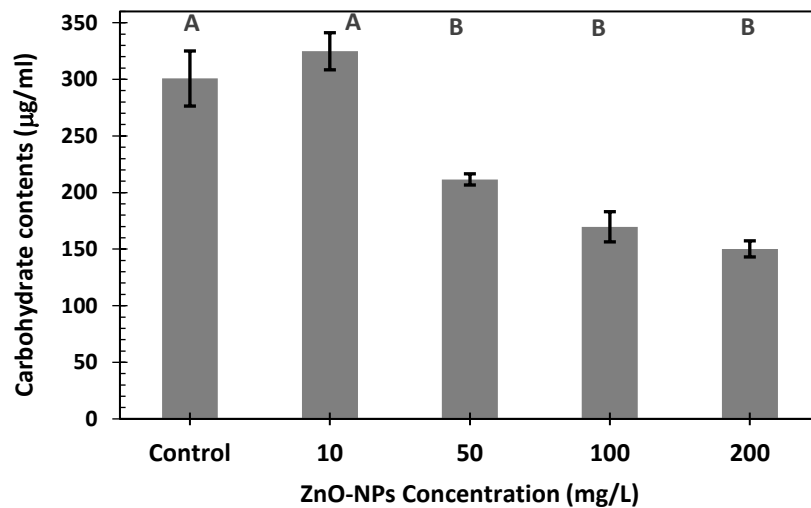


Fig. 3. Influence of different concentrations of ZnO-NPs on the carbohydrate content of *S. opoliensis* after 96 Hrs. of exposure. Different letters denote significant differences among treatments compared to control using pairwise comparison Error bars represent the standard error ($\pm\text{SE}$).

In contrast, the content of protein was significantly increased in *S. opoliensis* cells after ZnO-NPs treatment compared to the untreated cells (Fig. 4). Compared to the control sample ($P \leq 0.05$), protein content at 10 mg/L was increased by 35.8% and more increased at 50 and 100 mg/L by 47% and 59.4%, respectively. Remarkably, the highest protein content (73.83%) was achieved at 200 mg/L. An increase in protein content is considered as an indication of an active defense mechanism to prevent algae cells from damage by abiotic stress (Sabatini *et al.* 2009; He *et al.* 2017 and Hamed *et al.*, 2017). An increase in protein content has also been reported by (He *et al.*, 2017 and Fazelian *et al.*, 2020) for the treated *Scenedesmus obliquus* cells with ZnO and Fe₂O₃ NPs.

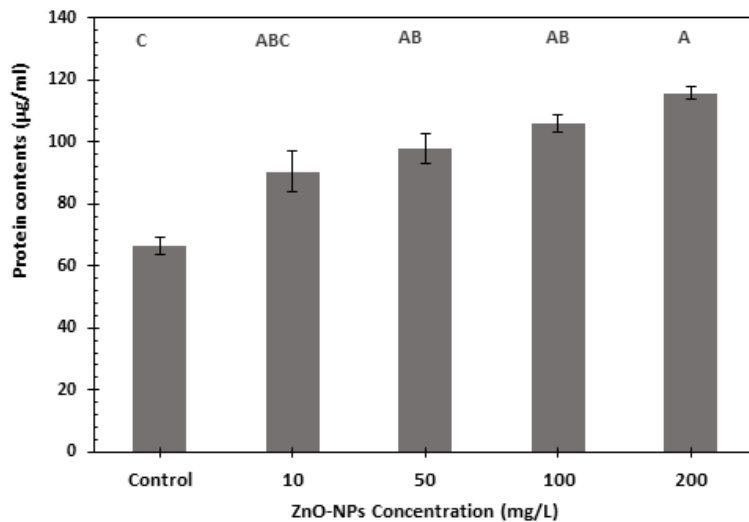


Fig. 4. The effect of different concentrations of ZnO-NPs on the protein content of *S. opoliensis*. Different letters represent significant differences among different ZnO-NPs concentrations compared to the control condition by pairwise comparison. Error bars represent the standard error (\pm SE).

A similar observation was found in lipid content, where the total lipid content was significantly increased in cultures of *S. opoliensis* by increasing ZnO-

NPs concentration compared to the control condition (Fig. 5). For instance, lipid content was increased by 13.73% at 10 mg/L and further increased at 50 and 100 mg/L by 25.35% and 49.66%, respectively. Interestingly, the highest lipid content was observed at 200 mg/L by 116.22%. Previous studies indicated that lipid synthesis is stimulated by stress conditions (**Hamed *et al.*, 2017**). In this context, abiotic stress conditions induced the production of stress proteins that would shift starch production to lipid synthesis (**Li *et al.*, 2013** and **Sun and Huang 2017**). The induction in lipid content in green microalgae following NPs treatment was attributed to the generation of oxidative stress and which improved lipid production (**Hu *et al.*, 2008** and **He *et al.*, 2017**). **Pancha *et al.* (2014)** explained this increase to induction of other types of lipids such as glycol lipids (GLs) and phospholipids (PLs), which are important components of external and chloroplast membrane, along with the endoplasmic reticulum. **Kang *et al.* (2014)** reported that the stress conditions induce a higher lipid accumulation in oleaginous microorganisms and suggested the oxidative stress was the main reason.

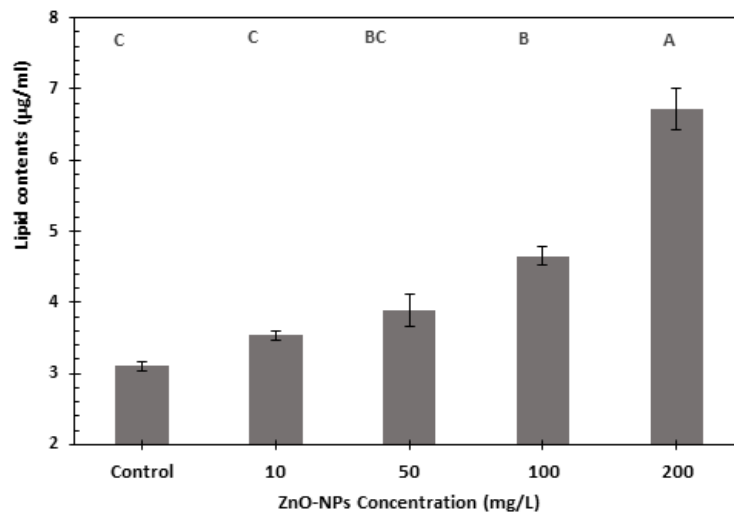


Fig. 5 The effect of different concentrations of ZnO-NPs on the lipid content of *S. opoliensis*. Letters represent the pairwise comparison between different ZnO-NPs concentrations. Error bars represent the standard error (\pm SE).

4. Effect on morphology

Detailed microscopic examination of treated *S. opoliensis* cells with ZnO-NPs (100, and 200 mg/L) showed obvious morphological changes and severe membrane damage in comparison to untreated cells. The untreated control cells showed uniform distribution of cell chloroplast with normal morphological features as shown in (Fig. 6, A). Whereas ZnO-NPs-treated, the cell was completely damaged and cell debris was observed (Fig. 6, F). A swollen or deformed cell and a colorless cell with no chlorophyll content were also detected after ZnO-NPs (Fig. 6, E). The algal cells morphology changes include cell stretching and shrinkage (Fig., B, and C). Also, irregular cell outlines and damaged cell surface (Fig. 6, D). Treated cells with ZnO-NPs. showed a larger cell size compared to control which could be attributed to lipid body accumulation and abnormal cell division in a few cells. Deformed cells, and irregular shapes, perhaps due to oxidative stress generated by ZnO-NP, which caused cell wall degradation and leakage of cellular material (Bhuvaneshwari *et al.*, 2015 and Kalamurthi *et al.*, 2019).

Conclusion

Our results demonstrated that ZnO-NPs had a considerable toxic effect on *Scenedesmus opoliensis*, especially at doses above 10 mg/L. ZnO-NPs significantly inhibited cell growth, Ch-*a* content, and carbohydrate content with a concentration-based response. On the contrary protein and lipid accumulation, were significantly increased after treatment with ZnO-NPs. The phenotypic examinations revealed that high ZnO-NPs concentration induced cell deformation. These results give an alarm to limit the excessive use of ZnO-NPs. which, has a negative impact on the food chain and the whole aquatic ecosystem.

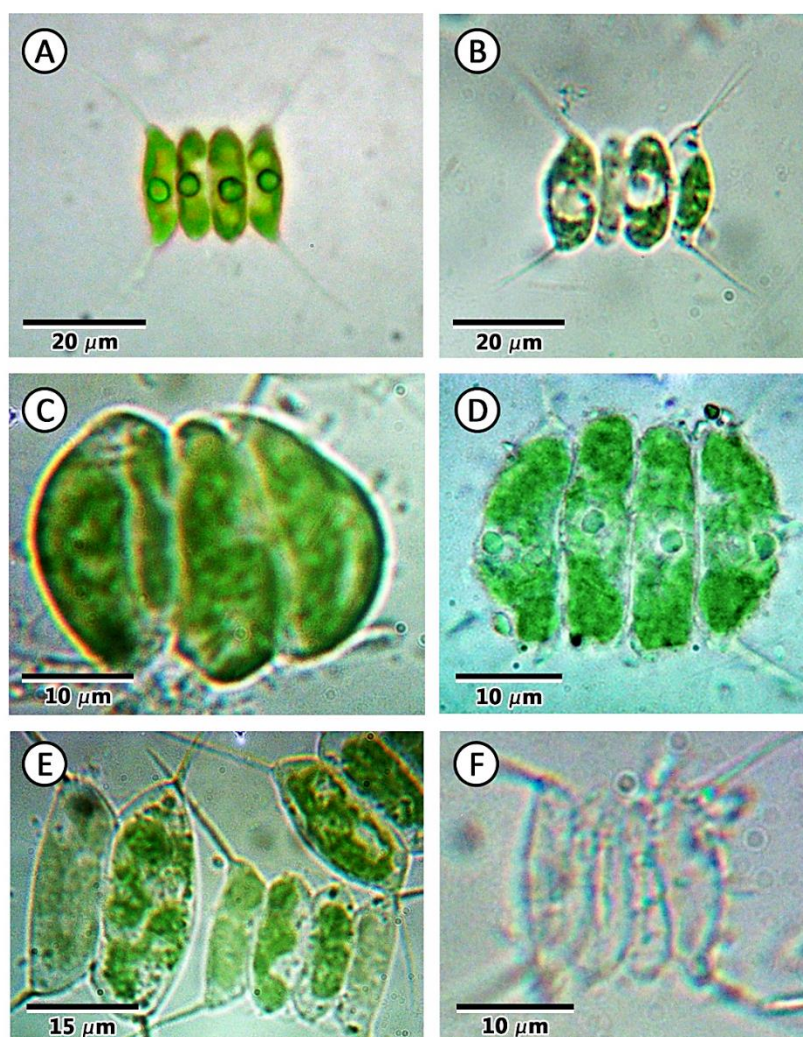


Fig. 6. Cells morphogenesis of *S. opoliensis* in response to different ZnO-NPs concentrations, (A): normal cells, (B): cells with enlarged empty pyrenoids, (C): Sticky cells, (D): damaged cell surfaces, (E); loss of chlorophyll and enlarged cells, (F) Cell death.

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النمو، و النواتج الأيضية الأولية ، و تشكل الخلايا في طحلب سينيديسماس اوبوليانسيس استجابة للإجهاد الناتج من جزيئات أكسيد الزنك النانوية

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تستخدم جزيئات أكسيد الزنك النانوية علي نطاق واسع في التطبيقات الصناعية و الزراعية، بالإضافة إلي مستحضرات التجميل. و مع ذلك فإن الاستخدام المفرط منها يمكن أن يؤدي إلي مشاكل بيئية مدمرة للنظام البيئي المائي. من بين الكائنات الحية، تعد الطحالب المجهرية الدقيقة من أكثر الكائنات المستخدمة كمؤشرات حساسة لتلوث المياه. في هذه الدراسة تم تقييم التأثيرات المختلفة لتركيزات من جزيئات أكسيد الزنك النانوية (10- 200 ملجم/ لتر) علي النمو، و النواتج الأيضية، و الشكل الظاهري لخلايا طحلب سينيديسماس اوبوليانسيس. أظهرت النتائج أن جزيئات أكسيد الزنك النانوية ادت إلي انخفاض معنوي في نمو الخلايا، و محتوى كلوروفيل أ، و محتوى الكربوهيدرات اعتمادا علي التركيز. على الجانب الآخر، زادت محتويات كل من البروتين والدهون مقارنة بالكنترول. كشفت الدراسات المورفولوجية عن حدوث تغيرات واضحة في خلايا الطحلب خاصة عند التركيزات العليا. تسلط الدراسة الحالية الضوء على السمية البيئية لجزيئات أكسيد الزنك النانوية، خاصة عند التركيزات العليا علي الطحالب بصفة خاصة ممثلة في سينيديسماس اوبوليانسيس و علي النظام البيئي بشكل عام.