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Effects of phenanthrene on growth parameters and antioxidant systems in the green microalga *Chlorella vulgaris*

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ABSTRACT

The effect of phenanthrene (PHE) as a polycyclic aromatic hydrocarbon (PAH) on the green microalgae *Chlorella vulgaris* was studied. After exposure of the algal cells to different concentrations (2, 10, 25 and 50 ppm) of phenanthrene for 7 days, a significant increase occurred in the activity of the antioxidant enzymes including ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD). In addition, PHE treatments resulted in an increase in total phenol and flavonoid contents as non-enzymatic antioxidant compounds. Intriguingly, the cell density, dry weight and fresh weight of algae were enhanced at 2 ppm of PHE in comparison to control. However, the growth parameters as well as the photosynthetic pigments content of *C. vulgaris* gradually decreased with the enhancing concentration of phenanthrene. Flow cytometric analysis showed no significant reduction in the viability cell in the samples exposed to 50 ppm of PHE for 24 h, while chlorophyll fluorescence was significantly reduced. Furthermore, distorted morphological symptoms of cell structure were observed through scanning electron microscopy (SEM) analysis in a number of cells exposed to 50 ppm of PHE. These findings revealed that *C. vulgaris* possesses significant resistance against phenanthrene as a PAH pollutant. Mainly, the algal cells fight the toxicity by the increasing activity of antioxidant systems in order to diminish the effects of reactive oxygen species (ROS).

Keywords: *Phycoremediation; Antioxidant systems; PAHs; Phenanthrene; Chlorella vulgaris.*

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are extremely persistent organic pollutants composed of at least two fused benzene rings [1]. PAHs can be distributed in the environment during the defective combustion of fossil fuels and organic materials, volcanic erosion, the operation of oil refineries and waste incineration [2, 3]. There is a serious anxiety about the adverse impacts of PAHs on the health of living organisms owing to their carcinogenic and genotoxic potentials [4, 5]. In fact, sixteen PAHs are recommended by the U.S. Environmental Protection Agency (US-EPA) as "Priority Pollutants" for monitoring in the environment [1, 2]. Phenanthrene (PHE) is a low-molecular weight PAH composed of three fused benzene rings [6] and it is mentioned in the environmental pollutants list of US-EPA [7].

PAHs can enter the aquatic ecosystems either by surface waters or through atmosphere [8]. The PAHs in polluted water bodies have serious negative effects not only on the growth and development of aquatic organisms but also on human health by transferring to the food chains [9, 10]. Therefore, the cleanup of the PAHs-contaminated ecosystems has attracted a great deal of attention. There are different remedial methods like adsorption, photolysis, chemical degradation and bioremediation for PAHs contamination [11]. Over the last few years, there has been a surge of interest in bioremediation techniques that utilize living organisms including algae, bacteria, and fungi to transform the environmental pollutants to less hazardous/non-hazardous forms [11, 12]. Algae as the main primary producers of aquatic ecosystems have a key role in removing toxic metals and other organic pollutants [13-15]. Phycoremediation (the utilization of algae for environmental remediation) is a promising issue since generated algal biomass from the contaminated environments can be used for several purposes such as biofuel production. Therefore, environmentally friendly wastewater treatment coupled with biofuel/bioenergy applications is considered as an ideal approach for ecological recovery [12, 16]. The ability of several algal species of different genera has been proved for the removal and biodegradation of PAHs [17-19] . Among different algae, the green freshwater microalga *Chlorella vulgaris* has outstanding capability to adapt to the various environmental circumstances because of its rapid growth potential. In fact, *C. vulgaris* is recognized as a suitable system for phycoremediation of pollutants in contaminated aquatic environments [20, 21].

Many preceding studies focused on the negative effects of PAHs on the plants and their responses to PAHs pollution and toxicity [22, 23]. It is well known that various environmental stresses in higher plants may trigger oxidative stress by causing the formation of reactive oxygen species (ROS). High concentrations of ROS can cause serious damages to different biomolecules and consequently result in metabolic disorders. To battle oxidative stress, plant cells have evolved two antioxidative defense mechanisms in the form of enzymes and non-enzymatic antioxidants [24-26]. The changes of antioxidant systems in organisms, for optimizing survival strategies, are considered as indicators for pollutants such as PAHs. However, little is known about the changes in antioxidant systems in microalgae and the response to PAHs contamination [24, 25]. Thus, the objective of

the current study was to examine the potential of *C. vulgaris* to grow under the heterotrophic condition in the presence of PHE. Furthermore, the function of antioxidant systems in the microalgae *C. vulgaris* was investigated in response to PHE treatments.

2. EXPERIMENTAL SECTION

2.1. Microalgae and culture conditions. The green microalga *Chlorella vulgaris* was supplied by the Culture Collection of Algae from Bushehr Shrimp Research Institute, Iran. The axenic cultures of *C. vulgaris* were maintained in 1 liter Erlenmeyer flasks containing BG11 medium. The pH of media was adjusted to 7.1 and cultures were incubated at a temperature of 25°C under 12:12 h light: dark period with continuous aeration. BG11 medium was prepared with the following mineral salt composition in distilled water: $NaNO_3$ (1.5 g L^{-1}), K_2HPO_4 (4.0 g L^{-1}), $MgSO_4.7H_2O$ (7.5 g L^{-1}), $CaCl_2.2H_2O$ (3.6 g L^{-1}), Citric acid (0.6 g L^{-1}), Ammonium ferric citrate green (0. 6 g L^{-1}), $EDTANa_2$ (0.1 g L^{-1}), Na_2CO_3 (2.0 g L^{-1}), H_3BO_3 (2.86 g L^{-1}), $MnCl_2.4H_2O$ (1.81 g L^{-1}), $ZnSO_4.7H_2O$ (0.22 g L^{-1}), $Na_2MoO_4.2H_2O$ (0.39 g L^{-1}), $CuSO_4.5H_2O$ (0.08 g L^{-1}), $Co(NO_3)$ $2.6H_2O$ (0.05 g L^{-1}) [27].

2.2. Experimental design and treatments. To carry out the growth assays, fifteen 250 mL conical flasks, each containing 100 mL BG11 medium, were autoclaved. Before algal inoculation, the proper amount of PHE (using a 1000 mg L^{-1} stock solution in acetone) was added into conical flasks to achieve PHE concentrations of 2, 10, 25 and 50 ppm in the culture media. Media without PHE were used as control. The treated flasks were used for algal inoculation after complete evaporation of acetone and relative dissolution of PHE on a shaker at 120 rpm for 48 h. Then, the algal cells in logarithmic growth phase were exposed to different treatments for 7 days. Algal samples were retrieved by centrifugation at 4000 rpm for 10 min at 4°C.

2.3. Measurement of growth parameters. A number of growth parameters including optical density, cellular density, fresh weight and dry weight were evaluated. Microalgal growth was determined daily by optical density with a spectrophotometer at 600 nm. In addition, during the exponential growth phase, linear relationship between optical density (OD_{600}) and cell density was determined by cells counting using a hemocytometer. The regression equation between cell density (y $\times 10^6$ mL^{-1}) and OD_{600} (x) was calculated using the standard curve as y = 42.025x-1.6709 (R^2=0.9976). At the end of the experiments, algal cells were harvested by centrifugation at 4000 rpm for 10 min. Consequently, the microalgal pellets were washed and fresh weight and dry weight were determined (after drying at 37°C for 24 h).

2.4. Scanning electron microscopic analysis. After exposure of cells to 50 ppm PHE for 24 h, the algal samples were centrifuged at 4,000 rpm for 10 min at 4°C. The cell pellets were washed three times with BG11 medium and were freeze-dried for 4 h. After gold sputtering, the morphology of treated and untreated algal cells was observed using a scanning electron microscopy (SEM, MIRA3 FEG-SEM).

2.5. Flow cytometric analysis. Flow cytometric assessments were performed to examine the influence of phenanthrene on the viability of *C. vulgaris* cells treated with 50 ppm PHE for 24 h in comparison with control. Briefly, about 1.0×10^6 cells were collected by centrifugation (4000 rpm, 4°C, 5 min), washed with phosphate buffer solution (PBS, pH 7.0), and exposed to 5 µl propidium iodide (PI) for an incubation period of 30 min in the dark. The fluorescent emission of the samples was obtained from ~10,000 events per cell sample in FACScalibur flow cytometer (Becton Dickins on Immunocytometry Systems, San Jose, CA, USA) FL2 channel and chlorophyll fluorescence was collected in FL3 channel.

2.6. Assessment of total protein and enzymatic antioxidant activities. The algal cells were frozen in liquid nitrogen and homogenized after adding the cold phosphate buffer (PBS, 50 mM, pH 7.0) in frozen state. The homogenates were centrifuged at 10000 ×g at 4°C for 10 min. The supernatant was immediately used for determination of the total soluble protein content [28] as well as the assessment of antioxidant enzymes activities including superoxide dismutase (SOD), peroxidase (APX) and catalase (CAT). SOD activity was evaluated by assessing the inhibition of nitro-blue-tetrazolium (NBT) photoreduction by algal extracts [29]. A reaction mixture (3 mL) was made up of 2.55 mL sodium phosphate solution (1 M, pH 7.8), 100 µl NBT (1.5 mM), 200 µl NaCN (0.3 mM) EDTA (1 M), 50 µl of riboflavin (0.12 mM) and 150 µl of enzyme extract. The reaction was initiated by adding 50 µl of riboflavin (0.12 mM) under 5000 Lux of illumination for 12 minutes. Afterward, the absorbance of the solutions was recorded at 560 nm. The required amount of the enzyme leading to a 50% protection of NBT photoreduction was defined as one unit of SOD activity. CAT activity was estimated according to the methods of Chance and Maehly [30]. A reaction mixture was prepared using 1.1 mL potassium phosphate buffer (50 mM, pH 7.0), 700 µl H_2O_2 (10 mM) and 200 µl of enzyme extract. The activity of CAT was recorded at 240 nm by following the decomposition of H_2O_2 for 3 min and was calculated on the basis of the extinction coefficient of 27 M^{-1} cm^{-1}. One unit of enzyme activity was defined as the amount of enzyme required for the reduction of 1 µM H_2O_2 per minute. The activity of APX was determined by recording the reduction in absorbance at 290 nm during oxidation of ascorbic acid for 3 minutes [31]. A reaction mixture was prepared using 550 µl potassium phosphate buffer (50 mM, pH 7.0), 400 µl ascorbate, 100 µl hydrogen peroxide, 400 µl EDTA and 150 µl of enzyme extract in a total volume of 2 mL. The reaction was started by adding the enzyme extract or hydrogen peroxide, and the absorbance decrease was recorded in 30 sec intervals. The quantity of enzyme required for the oxidation of 1 µmol ascorbic acid min^{-1} was defined as one unit of activity.

2.7. Evaluation of total phenol and flavonoid content. The algal cells were homogenized in 100% methanol and incubated at 4°C for 24 hours in the dark. The homogenates were centrifuged at 10000 ×g for 10 min and then the supernatant was used for the assays. For measurement of total flavonoid content of the algal extracts, 500 µl of supernatant, 1.5 mL of 100% methanol, 100 µl of 10%

aluminum chloride solution, 100 µl of 1 M potassium acetate, and 2.8 mL of distilled water were added to 500 µl of extract. The absorbance of the mixture was read at 415 nm after 40 minutes. Quercetin was used for the preparation of calibration curve (20-200 mg L^{-1}). The total flavonoid content of the extracts was reported as milligram quercetin equivalent per fresh weight of microalgae ((QE) g^{-1} FW) [32]. The total phenol concentration in the algal extracts was obtained using Folin-Ciocalteu procedure as described by Meda et al. [33]. Accordingly, 100 µl of the algal extract was mixed with 2.8 mL of deionized water, 100 µl of Folin-Ciocalteu reagent and 2 mL of 2% sodium carbonate aqueous solution in a test tube. The samples were incubated for 30 min in dark at room temperature and then the absorbance was read at 720 nm. Gallic acid was used as a standard and the total phenol content of the extracts was expressed as milligram gallic acid equivalent per fresh weight of microalgae (mg GAE g^{-1}).

2.8. Assessment of photosynthetic pigments. Photosynthetic pigments including chlorophyll a, b and total carotenoids were extracted from algal cells by 100% methanol at $4^{\circ C}$ for 24 h in the darkness. After centrifugation for 10 minutes at 10000 ×g, absorption of the supernatant was measured at 470, 665, and 653 nm with a UV/V spectrophotometer. The amounts of the pigments were calculated using adjusted equations of Lichtenthaler [34].

2.9. Statistical analysis. All statistical analyses were carried out in three replications. One-way analysis of variance (ANOVA) was directed with Duncan multiple comparison tests by SPSS 21 software. The confidence level was taken at $P < 0.05$.

3. RESULTS SECTION

3.1. The effect of PHE on the growth of *C. vulgaris*. Assessing cell number, fresh weight and dry weight of *C. vulgaris* confirmed the significant impact of different concentrations of PHE on the algal cells (Figures 1, 2). Intriguingly, the cell density of algae was enhanced at 2 ppm concentration of PHE in comparison with the control (Figure 1). Increase in the cell density and dry weight of *C. vulgaris* at low concentrations of crude oil were previously reported [35]. The higher growth rate of *C. vulgaris* in the non-toxic crude oil contaminated medium may indicate the ability of the microalgal species for consuming the organic compounds as nutrient [12]. However, growth parameters of *C. vulgaris* were relatively the same with control sample at 10 ppm of PHE during seven days of growth (Figure 2). In contrast, by the increasing concentration of phenanthrene to 25 and 50 ppm, all of the growth parameters were significantly reduced (compared to the control sample). Although cell density of microalgae was very limited at 25 and 50 ppm of PHE for 72 h; but it was notably increased from the fourth day of growth (Figure 1). As shown in Fig. 2, 25 and 50 ppm of PHE had negative influences on a dry weight (with 36% and 50% reduction) and fresh weight (with 27% and 41% reduction) of microalgae, respectively ($P < 0.05$). In agreement with our data, the inhibitory impacts of PAH pollutants have been reported on the growth of *Chlorella* and some other microalgae [17, 19].

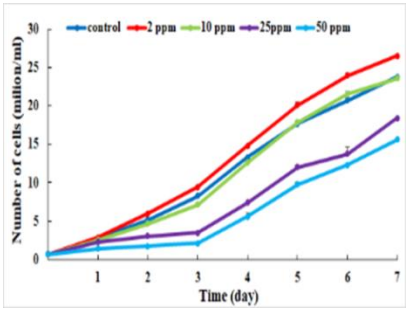


Fig. 1. Growth of *C. vulgaris* during 7-day-exposure to different concentrations of PHE.

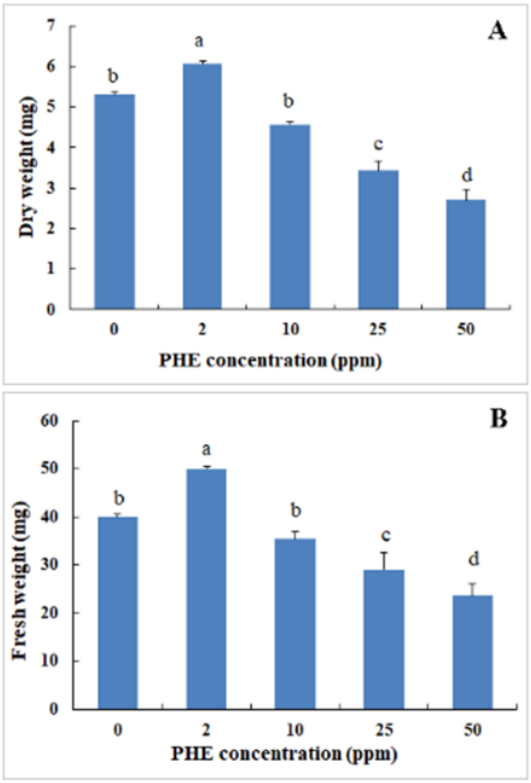


Fig. 2. The effect of different concentrations of PHE on dry weight (A) and fresh weight (B) of *C. vulgaris* after 7 days. Different letters indicate significant differences according to Duncan Test at $P < 0.05$. The error bars represent standard deviation of the mean of three replications.

3.2. Morphology of the cells. The SEM image showed the intact structural features of the microalgal cell in the control sample. In contrast, after 24 h of exposure to PHE (50 ppm) distorted morphological symptoms of the cell structure were revealed in a number of cells (Figure 3). The similar morphological changes as well as cell wall damages were reported in *C. vulgaris* following the exposure to different concentrations of zinc oxide nanoparticles using FESEM microscopic analyses [15].

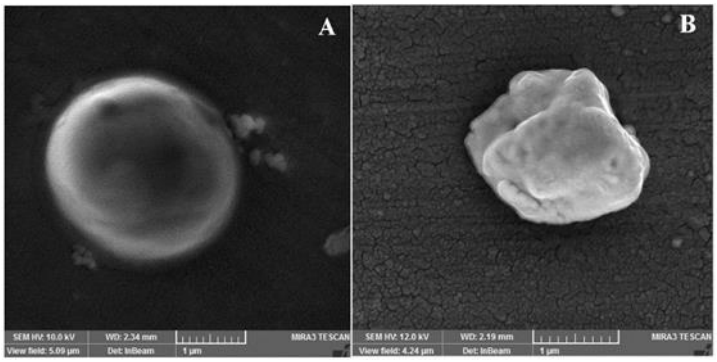
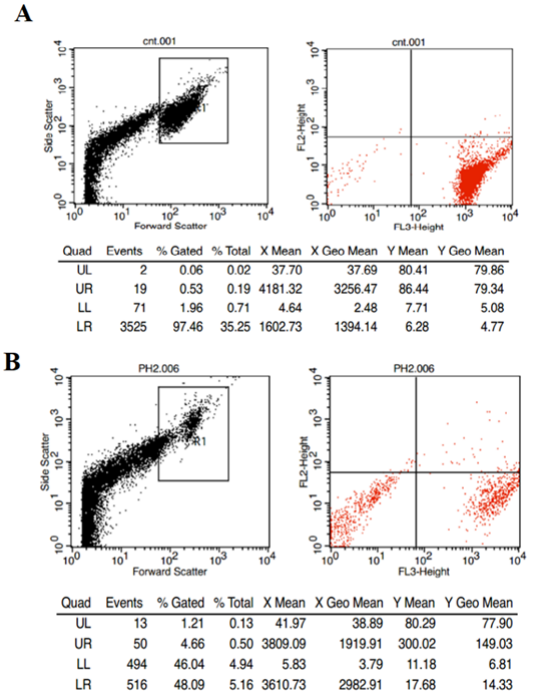


Fig. 3. Scanning electron images of *C. vulgaris*: (A) control sample; (B) treated with 50 ppm PHE.

3.3. Flow cytometric analysis. The cytotoxic effects of 50 ppm PHE on the cells of *C. vulgaris* were analyzed using flow cytometry. In this technique, red-fluorescent nucleic acid dye (PI) can enter the dead cells and stain the nucleic acids. PI binds to double-stranded DNA and when is excited at 488 nm produces red fluorescence [15]. Accordingly, PI can be used to quantify viable cells in a cell population. In flow cytometry diagrams, upper left and right quadrants illustrate the percentage of dead cells and lower left and right quadrants display the percentage of living cells and lower right quadrant shows chlorophyll fluorescence (Figure 4). The cell viability of the reference sample was almost 100% during the 24 h of culture and all of the cells showed chlorophyll fluorescence (Figure 4 A). However, the percentage of viable cells in the samples exposed to 50 ppm of PHE was decreased to 94.13% of viable cells and 46.04 % of the cells didn't show chlorophyll fluorescence (Figure 4 B). Hitherto, flow cytometry also revealed the significant reduction in *C. vulgaris* viable cells treated with different concentrations of zinc oxide nanoparticles [15]. These findings indicated that the variation in the amount of chlorophyll fluorescence in the PHE-treated cells is associated with the response of the individual cells to the toxicant.



Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	2	0.06	0.02	37.70	37.69	80.41	79.86
UR	19	0.53	0.19	4181.32	3256.47	86.44	79.34
LL	71	1.96	0.71	4.64	2.48	7.71	5.08
LR	3525	97.46	35.25	1602.73	1394.14	6.28	4.77

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	13	1.21	0.13	41.97	38.89	80.29	77.90
UR	50	4.66	0.50	3809.09	1919.91	300.02	149.03
LL	494	46.04	4.94	5.83	3.79	11.18	6.81
LR	516	48.09	5.16	3610.73	2982.91	17.68	14.33

Fig. 4. Flow cytometry images: (A) control sample showed nearly 100% of cell viability; (B) cell viability of *C. vulgaris* treated with 50 ppm FLU decreased to ~ 94 % after 24 h.

3.4. The effect of PHE on the activity of antioxidant enzymes. Environmental pollutants such as PAHs may cause oxidative stress in algal cells and produce a high level of reactive oxygen species (ROS) [24]. The overproduction and accumulation of ROS in the environment can result in structural and physiological damages in cells. The defensive system of algae fights the excess ROS by a complex antioxidant system composed of non-enzymatic (such as flavonoids and phenols) and enzymatic antioxidants (such as SOD, APX, and CAT) [21, 36] .

The effect of different concentrations of PHE on antioxidant enzymes activities was assessed in *C. vulgaris* after 7 days of exposure (Figure 5). APX inhibits the elevated levels of H_2O_2 in photosynthetic organisms and its activity goes up in response to a variety of biotic and abiotic stresses [37]. The activity of APX was significantly enhanced with the increasing concentration of PHE from 10 to 50 ppm ($P < 0.05$) (Figure 5 A). Similar increment in the activity of APX was also reported in *C. vulgaris* following the exposure of algal cells to different concentrations of heavy metals [13, 14, 25, 38].

CAT as a key enzyme involved in the detoxification of ROS catalyzes the dismutation of H_2O_2 [39]. The highest significant enhancement in the activity of CAT was measured as a consequence of algal exposure to 50 ppm PHE for 7 days (Figure 5 B). In agreement with our data, the cells of *Sochrysis zhanjiangensis* and *Platymonas subcordiformis* treated with a pyrene derivative as a PAH increased the activity of CAT [14].

SOD is required for destruction of superoxide radical and converts it to oxygen and H_2O_2 [24]. Rising concentrations of phenanthrene from 10 to 50 ppm caused a remarkable increase in SOD activity compared to the control ($P < 0.05$). However, there was not a significant difference in SOD activity between the control cells and exposed cells to 2 ppm of phenanthrene (Fig. 5 C). Similarly, SOD activity was increased in *C. vulgaris* after treatment with copper and cadmium (1.5 µM) at exponential growth phase [13].

Increase in the activity of APX, CAT and SOD, in high concentrations of PHE can be regarded as a defensive response to the production of cellular ROS. In actual fact, some microalgae are able to tolerate the toxic impacts of the organic contaminant using a well-coordinated antioxidant enzymes system [38].

3.5. The effect of PHE on total phenol and flavonoid contents. Phenol and flavonoid compounds play an important role as non-enzymatic antioxidant to overcome oxidative stress in plants by donating an electron or hydrogen [24, 26]. In the current work, the effects of different concentrations of PHE on total phenol and flavonoid contents of *C. vulgaris* are studied (Figure 6). A significant increase in the total phenol and flavonoid contents was observed in *C. vulgaris* after 7 days of exposure to 25 and 50 ppm of PHE in comparison to the control (Figure 6 A). So far, little attention has been paid to total phenol and flavonoid contents in plants and particularly in microalgae as a result of PAHs stress. In agreement with our data, a rise in total phenol content has been observed in *Spirogyra setiformis* due to exposure to cadmium (Cd^{2+}) [40]. It could be concluded that increases in total phenol and flavonoid contents in PHE-treated groups are occurred for eliminating ROS and reducing their harmful effects.

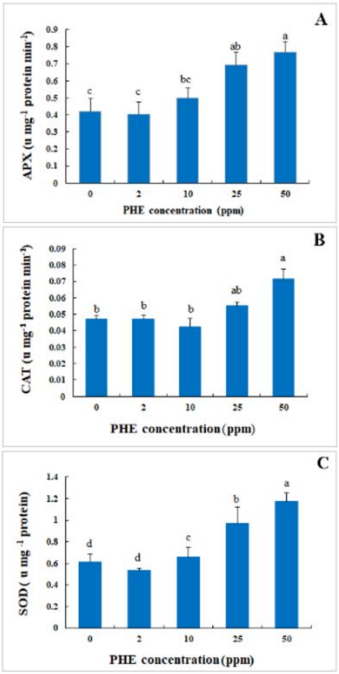


Fig. 5. The effects of different concentrations of PHE on the activities of APX, CAT and SOD in *C. vulgaris* after exposure for 7 days. Different letters indicate significant differences according to Duncan Test at $P < 0.05$. The error bars represent standard deviation of the mean of three replications.

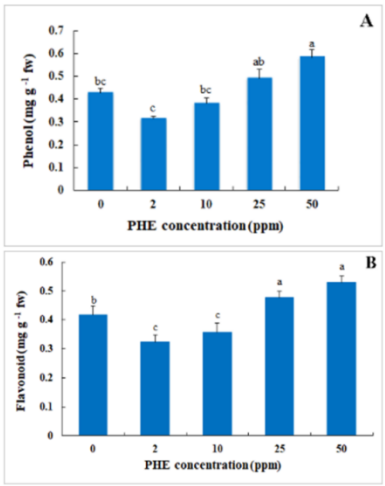


Fig. 6. Effect of various concentrations of PHE on total phenol and flavonoids (A, B) in *C. vulgaris* cells. Different letters indicate significant differences according to Duncan Test at $P < 0.05$. The error bars represent standard deviation of the mean of three replications.

3.6. The effect of PHE on photosynthetic pigments content. Photosynthetic pigments content in *C. vulgaris* was determined after a 7-day-exposure to different concentrations of PHE. Subsequently, the content of chlorophyll a in the treated cells with 25 and 50 ppm of PHE were decreased in comparison to the control ($P < 0.05$) (Figure 7). The amount of chlorophyll b was also significantly reduced after 7 days of exposure to 50 ppm of PHE. However, the changes in chlorophyll a, chlorophyll b, and total chlorophyll contents were not significant after the treatment with 2 and 10 ppm of PHE in comparison to the control ($P < 0.05$). In addition, different concentrations of PHE showed no significant influences on carotenoids content of *C. vulgaris* (Figure 7). The decline in chlorophyll contents was obtained for *C. vulgaris* as a consequence of exposure to different concentrations of heavy metals [14, 21]. The reduction of chlorophyll contents in plant cells is actually a biomarker for oxidative stress [41, 42]. High ROS levels may lead to a decrease in chlorophyll levels and photosynthesis. This restriction may be a protective response to limit ROS byproducts production in chloroplasts [43]. As a result, the potential phytotoxicity of pollutants can be evaluated by means of the decreasing content of photosynthetic pigments [44, 45].

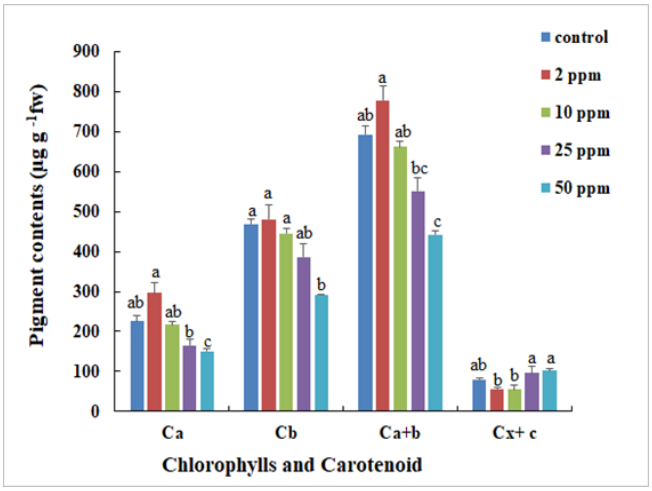


Fig. 7. Effect of different concentrations of PHE on photosynthetic pigments content in *C. vulgaris*. Different letters indicate significant differences and letter(s) are not used due to insignificant differences according to Duncan Test at $P < 0.05$. The error bars represent standard deviation of the mean of three replications.

4. CONCLUSIONS

This is the first study of the effects of PHE on the growth parameters, morphological characteristics and antioxidative responses in the green microalga *C. vulgaris*. Distorted morphological features of the cell structure were confirmed through SEM analysis in the cells exposed to 50 ppm of PHE for 24 h. Concurrently, flow cytometry showed a small reduction in cell viability in the treated algal cells. However, the results conclusively revealed that *C. vulgaris* owns considerable resistance against the PAHs pollutants by the gathering antioxidant systems. The increasing activity of antioxidant enzymes including APX, SOD and CAT, as well as the rising contents of total phenol and flavonoid could confirm the activation of antioxidative defense mechanisms to overcome oxidative stress emerged by phenanthrene. As a final outcome, the potential of the algal species to fight against the PHE pollutant makes microalgae particularly attractive for phycoremediation studies.

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