

Original Article**Phytotoxicological Effects of Bulk-NiO and NiO Nanoparticles on Lesser and Giant Duckweeds as Model Macrophytes: Changes in the Plants Physiological Responses**

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ABSTRACT

Background: Although the production and usage of nanoparticles and their entrance to the ecosystem have increased in last decades, information about their negative impacts on organisms is scarce. We concentrated on the comparison of the toxicological effects of different concentrations of bulk-nickel oxide and nickel oxide nanoparticles (NiO-NPs) on lesser (*Lemna minor* L.) and giant (*Spirodela polyrhiza* (L.) Schleid.) duckweeds as two model macrophytes.

Methods: The morphology of nickel oxide nanoparticles was studied by scanning electron microscopy (SEM) and transition electron microscopy (TEM). The plant's growth, photosynthetic pigments content, the contents of total phenols, flavonoids and MDA, and the activities of some antioxidant enzymes were investigated as indices to assess the toxicological effects of the NPs on two plant species.

Results: The content of photosynthetic pigments in both of the plant species was significantly reduced by high concentration of NiO-NP. Increasing the concentration of the contaminants in the plant species was led to the remarkable enhancement of total phenol and flavonoid and MDA contents. Moreover, increasing the activity of the plant's antioxidant enzymes could reflect high reactive oxygen species (ROS) production after the plant's treatments with the contaminants.

Conclusion: The negative effects of the NiO-NPs, especially in high concentrations, on *L. minor* and *S. polyrhiza* were more than those of Bulk-NiO.

Keywords: Duckweed, Environmental Pollution, Nickel Oxide Nanoparticles, Plant Physiological Responses, Phytotoxicity, Risk Assessment.

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INTRODUCTION

The increasing level of production and widespread and progressive usage of nanoparticles (NPs) in different fields has unavoidably led to the entrance of these materials into ecosystems [1]. Because of low discharge concentration of nanomaterials, clear evidence about their damages in the ecosystems are infrequent [2]. Therefore, nanomaterials interaction with organisms and their potential hazards to biological systems should urgently be distinguished in order to design nanomaterials with minimum adverse impacts.

With regards to nanotechnology grows, toxicological effects of nanomaterials have been widely studied in different micro and macro organisms in recent years [3-5]. Plants as an indispensable and essential part of ecosystems may subject to nanomaterials pollutants and therefore may

be involved in their fate and their importance to the food chains [6]. In contrast, the negative effects of the nanomaterials on plants could be related to reactive oxygen species (ROS) formation, aggregation and absorption to cell walls and release of toxic ions [6,7].

The aquatic environment is the ultimate destination of released NPs and many studies have focused on the toxicity of NPs in aquatic organisms such as aquatic plants [8,9]. Due to small size, the simple structure and morphology, easy cultivation and sensitivity to different classes of the pollutants, duckweeds from Lemnaceae family have received broad application in ecotoxicology like nanotoxicological investigations [10-12].

Nickel oxide (NiO) NPs have a wide range of applications including production of microwave absorbing materials, commercial batteries, magnetic

recording media and formation catalysts [13]. Moreover, the materials have received considerable attention for wastewater treatment because of its chemical magnetic properties [14].

Although there are some reports on the different effects of nanomaterials to aquatic organisms, most of the available information are contradictory and vague [14]. Hence, the aim of the present study was to evaluate the toxicological and physiological effects of NiO-NP and Bulk-NiO on lesser (*Lemna minor* L.) and giant (*Spirodela polyrhiza* (L.) Schleid) duckweeds. Some biochemical and physiological parameters to be assessed as indicators of nanotoxicology are plants growth, photosynthetic pigments content, the cell membrane disruption by assessing the creation of malondialdehyde (MDA), total phenol and flavonoid contents and activities of some antioxidant enzymes (peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD)).

MATERIAL AND METHODS

Nickel (II) Oxide and Nickel Oxide Nanoparticles

Nickel (II) oxide was purchased from Merck, Germany and used as Bulk-NiO. Nickel nanoparticles (NiO-NPs) were supplied from Iranian Nanomaterials Pioneers Company (Iran). Field emission scanning electron microscopy (FE-SEM) was carried out by a Mira microscope (Mira3, Tescan, Czech Republic) to obtain the morphology of the NiO-NPs and the diameters of nanoparticles were measured on the SEM images. Digimizer software was utilized to determine the size distribution of the nanoparticles [15]. Transmission electron microscopy (TEM Philips 300 KV) was used for study of structure and shape for obtained nanoparticles.

Plant Materials and Treatment Methods

Lemna minor was gathered from Ali Jan near Bostanabad, in northwest of Iran and *Spirodela polyrhiza* was obtained from Anzali lagoon, in north of Iran. Their surfaces were washed carefully using distilled water. *L. minor* and *S. polyrhiza* were acclimatized separately for three weeks in two large aquariums containing special growth mediums [16,17] at 23 ± 2 °C under continuous light ($65 \mu\text{mol}$ photosynthetic active radiation ((PAR) $\text{m}^{-2} \text{s}^{-1}$)).

Two stock suspensions of 500 mg/L Bulk-NiO and NiO-NPs were obtained by their addition to the culture medium. The suspensions were treated by sonication (Soniprep 150, model: MSS150.CX35, UK; 50 Hz, 10-second pulse and 5-second interval)

for 10 min and used to prepare the various concentrations of Bulk-NiO and NiO-NPs (0, 1, 10 and 50 mg/L). In all of the experiments, the plants (2 g) were transferred into 250 mL beakers containing 200 mL of the culture medium with different concentrations of Bulk-NiO and NiO-NPs (0, 1, 10 and 50 mg/L). The temperature was kept constant in the incubator (Sanyo, Ogawa Seiki Co., Japan) during the experiments.

The Growth Rate

Relative frond number (RFN) was applied as the suitable indicators of potential toxicity to determine the plant's growth rate. RFN was measured for two plant species using Eq. (1) [18]:

$$\text{RFN} = \left[\frac{\text{frond number at day } n - \text{frond number at day 0}}{\text{frond number at day 0}} \right] \quad (1)$$

$n = 0, 4, 8, 12, 16, 20.$

Photosynthetic Pigments Content

In order to the extraction of the plant's photosynthetic pigments, 100 mg freshly sampled leaves of two plants were ground in 100% acetone, separately. The content of Chlorophylls and carotenoids was measured spectrometrically at 662, 645 and 470 nm for the maximum absorption of chlorophyll "a" (Chl a), chlorophyll "b" (Chl b) and carotenoids, respectively. Pigments content was measured according to the equations described [19].

Biochemical Assays

Antioxidant Enzymes Assay

For investigation of the effects of examined materials on antioxidant enzymes activities, the plants were treated for 7 d by different concentrations of Bulk-NiO and NiO-NP suspensions (0, 1, 10 and 50 mg/L) in the nutrient solution. 0.25 g of fresh plants tissues were homogenized in some 3 mL of 0.1 mol/L phosphate buffer solution (pH 7) containing 0.2% polyvinylpyrrolidone (PVP) to gain the crude plant extracts. The homogenates were centrifuged at 4000 rpm for 15 min at 4 °C and the resulting supernatants were used to measure the activities of antioxidant enzymes and the protein content.

The SOD activity was assayed by the means of photoreduction prohibition nitrobluetetrazolium (NBT) [20]. The reaction buffer contained 2.65 ml of 67 mmol/L potassium phosphate buffer solution (pH 7.8), 0.1 mL of 1.5 mmol/L NBT, 0.2 mL of 0.1 mmol/L EDTA solution containing 0.3 mmol/L sodium cyanide, 50 μL of 0.12 mmol/L riboflavin, and a suitable aliquot of enzyme extract. The reaction mixture was illuminated for 15 min at the light intensity of 5000 Lux. The absorbance was measured

at 560 nm. One unit of SOD equals to the volume of enzyme stopping 50% of the NBT depletion under the analysis state. The control assay was done in the absence of plants extract to prevent possible auto-oxidation of the substrates.

The POD activity was calculated following the polymerization of guaiacol to tetraguaiacol using the method [21]. The amount of enzyme that can generate $1\ \mu\text{mol L}^{-1}$ tetraguaiacol min^{-1} [$\epsilon=26.6$ ($\text{mmol L}^{-1})^{-1}\ \text{cm}^{-1}$] equals to the level of one unit of POD activity.

In addition, CAT activity was measured by following the dismutation of H_2O_2 at 240 nm for 3 min using the UV absorbance method. One unit of CAT was the amount of enzyme for the dismutation of $1\ \mu\text{mol/L}$ H_2O_2 per min. The extinction coefficient for H_2O_2 at 240 nm was considered $39.4\ \text{M}^{-1}\ \text{cm}^{-1}$ [22]. Protein content was determined according to the method [23], using bovine serum albumin (BSA, Sigma Aldrich) as a standard protein.

Phenol and Flavonoid Contents

Phenol content of the plants methanol extracts was determined by using Folin- Ciocalteu method [24]. Standard curve for determination of phenolic contents was prepared using different concentration of gallic acid and the absorbance was measured at 720 nm. 2 mg of each extract was dissolved in absolute methanol (1 mL). Then, 200 μL of each crude sample was taken in a test tube and added 2% Na_2CO_3 (100 μL). Subsequently, 50% Folin-Ciocalteu reagent (100 μL) was added to the tubes and the tubes were kept in the room temperature and dark place for 30 min. The absorbance was measured for all solutions by spectrophotometer at 720 nm. Phenol content was expressed as μg gallic acid per mg extract ($\mu\text{g mg}^{-1}$ extract).

The aluminum chloride colorimetric assay was used for determination of flavonoid content of the plant's extracts [25]. 250 μL of each extracts with suitable dilution rate was placed in to separate test tubes and 75 μL NaNO_3 (5 %), 150 μL AlCl_3 (10%) and 500 μL NaOH (1 M) were added to each tube. Finally, volume was making up to 2.5 mL with distilled water. Orange yellowish color was developed after a proper time. The absorbance was determined at 507 nm. The calibration curve was plotted using standard quercetin. Flavonoids content of the extracts was expressed as ng quercetin per mg of extract (ng mg^{-1} extract).

Malondialdehyde (MDA) Rating

The formation of malondialdehyde (MDA) was evaluated for estimation of lipid peroxidation. The

plant samples were homogenized using 1mL of 20% trichloroacetic acid and the crude extracts were mixed with 1 mL of 0.67% thiobarbituric acid solution. After heating for 30 min and rapid cooling, the absorbance of the supernatant was measured at 532 nm. The MDA concentration was determined using an extinction coefficient of $155\ \text{mmol/L cm}^{-1}$ [26].

Statistical Analysis

Data with four replicates were statistically analyzed by one-way analysis of variance (ANOVA), followed by Tukey- Kramer multiple comparisons test using GraphPad software (GraphPad Software, Inc. USA). The results were described as mean \pm standard deviation (SD). Significant difference was reported when the probability was less than 0.05.

RESULTS

Characterization of the Treated Materials

SEM images of Bulk-NiO and NiO nanoparticles were shown in Fig. 1a and 1b, respectively. The size of examined nanoparticles was in the nanometer range and the major particle size distribution of nanoparticles was in the range of 10-20 nm (Fig. 1b and 1c). The nearly spherical shape of nanoparticles was clearly observed in TEM image (Fig. 1d).

The Effects on the Growth of the Plant

The treatment effects of different concentrations of Bulk-NiO and NiO-NPs on RFN of *L. minor* and *S. polyrhiza* are illustrated in Table 1. RFN was decreased by increasing the concentrations of Bulk-NiO and NiO-NPs and all examined concentrations of NiO-NPs had significant negative effects on RFN. It is while, among 3 treated concentrations of Bulk-NiO, only 50 mg/L was led to the notable reduction of RFN. For instance, after 20 d of exposure to 50 mg/L of Bulk-NiO and NiO-NPs, RFN was significantly reduced to 29.5% and 41.9%, respectively. Therefore, the negative effects of NiO-NPs on RFN of *L. minor* were more than the effects of Bulk-NiO.

After 20 d treatment of *S. polyrhiza* with various concentrations of Bulk-NiO, only the concentration of 50 mg/L was led to remarkable reduction of RFN in *S. polyrhiza* (reduction to 31.2%) as compared to the control. In contrast, RFN was decreased up to 17.7% and 46.4% after 20 d treatment with 10 and 50 mg/L of NiO-NP, respectively. Low concentration of Bulk-NiO and NiO-NP (1 mg/L) had no significant negative effect on RFN of two plant species.

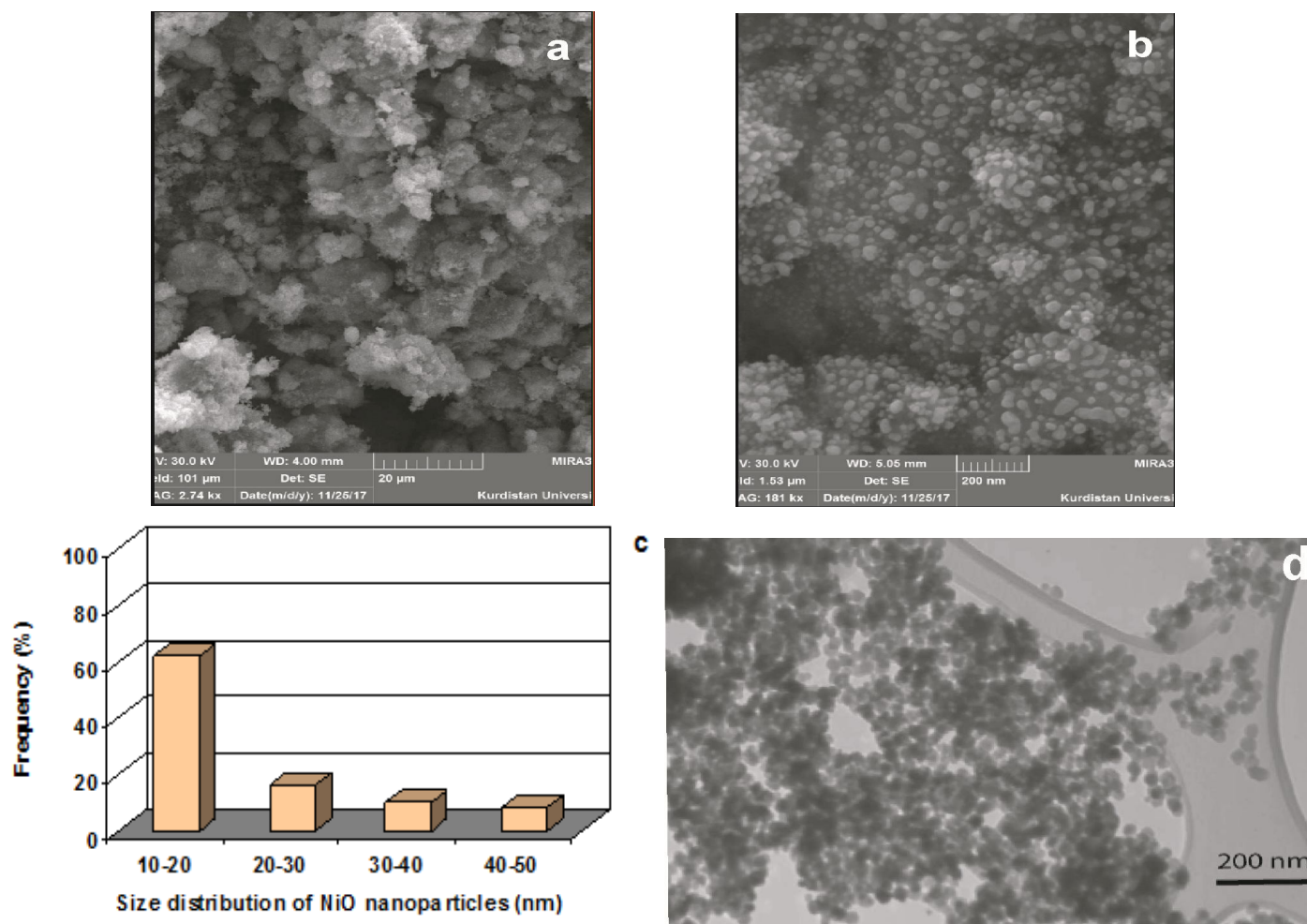


Figure 1. SEM images of a) Bulk-NiO, b) NiO-NPs; c) size distributions of NiO-NPs and d) TEM image of NiO-NPs.

Table 1. Effect of three concentrations of NiO-NP and Bulk-NiO (1, 10 and 50mg/L) on relative frond number (RFN) of *L. minor* and *S. polyrhiza* plants.

Day	RFN of <i>L. minor</i>				RFN of <i>S. polyrhiza</i>											
	NiO-NPs (mg/L)				Bulk-NiO (mg/L)				NiO-NPs (mg/L)				Bulk-NiO (mg/L)			
	0	1	10	50	0	1	10	50	0	1	10	50	0	1	10	50
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4th	3	2.5	1.9	1.1	3	2.8	2.3	1.7	2.75	2.3	1.7	1.52	2.75	2.61	2.2	1.63
8th	4.5	4	2.85	2.1	4.5	4.02	3.2	2.8	3.12	2.61	2.2	1.74	3.12	2.89	2.61	2.15
12th	6.5	5.3	4.5	2.9	6.5	5.87	5.5	4.2	3.66	3.3	2.55	1.84	3.66	3.32	2.86	2.33
16th	7.65	6.97	5.8	2.88	7.65	7.22	6.9	5.3	4.12	3.85	3.2	2.21	4.12	3.74	3.21	2.78
20th	7.95	7.56	6.2	2.86	7.95	7.79	7.69	5.6	4.8	4.55	3.7	2.57	4.8	4.7	4.57	3.3

The Effect on Photosynthetic Pigments Content

After exposure of the plants to 1, 10 and 50 mg/L of Bulk-NiO and NiO-NP, the pigments content was determined. After 7 d exposure of *L. minor* by high concentration of NiO-NP (50 mg/L), the amounts of all photosynthetic pigments were significantly decreased compared to the control sample (Table 2). In contrast, 50 mg/L treatments of Bulk-NiO were led to remarkable increase of Chl *a* and Chl *b* and

carotenoids near to 39.2%, 53.6%, and 55.5%, respectively.

In accordance with the results of the NiO-NPs and Bulk-NiO treatments in *L. minor*, the amounts of Chl *a* and carotenoids of *S. polyrhiza* were notably decreased after 7 d exposure to 50 mg/L of NiO-NP (Table 2). The amounts of all pigments content were enhanced by the treatment with 50 mg/L of Bulk-NiO. Low concentration of two groups of examined contaminates (1 mg/L) had no significant effects on photosynthetic pigments of *S. polyrhiza* ($P > 0.05$).

Table 2. Contents of chlorophyll a, b and total carotenoids in control *L. minor* and *S. polyrhiza* plants and the plants exposed to 1, 10 and 50 mg/L of NiO-NP and Bulk-NiO for 7 d (Mean \pm SD, n=4, *Significant difference at $P<0.05$, ** Significant difference at $P<0.01$, *** Significant difference at $P<0.001$).

Concentration of contaminants (mg/L)	Pigments content of <i>L. minor</i> (mg g ⁻¹ FW)			Pigments content of <i>S. polyrhiza</i> (mg g ⁻¹ FW)			
	Chl a	Chl b	Crotenoids	Chl a	Chl b	Crotenoids	
0 (control)	9.48 \pm 0.25	5.6 \pm 0.30	2.7 \pm 0.24	36.8 \pm 1.90	12.6 \pm 0.65	5.3 \pm 0.10	
Bulk-NiO	1	9.68 \pm 0.09	5.84 \pm 0.10	2.81 \pm 0.17	37.3 \pm 2.00	12.78 \pm 0.49	5.42 \pm 0.11
	10	11.3 \pm 0.13***	6.36 \pm 0.18	3.1 \pm 0.10	41.03 \pm 1.11	14.3 \pm 0.17**	6.1 \pm 0.12***
	50	13.2 \pm 0.12***	8.6 \pm 0.40***	4.2 \pm 0.09***	45.6 \pm 2.20**	17.1 \pm 0.45***	6.7 \pm 0.13***
NiO-NP	1	10.12 \pm 0.09*	6.1 \pm 0.06*	2.9 \pm 0.21	41.6 \pm 0.90	13.01 \pm 0.36	5.6 \pm 0.14
	10	15.3 \pm 0.19***	8.37 \pm 0.28***	3.6 \pm 0.17**	48.8 \pm 2.50***	16.8 \pm 1.10***	6.4 \pm 0.10***
	50	8.68 \pm 0.23**	5.05 \pm 0.02*	2.2 \pm 0.17*	30.1 \pm 2.20***	19.3 \pm 0.70***	4.69 \pm 0.30*

Enzymatic Analysis

The activity of SOD, POD, and CAT was assayed at different concentrations of Bulk-NiO and NiO-NP. The effects of their different concentrations on the SOD, POD and CAT activities are illustrated in Fig. 2. According to Figure 2a, enhancement in the concentrations of the contaminants in each of two examined groups (Bulk-NiO and NiO-NP) was led to the notable increase in SOD activity in that group. The treatments of 10 and 50 mg/L of NiO-NP significantly increased the SOD activity in both examined plant species. For instance, after 7 d treatments of 50 mg/L of NiO-NP, SOD activity was enhanced near to 2.4 and 2.2-fold in *L. minor* and *S. polyrhiza*, respectively, as compared with the control samples. However, only 50 mg/L of Bulk-NiO could significantly induce the enzyme activity after 7 d exposure of the plants.

POD activity showed a pattern almost similar to the activity of SOD. Its activity was increased by augmentation of NiO-NP and Bulk-NiO concentrations in both plant species except for the treatment of 50 mg/L of NiO-NP in *L. minor* (Fig. 2b). POD activity in *L. minor* treated with 50 mg/L of NiO-NP was suppressed. The treatment of NiO-NP and Bulk-NiO in low concentration (1 mg/L) had no notable effect on POD activity in two plant species ($P>0.05$). The only high concentration of NiO-NP could induce the CAT activity in both plant species (Fig. 2c). Other concentrations of NiO-NP along with all treated amounts of Bulk-NiO had no remarkable effects on the plant's CAT activities (Fig. 2c).

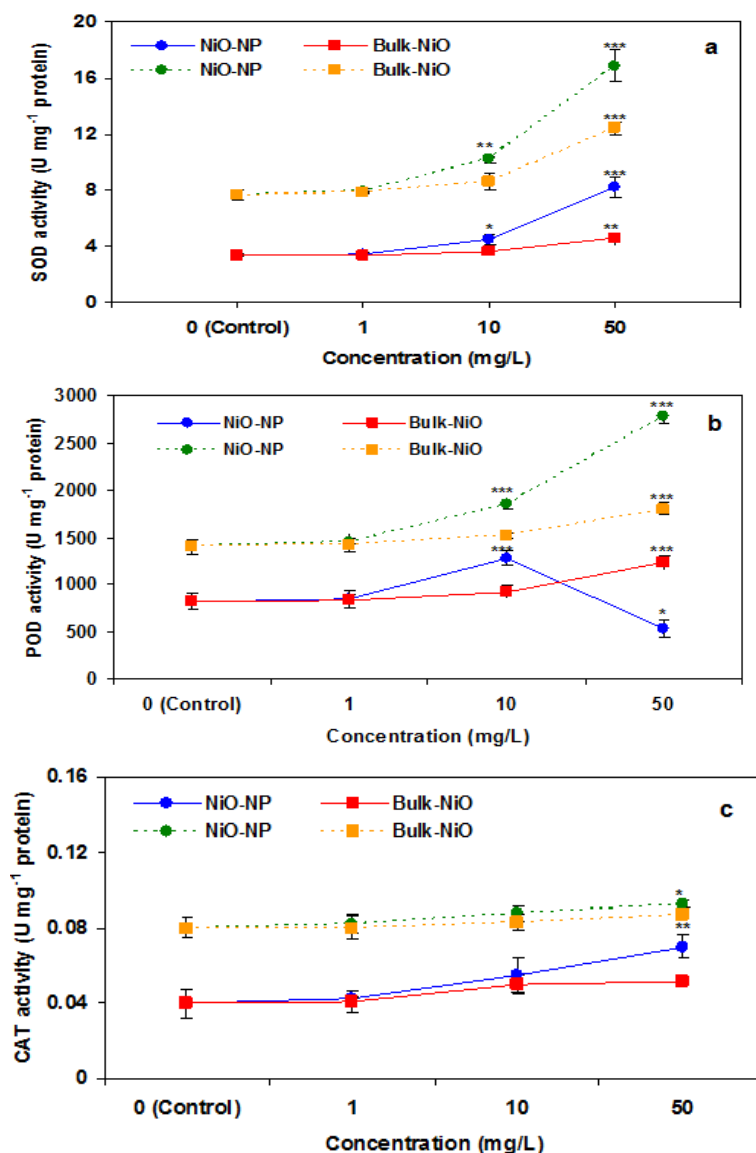


Figure 2. The activities of (a) SOD, (b) POD and (c) CAT in control *L. minor* and *S. polyrhiza* plants and the plants exposed to different concentrations of NiO-NP and Bulk-NiO (1, 10 and 50mg/L) for 7 d (Mean \pm SD, n=4, * Significant difference at $P<0.05$, ** Significant difference at $P<0.01$, *** Significant difference at $P<0.001$, Solid lines and Dashed lines related to *L. minor* and *S. polyrhiza* samples, respectively).

Phenol and Flavonoid Contents

After 7 d exposure of the plants with both NiO-NPs and Bulk-NiO, the phenol content was notably raised by enhancement of the contaminants concentration (from 1 to 50 mg/L) in an elevating gradient manner (Fig. 3a). Treatment of 10 and 50 mg/L of NiO-NP in two plant species was led to the statistically significant enhancement of the phenol contents. The content was augmented just by the treatment of high concentration (50 mg/L) of Bulk-NiO. Treatment of NiO-NPs produced more ROS in two examined plant species and had considerable negative impacts on those plants.

Similar to the pattern of phenol content increment, flavonoids content was increased by increasing the concentration of treated contaminants (Fig. 3b). The content in *L. minor* and *S. polyrhiza* was raised near to 3.8 and 3.3-fold after treatment of 50 mg/L NiO-NP, respectively. These amounts were 1.9 and 2.6-fold for the treatment of high concentration of Bulk-NiO.

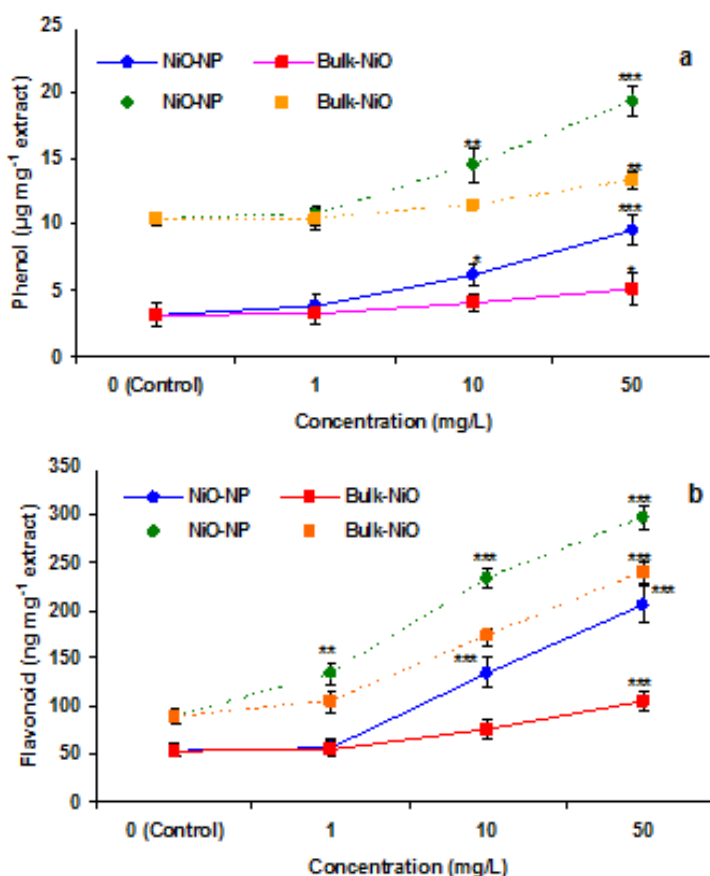


Figure 3. The effect of different concentrations of NiO-NP and Bulk-NiO (1, 10 and 50mg/L) on the plants a) Phenol and b) flavonoid content after 7 d treatment (Mean \pm SD, n=4, * Significant difference at $P<0.05$, ** Significant difference at $P<0.01$, *** Significant difference at $P<0.001$, Solid lines and Dashed lines related to *L. minor* and *S. polyrhiza* samples, respectively).

The Effect on Membrane Integrity

In order to determine the effects of NiO-NP and Bulk-NiO (1-50 mg/L) on membrane integrity of *L. minor* and *S. polyrhiza*, MDA content was measured. By increasing the concentration of two groups of examined contaminants, MDA content was improved in the plants. Accordingly, no notable changes in the MDA content were observed after 7 d treatment of two plant species with 1 mg/L of NiO-NP and Bulk-NiO. In contrast, high concentration of NiO-NP was led to statistically significant augment of MDA content in two examined plants (Fig. 4). However, among different treated concentrations of Bulk-NiO in *L. minor* and *S. polyrhiza*, only the treatment of 50 mg/L in *S. polyrhiza* increased MDA content compared to the control sample ($P<0.05$).

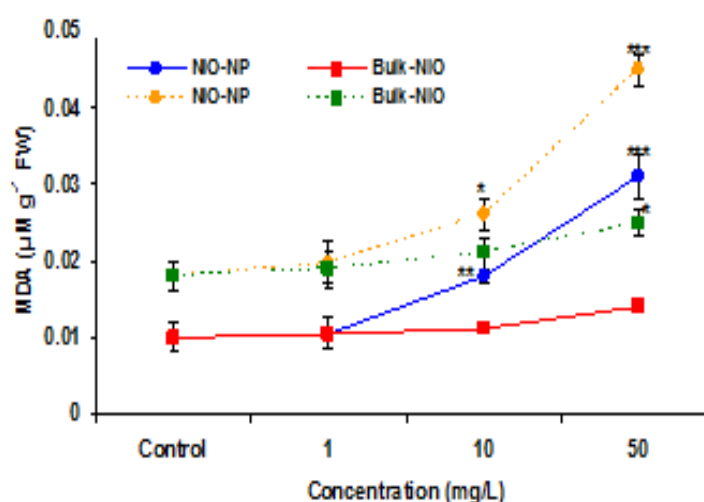


Figure 4. The effect of different concentrations of NiO-NP and Bulk-NiO (1, 10 and 50mg/L) on the plants MDA content after 7 d treatment (Mean \pm SD, n=4, * Significant difference at $P<0.05$, ** Significant difference at $P<0.01$, *** Significant difference at $P<0.001$, Solid lines and Dashed lines related to *L. minor* and *S. polyrhiza* samples, respectively).

DISCUSSION

Due to high production and usage of nanomaterials in recent years, their entrance in ecosystem is unavoidable and existence of these materials is one of the reasons of environmental pollution. Therefore, nanomaterials interaction with organisms should be studied for evolution of their negative impacts on biological systems.

Treatment of the plants with NiO-NPs was led to adverse impact in two examined plant species. The negative effects of the nanoparticles on plants could be related to the release of ions from them and/or their direct interactions with plants [27]. Inhibition of frond multiplication and reduction of plant fresh weight were reported for the *L. minor* and *S.*

polyrhiza treated with different concentrations of Ni ion [28]. Moreover, nanoparticles can be possibly adhered on to the root surface of plants and inhibited root growth. Plant root growth inhibition can subsequently block water transport pathways and decrease mineral absorption, thus affecting the growth of the whole plant [29].

About the effects of different concentrations of NiO-NPs and Bulk-NiO on photosynthetic pigments content of the plants, the treatment of high concentrations of NiO-NP, was led to the significant reduction of the content of photosynthetic pigments. The dissolution of Ni ions may play a significant role in the toxicity of nanoparticles, the inadequacy of chloroplasts in response to surplus Ni^{2+} [28]. Accordingly, Ni^{2+} strongly influences the thylakoid systems in some species of Lemnaceae family and it was led to the chloroplasts transform into chloro-amyloplasts, amylo-chloroplasts or even amyloplasts with some stroma and a rudimentary thylakoid system. Therefore, the content of photosynthetic pigments especially Chlorophyll a was decreased after application of Ni^{2+} [28].

Nanomaterials induce toxicity by ROS generation and consequent production of oxidative stress in many biological systems. The overproduction of ROS is regarded as one of the principal causes of cellular damages [30]. In fact, ROS induce the biological defense system and antioxidant responses against to reactive intermediates and for damage repair. On the other hand, dissolution of metal ions from nanomaterials and/or their direct interactions with organisms are other possible mechanisms of nanotoxicity [30]. The activities of antioxidant enzymes such as SOD, POD, and CAT are usually changed in response to oxidative stress and these changes have been proposed as biomarker of oxidative stress [31]. SOD activity increased by enhancement of treated concentration of Bulk-NiO and NiO-NPs, a similar trend of the results has been previously reported in the case of different plant species treated with Ni^{2+} [32]. SOD catalyzes the conversion of superoxide anion radicals to molecular oxygen and hydrogen peroxides which then are detoxified by CAT, POD or other antioxidative enzymes [11].

POD activity also increased by increasing the concentration of two groups of the pollutants, but its activity was suppressed just in *L. minor* treated with 50 mg/L of NiO-NP. It could be due to the weakness of the plant defense system in scavenging ROS produced in the high concentration of NiO-NP. In fact, the reduction of POD activity at concentration of 50 mg/L NiO-NP could be the outcome of high

ROS production which might decompose enzyme structure. Some previous studies are consistent with our obtained results such as the falling in POD activity in *L. minor* treated with high concentration of ZnO-NPs and Si-NPs after a raise in SOD activity [33,34]. In contrast, low concentration (1 mg/L) of NiO-NP and Bulk-NiO was not induce POD activity in the examined plants. Possibly, the amounts of ROS produced during such treatment in the plants were not enough to induce the POD activity.

About the effects of NiO-NP and Bulk-NiO, the results showed that only high concentration of NiO-NP could induce the CAT activity in both plant species. It means ROS generation was stimulated by high concentration of NiO-NP. Some results have been published in agreement with the present study. For instance, CAT activity was raised in *L. gibba* treated with Ag and ZnO-NPs [9].

Phenol and flavonoid contents can be influenced by different stress situations such as plants exposure to different contaminants. In that conditions, phenolic compounds operate for conservative purposes against stress and detoxification of ROS in plant cells [34]. Treatment of two plant species with high concentrations of NiO-NP (10 and 50 mg/L) was led to the statistically significant enhancement of the phenol and flavonoid contents in two plant species were increased by enhancement of the NiO-NP and Bulk-NP concentrations. The enhancement of total phenol and flavonoids content has been occurred for detoxifying and removing of ROS as a defense mechanism in resistance to toxicity. Increments in phenolic and flavonoid contents were previously reported for *Brassica nigra* after treatments with ZnO-NPs [35].

MDA content was improved in the plants by increasing the concentration of two groups of examined contaminants. This gradient increment could be due to the escalated oxidative stress and lipid peroxidation. MDA content was correlated with ROS accumulation and ROS incursion as a result of poisoning the plants by contaminants was led to the cell membrane destruction [34].

CONCLUSION

The biochemical and plant physiological responses of *L. minor* and *S. polyrhiza* to NiO-NPs and Bulk-NiO were investigated in the present study. Phytotoxic effects of the examined contaminants on the plants were proved by reducing growth of plants as well as photosynthetic pigments content, in contrast, to increasing in total phenol and flavonoid contents and MDA. The negative effects of the NiO-NPs on *L. minor* and *S. Polyrhiza* were more than

those of Bulk-NiO. The content of photosynthetic pigments in both of the plant species was significantly reduced by high concentration of NiO-NP. The reduction of the pigments content could be one of the important reasons for the other negative responses such as reduction of the growth. RFN of the plants was reduced by increasing the concentrations of NiO-NPs and Bulk-NiO. Moreover, increasing the concentration of the contaminants in the plant species was led to the remarkable enhancement of total phenol and flavonoid and MDA contents. In the case of antioxidant enzymes activities, inductions of SOD activity by enhancement of the concentration of the contaminants verify SOD significance in the permanence of the plants to the treated contaminants. POD and CAT activities were increased at 50 mg/L of NiO-NP except the treatment of high concentration of NiO-NP that was led to the reduction of POD activity. It could be due to the high ROS production which might decompose enzyme structure. Research on examined NPs aggregation in the natural aquatic environment, sediment toxicity, and their bioaccumulation may provide additional valuable information.

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