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Original Research

Investigating the Toxicity of Phenol-Loaded and Phenol-Free TiO_2 and ZnO Nanoparticles Using Bioassay Experiments

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Abstract

This study investigates the potential toxicity of TiO_2 and ZnO nano-particles (nano-TiO_2 and nano-ZnO) against *Daphnia magna* (*D. magna*) neonates before and after absorbing phenol. Although limited scientific investigation is conducted on possible hazards of nano-particles (NPs), no research has been carried out either on the toxicity of nano-ZnO and TiO_2 in combination with other materials or on their ability to release the hazardous substances adsorbed on their surface in cells.

Daphnia magna neonates exposed to different concentrations of phenol-free and phenol-loaded NPs and resulting mortality rates were recorded after 12 to 96 h. The results of mortality rates were applied to SPSS ver. 16.0 in order to calculate median lethal concentrations (LC_{50}) of NPs.

The results of experiments showed that phenol-free TiO_2 was "practically nontoxic" according to EPA overview (48 h LC_{50}: 2705 $mg \cdot L^{-1}$). But after exposure to phenol, its 48 h LC_{50} reached 414 $mg \cdot L^{-1}$, which means a 6-time increment in toxicity. 48 h LC_{50} of phenol-loaded and phenol-free nano-ZnO was 2.14 and 2.18 $mg \cdot L^{-1}$, respectively.

This study showed, in contrast to nano-TiO_2, that no significant difference is found between the toxicity of nano-ZnO before and after exposure to phenol. Researchers considered the amount of toxin absorbability of NPs to be one of the most important factors influencing the change in NP toxicity.

Keywords: LC_{50}, NPs, combined toxicity, *Daphnia magna*, exposure to phenol

Introduction

Zinc oxide and Titanium oxide are two of the important nano-particles (NPs) used on an industrial scale in many countries [1]. ZnO (nano-ZnO) NPs have great potential to enter aqueous media. In fact, Italian researchers showed that 25% of sunscreen creams consumed to protect skin are washed out through bathing and swimming; this figure corresponds to 250 tons of these materials entering aqueous environments [2]. Considering the similar application of nano-ZnO and TiO_2 in cosmetic and household products, TiO_2 might also have a great potential to enter aqueous media [3]. According to Lee et al. [4], nano-TiO_2 is also produced as a Titanium tetrachloride ($TiCl_4$) byproduct with $TiCl_4$ having an efficient and cost-effective application in wastewater as coagulant. On the other hand, many parti-

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cles made as NPs have useful catalytic and adsorptive properties compared to their bulk state [2].

Phenol is one of the most toxic compounds [5], and its concentration in the wastewater of some industries such as in the petroleum refinery where it is disposed to the environment reaches more than 1000 mg·L^{-1} [6]. In addition, NPs are increasingly used in material chemistry and refining [7]. Considering the highly adsorptive properties of NPs, their presence in various ecosystems, widespread presence of phenol in industrial wastewaters, and mixing of various industrial wastewaters to equalize wastewaters, the adsorption of phenol on NPs is a possibility.

This research aims to investigate the probable changes occurring in NP toxicity due to contact with poisons. Researchers exposed ZnO and TiO_2 NPs to phenol and determined the value of their median lethal concentration (LC_{50}) after 12-96 h using *D. magna*. The change in level of toxicity could be determined by comparing LC_{50}s of phenol-loaded and phenol-free NPs. Although limited scientific investigation is conducted on possible hazards of NPs, no research has been carried out either on the toxicity of nano-ZnO and TiO_2 in combination with other materials or on their ability to release the hazardous substances adsorbed on their surface in cells.

Materials and Methods

The laboratory of Isfahan University (Isfahan-Iran) supplied the researchers with ZnO and TiO_2 NPs. The laboratory reported the size of the NPs to be 50-70 nm as measured by the dynamic light scattering (DLS) method (Malvern Zetasizer ZS, Malvern UK). Stock solution (10 g L-1, pH: 6.5) of NP was placed in an ultrasonic instrument for 30 minutes at 65ºC. In order to prepare phenol-loaded TiO_2, a mixture of phenol and NPs containing 10 g·L^{-1} of each substance was stirred by a magnetic stirrer in a sealed volumetric flask using the batch method for 30 h. The pH of nano-suspensions was adjusted to 5.8. Dynamics of phenol uptake by NPs was determined by sampling at 1-30 h. After these time periods the amount of phenol residue in the solution was measured by a spectrometer apparatus (Perkin Elmer-lambda 25-UV/VIS spectrometer) in accordance with the standard number C-6420 of the Standard Methods [8]. The amount of phenol adsorbed on per gram of NPs was calculated by comparing phenol concentrations before and after contact. The phenol-loaded nano-ZnO was also prepared accordingly. In addition, to determine the possible toxic effects of desorbed phenol, the effective amount of phenol released from the applied NP to the culture media under test conditions and contained in the phenol-loaded NP suspensions was determined in 6, 12, 24, 48, 72, and 96 h. The maximum concentration of released phenol was added into control samples for detecting its probable toxicity. In the control samples there were no NPs.

In order to eliminate the un-adsorbed phenol, the mixture of NPs and phenol was centrifuged at 2000 rpm for 5 minutes before the excess clear solution was decanted. NPs and phenol residue in each vial were diluted by de-ionized water and centrifuged again. This process was repeated until the concentrations of phenol sample in the culture medium in each test approached 0.1 mg·L^{-1}. The same amount of phenol was added to the plain samples without affecting daphnia's mortality.

Mortality due to TiO_2 NPs was tested at 9 concentrations within the range of 50 to 8,000 mg·L^{-1}. For nano-ZnO, 9 concentrations in the range of 0.5 to 5 mg·L^{-1} were applied. A toxicity test was performed according to the standard number 8,711 of the Standard Methods [8]. To determine the acute toxicity by daphnia, a 48 h exposure is an acceptable time period [8]. However, in the current research exposure times of 12, 24, 48, 72, and 96 h were applied in order to study the time effect. Since the NPs are able to precipitate, the deposited NPs were stirred once every 8 h to increase the contact between the particles and daphnia.

Preparation and growth of *D. magna* was performed according to the standard number 8711 of the Standard Methods [8]. After preparation of test solutions, the neonates that had been released from the bearing embryos during the past 24 h at 20-25ºC were inoculated into each plate with the same number (at least 10).

The experiment for each concentration was repeated 3 times. Mean values for experiments were determined. In order to determine the relationship between the amounts of toxicity and concentration, Pearson correlation coefficient was calculated by SPSS ver.16.0 software. The results of mortality rates were applied to SPSS ver 16.0 in order to calculate phenol-loaded and phenol-free NP LC_{50} based on probit. The results obtained from probit analysis were used to plot diagrams in Microsoft Excel 2007. Non-observed effect concentration (NOEC) was also determined by probit analysis via calculation of concentration in which the mortality rate was 10%. In addition, the 100% mortality rate was determined by calculation of a concentration in which the mortality rate was 99%, using probit analysis. The LC_{50}s of NPs in the case of phenol-free and phenol loaded were compared at different conditions in confidence intervals of 95% using t-test.

Results and Discussion

DLS results showed that the size of NPs have not been changed after preparing their phenol-loaded suspensions (Figs. 1a and b). According to these Figures the size of nano-ZnO and TiO_2 were 69.0 and 53.6 nm, respectively; however, after 48 h results of this method showed an agglomeration among phenol-loaded and phenol-free NPs. (after 48 h, the average size of phenol-free nano-ZnO and TiO_2 were 151 and 258 nm respectively; that is, increasing the Polydispersity index (PDI) and multiple peaks in Figs. 1c and 1d proved that NPs agglomerate in the suspensions). These results demonstrated that agglomeration of nano-TiO_2 is higher than nano-ZnO. The results of phenol-loading show a similar occurrence with a slighter agglomeration (figures were not shown).

Table 1. *D. magna* death ratio (percent) of the three performed experiments due to exposure to phenol-loaded and phenol-free nano-ZnO and nano-TiO_2 at different periods of time.

NPs	Parameter	Phenol-free nano-ZnO					Phenol-loaded nano-ZnO				
Nano-ZnO	Time / Concentration ($mg \cdot L^{-1}$)	12-h	24-h	48-h	72-h	96-h	12-h	24-h	48-h	72-h	96-h
	0.5	0.0	0.0	0.0	20.0	33.3	0.0	3.3	3.3	17.0	27.0
	0.8	0.0	10.0	20.0	23.3	57.0	0.0	13.3	23.3	27.0	60.0
	1	10.0	13.3	20.0	30.0	83.3	13.3	17.0	27.0	30.0	87.0
	1.5	17.0	20.0	23.0	57.0	100.0	23.3	23.3	27.0	50.0	100.0
	2	20.0	26.6	27.0	90.0	100.0	30.0	27.0	30.0	83.0	100.0
	2.5	33.3	40.0	57.0	100.0	100.0	43.3	50.0	50.0	100.0	100.0
	3	60.0	70.0	87.0	100.0	100.0	73.3	77.0	80.0	100.0	100.0
	4	80.0	87.0	100.0	100.0	100.0	93.3	90.0	97.0	100.0	100.0
	5	87.0	100.0	100.0	100.0	100.0	97.0	100.0	100.0	100.0	100.0
Nano- TiO_2	Parameter	Phenol-free nano-TiO_2					Phenol-loaded nano-TiO_2				
	Time / Concentration ($mg \cdot L^{-1}$)	12-h	24-h	48-h	72-h	96-h	12-h	24-h	48-h	72-h	96-h
	50	0.0	0.0	0.0	0.0	0.0	0.0	0.0	23.3	23.3	33.3
	200	0.0	0.0	0.0	0.0	0.0	0.0	13.3	33.3	46.6	50.0
	500	0.0	0.0	0.0	0.0	0.0	10.0	33.3	66.6	66.6	73.3
	1000	0.0	0.0	0.0	3.3	3.3	40.0	76.6	96.6	100.0	100.0
	1500	0.0	0.0	3.3	3.3	16.6	66.6	86.6	100.0	100.0	100.0
	2500	0.0	6.6	16.6	20.0	46.6	86.6	100.0	100.0	100.0	100.0
	4000	3.3	40.0	56.6	60.0	86.6	100.0	100.0	100.0	100.0	100.0
	6000	20.0	66.6	80.0	86.6	93.3	100.0	100.0	100.0	100.0	100.0
	8000	53.3	90.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 2. LC_{50} ($mg \cdot L^{-1}$) of phenol-free and phenol-loaded nano-TiO_2 at different periods of time using *Daphnia magna* with 95% confidence intervals.

NPs / Time	Phenol-free nano-ZnO	Phenol-loaded nano-ZnO	Phenol-free nano-TiO_2	Phenol-loaded nano-TiO_2
12-h	3.00 (2.69-3.37)	2.51 (2.26-2.980)	6106 (5435-6945)	1387 (1093-1780)
24-h	2.58 (2.35-2.85)	2.40 (2.17-2.70)	3478 (2740-4386)	781 (571-1032)
48-h	2.14 (1.77-2.62)	2.18 (1.80-2.67)	2705 (2222-3348)	414 (371-458)
72-h	1.25 (1.04-1.46)	1.31 (1.09-1.54)	2515 (2196-2909)	355 (316-395)
96-h	0.69 (0.63-0.74)	0.69 (0.64-0.74)	1838 (1296-2648)	330 (294-367)

Death percentages of *D. magna* population at specified concentration and time due to exposure to NPs are shown in Table 1.

The LC_{50}s of phenol-free nano-ZnO and nano-TiO_2 after 12 to 96 h are illustrated in Table 2. According to Figs. 2a and b, both nano-ZnO and nano-TiO_2 suspensions exhibit a concentration-dependent toxicity for *D. magna.* Regarding the slope of curves, visually it can be inferred that this dependency increases over time (from 12 to 96 h). Measurement of effective amounts of phenol released from the applied NMs to the culture media in 6-96 h showed that the maximum concentration of released phenol is 0.3 $mg \cdot L^{-1}$,

but according to our experiments adding this concentration into control samples was not toxic to daphnia even after 96 h exposure.

The probit analysis results of phenol-loaded NPs are illustrated in Figs. 2c and d in which a concentration and time-dependent toxicity can be seen for phenol-loaded nano-ZnO and nano-TiO_2.

In the case of phenol-free NPs, the slope of curves suggests that the concentration dependency of toxicity increases over time (from 12 to 96 h). Measurement of effective amounts of phenol released from the applied NMs to the culture media in 6-96 h showed that the maximum concentration of released phenol is 0.3 mg·L^{-1}; however, according to these experiments adding this concentration into control samples was no longer toxic to daphnia after 96 h exposure.

Table 2 presents the results for LC_{50} of phenol-loaded and phenol-free nano-ZnO and TiO_2. This research showed no difference between toxicity of phenol-loaded and phenol-free nano-ZnO. Since the LC_{50} of phenol-free nano-ZnO is within the range of 3.00 mg·L^{-1} in 12 h to 0.69 mg·L^{-1} in 96 h and its LC_{50} changes slightly after exposure to phenol and alters to 2.51 mg·L^{-1} in 12 h to 0.69 mg·L^{-1} in 96 h, this change is not proved to be statistically significant ($P>0.05$).

Findings reveal that the toxicity of nano-TiO_2 increases after exposure to phenol. Table 2 demonstrates the 48 h LC_{50} of nano-TiO_2 of higher than 2,705 mg·L^{-1} to decrease to 414 mg·L^{-1} after exposure to phenol. This change is statistically significant ($p<0.05$).

According to the experiments, 48 h LC_{50} of nano-ZnO was determined to be 2.4 mg·L^{-1}. In addition, NOEC after

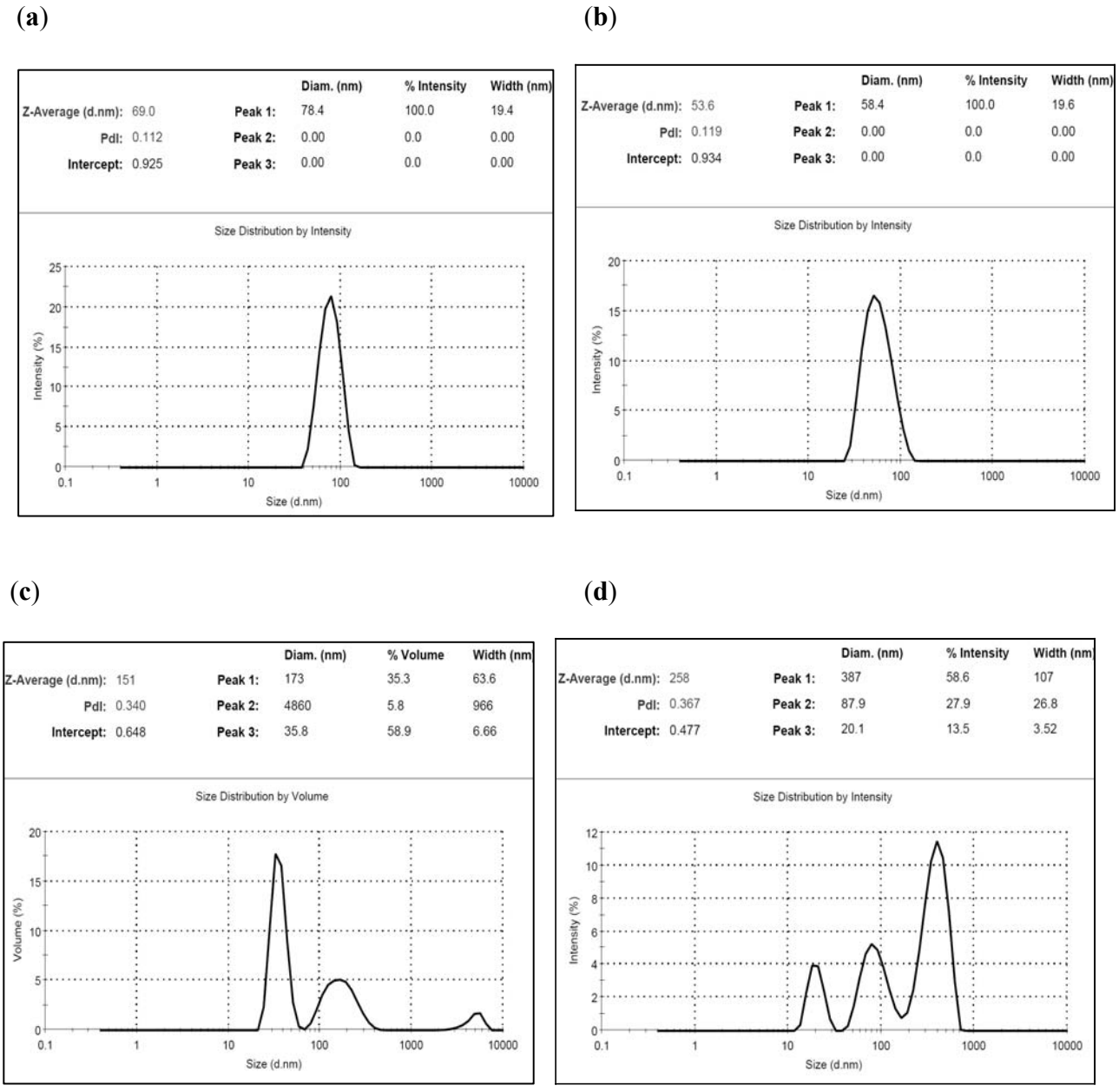

Fig. 1. Particle size of phenol-loaded NPs before being applied to the test culture (a: nano-ZnO and b: nano-TiO_2) and after 48 h in suspension (c: nano-ZnO and d: nano-TiO_2) of Malvern instrument DLS.
Z-average – The average size of NPs.

Table 3. NOEC and 100% mortality values ($mg \cdot L^{-1}$) for *Daphnia magna* due to exposure to phenol-free and phenol-loaded nano-ZnO and TiO_2 at different periods of time.

NPs	Phenol-free nano-ZnO		Phenol-loaded nano-ZnO		Phenol-free nano-TiO_2		Phenol-loaded nano-TiO_2	
Time	NOEC (mg/L)	100% mortality* (mg/L)	NOEC (mg/L)	100% mortality* (mg/L)	NOEC (mg/L)	100% mortality* (mg/L)	NOEC (mg/L)	100% mortality* (mg/L)
12-h	1.22	>6.24	1.10	>5.08	3351	>11108	476	>3041
24-h	1.11	>5.26	0.84	>5.22	1488	>7090	156	>1916
48-h	0.86	>4.46	0.58	>5.10	1253	>5342	41	>1090
72-h	0.42	>2.75	0.4	>2.95	1150	>4993	25	>954
96-h	0.25	>1.48	0.33	>1.36	653	>3990	25	>878

* x>99% mortality in Probit analysis

48 h was determined to be 0.86 $mg \cdot L^{-1}$ and 100% mortality was observed at concentrations higher than 4.46 $mg \cdot L^{-1}$ (Table 3). These values are much lower than the results obtained by Heinlaan et al. [1]. This difference is not only due to the genetic and individual differences between the daphnia used, but also may be related to differences in the chemical conditions of daphnia's environment and the properties of NPs [9]. It also has been shown that the toxicity of NP mostly depends on the amount of metal ions released from it [10].

In contrast to nano-ZnO, the conducted experiments in the present study did not show a high toxicity for nano-TiO_2; therefore, no mortality was observed in daphnia at a concentration of 1,250 $mg \cdot L^{-1}$ after 48 h (Table 3). Based on the overviews of the Environmental Protection Agency [11], compounds with no mortality in such high concentration levels are categorized as "practically nontoxic" substances.

This research is congruent with the findings of previous studies in concluding that the toxicity of nano-TiO_2 is much

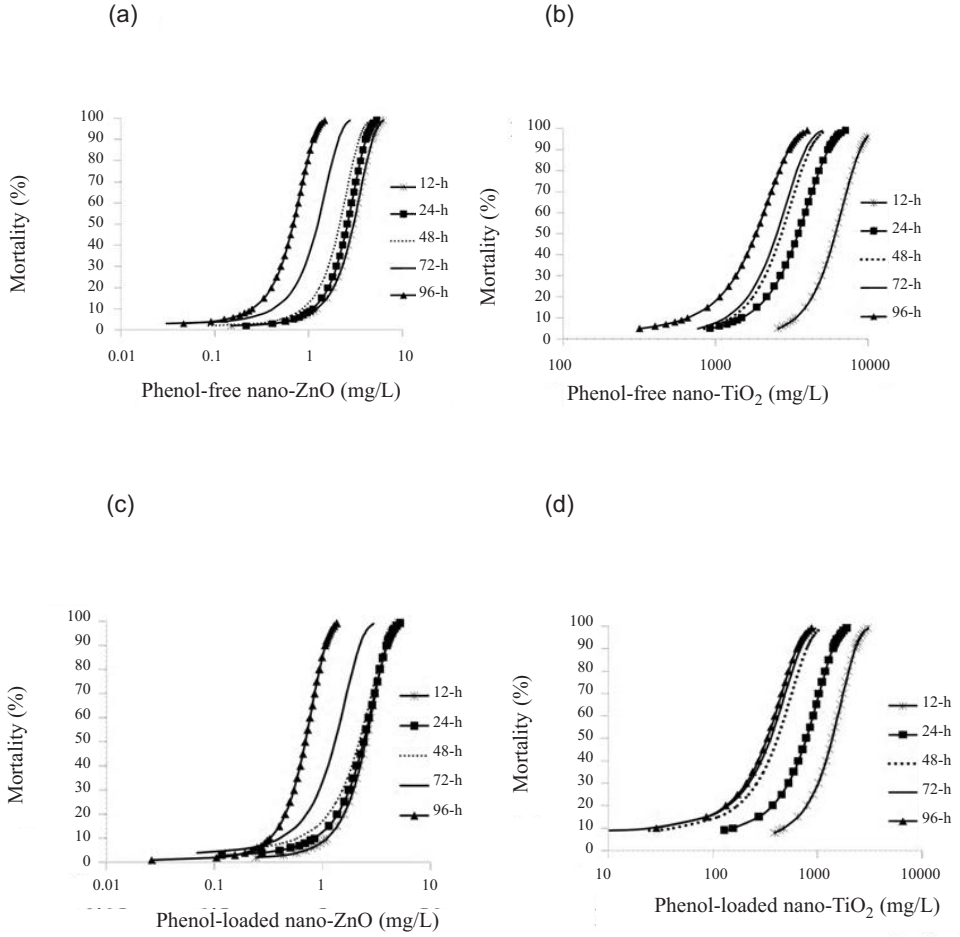

Fig. 2. Mortality rate for *Daphnia magna* at the various concentrations of phenol-free NPs (a and b) and phenol-loaded NPs (c and d) at different periods of time.

less compared to the toxicity of other NPs [1]. However, results of this investigation show that the toxicity of nano-TiO_2 increases by a factor of more than 6 due to the adsorption of phenol. Phenol adsorbs on the NPs through chemisorptions [12], in which case an O (alcohol) – metal (surface) bond and an H-bond with surface oxygen is formed [13].

One of the toxicity mechanisms of NPs is thought to be the generation of reactive oxygen species (ROS) such as OH and H_2O_2 radicals [14]. Other mechanisms of toxicity include releasing metal ions and their speciation; therefore, toxicity of these metal ions increases with their solubility. Nevertheless, since it is not expected that metal ions change during exposure of NPs to phenol, toxicity change could not be attributed to this mechanism. Therefore, the most prominent mechanism that has been proved in this context is the bioavailability of phenol adsorbed to NPs. It is expected, according to this mechanism, that phenol adsorbed on TiO_2 NPs is either released through passing the digestive system or via intracellular reactions and thus causes an increase in toxicity [15, 16].

Conclusions

No research has been conducted on toxicity of ZnO and TiO_2 NPs after exposure to poisons and other hazardous substances. The study on the toxicity of phenol-loaded and phenol-free nano-TiO_2 concludes that the toxicity increases after phenol is adsorbed. Since the rate of phenol adsorption on nano-ZnO is much lower than that of nano-TiO_2, and toxicity of nano-ZnO does not change significantly after exposure to phenol, it could be concluded that the major factor influencing occurrence of the above phenomenon is the high adsorption rate of toxins by NPs. These experiments indicate that when applying NPs in different media and disposing of NP waste materials, issues related to bioavailability must be taken into consideration. This study suggests that future research is required to avoid potential health and environmental effects of NPs in these ecosystems.

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