Ecotoxicity study of iron oxide nanoparticles on Chlorella sp. and Daphnia magna

Chlorella sp. ve Daphnia magna üzerinde demir oksit nanopartiküllerinin ekotoksisite çalışması

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Bu makaleye şu şekilde atıfta bulunabilirsiniz (To cite this article): Ertit Taştan B., Kars Durukan I. Ve Ateş M., “Chlorella sp. ve Daphnia magna üzerinde demir oksit nanopartiküllerinin ekotoksisite çalışması”, Politeknik Dergisi, *(*): *, (*).

Erişim linki (To link to this article): http://dergipark.org.tr/politeknik/archive

DOI: 10.2339/politeknik.581107
Ecotoxicity Study of Iron Oxide Nanoparticles on Chlorella Sp. and Daphnia Magna

ABSTRACT

Nanoparticles have great impact due to their tremendous industrial applications. However, their applications have produced toxicity effects on the aquatic environments and their detailed analyses are not clearly understood. Iron oxide nanoparticles (Fe₂O₃ NPs) are being used extensively in many industries but are considered highly toxic to aquatic species residing in surface waters. This paper demonstrates the acute toxicity of α-Fe₂O₃ and γ-Fe₂O₃ NPs to Chlorella sp. and D. magna. The growth of microalgal species was investigated. The growth of microalgae decreased with increased concentration of the α-Fe₂O₃ and γ-Fe₂O₃ NPs concentrations but did not show a significant toxic effect. The Fe₂O₃ concentration value was 500 mg/L and LD₅₀ concentration value was 1000 mg/L for α-Fe₂O₃ treated daphnids in 72 h, respectively. The findings demonstrate the significant evidence in understanding acute toxicity of Fe₂O₃ NPs for environmental protection as part of risk assessment strategies.

Keywords: α-Fe₂O₃, γ-Fe₂O₃, nanoparticles, nanotoxicity, Chlorella sp., Daphnia magna.

1. INTRODUCTION

Metal oxide nanoparticles are attracting great interest due to their tremendous applications in engineering, water treatment, medicine and cosmetics (1). Among these oxides, iron oxides are very important materials, composed of three different forms: hematite (α-Fe₂O₃), maghemite (γ-Fe₂O₃), and magnetite (Fe₃O₄) (2). α-Fe₂O₃ is a thermodynamically stable structure at ambient conditions. It has more rigid structure than diamond and offers high resistance to corrosion. Additionally, it is low cost (3, 4). γ-Fe₂O₃ is magnetic, which has an isometric crystalline structure. At room temperature, γ-Fe₂O₃ is ferromagnetic, but if smaller than 10 nm, they are considered as superparamagnetic.

At high temperature, γ-Fe₂O₃ is unstable and loses its sensitivity over time. Iron oxide nanomaterials are widely used in drug delivery (5), biosensing (6), cell labeling (7), magnetic trapping (8), and magnetic resonance imaging (9), biomolecular magnetic hyperthermia (10). Iron oxide nanomaterials are generally regarded as non- or low-toxic (11-13). However, recent studies have revealed that iron oxide nanomaterials have potential adverse effects due to their potential escape into the environment (14). Super paramagnetic iron oxide nanoparticles show less cytotoxicity (15-17) also found that ferrie oxide nanoparticles have produced potential lung and systemic cumulative toxicity in rats, and intravascular iron oxide nanoparticles that may induce human endothelial inflammation and dysfunction. Moreover, iron oxide
nanomaterials could serve as significant carriers of toxic chemicals (18; 19) and increase exposures to adsorbed pollutants. An acute toxicity of citrate coated Fe3O4 NP was reported as 57.1 mg/L (48-EC50) and 31.7 mg/L (96-EC50) for Daphnia magna (D. magna), which were partially ascribed to dissolved iron ions and their ability to form reactive oxygen species (ROS) (20). The fate, transport and exposure pathways of manufactured Fe3O4NP were also investigated using pumpkin plants (Cucurbita maxima) (21). Additionally, a previous study was reported on the effects on the Fe3O4 (≥10 mg/L) NP on the development of mental toxicity of zebrafish (Danio rerio) embryos was studied on mortality, hatch- ingdelay, and malformation (22). Apart from that, another study was reported the toxic effects of Fe2O3 nanoparticles (35 nm) on the green alga Chlorella vulgaris treated 72 h to a nominal concentration range from 200 to 1600 µg/mL. In a previous paper, the authors showed an induction of oxidative stress and an alteration of photosynthetic activity based on absorbed CO2 fixation (22). Similarly, the toxicity of super paramagnetic iron oxide nanoparticles was investigated on Chlorella vulgaris cells during 72 hours to Fe3O4 to a range of concentrations from 12.5 to 400 µg/mL. Under these treatments, toxicity impact was indicated by the deterioration of photochemical activities of photosynthesis, the induction of oxidative stress, and the inhibition of cell division rate.

The aim of the current study was to investigate the effects of α-Fe2O3 and γ-Fe2O3 NPs on aquatic organisms; Chlorella sp. and D. magna. These species are chosen since they are widely used as food in aquaculture and they are two of the most frequently used species in aquaculture (23). We investigated the impacts of α-Fe2O3 and γ-Fe2O3 NPs on behavioral change and ecotoxicity, and determined the effective concentration (EC50) and lethal dose (LD50) values. The attachment and accumulation of α-Fe2O3 and γ-Fe2O3 NPs in aquatic organisms were investigated using scanning electron microscopy (SEM). Also the effects of particle size and solubility were comparatively examined on the toxicity of α-Fe2O3 and γ-Fe2O3 NPs to these commonly used phytoplankton and zooplankton species in aquaculture.

2. MATERIAL VE METHOD
2.1 nanoparticle characterization
The characteristic feature of nanomaterials, such as size, shape, size distribution, surface area, solubility, aggregation, etc. need to be evaluated before assessing toxicity or biocompatibility (24). Morphologies of the α-Fe2O3 and γ-Fe2O3 NPs were examined using transmission electron microscopy (TEM). Powered X-ray diffraction analysis (XRD) was carried out to characterize the crystal structure of the NPs. Hydrodynamic diameters and zeta potentials of the NPs were measured with a Zetasizer (25, 26).

2.2 Microalgae culture conditions
The microalgae Chlorella sp. was isolated from the water supply in Sorgun, Yozgat, Turkey (27). The medium BG 11 (28) was used to conduct algal growth inhibition assay based on the OECD 201 (29). The starting OD value of microagal cultures in the beginning of the experiment was about 0.2 in 100 mL. BG11 media in 250 mL Erlenmeyer flasks at 25 ± 2 °C under continuous illumination at 30% (1500 lx) at 100 rpm stirring rate.

D. magna, a planktonic crustacean was used as test species (30). The daphnids were maintained at a constant temperature of 20 ± 1 °C at 16:8 h light:dark cycle. The acute immobilization test was based on the OECD 202 (31).

2.3 Microalgabby Growth Inhibition
Exponentially growing algal cells were propagated in Erlenmeyer flasks containing α-Fe3O4 and γ-Fe3O4 at 50, 100, 250, 500 and 1000 mg/L of the BG11 medium separately. In addition, the control medium consisted of flasks without α-Fe3O4 or γ-Fe3O4 NPs. All experiments were carried out twice in triplicate.

2.4 Acute Immobilization
D. magna, a planktonic crustacean was used as test species (20). The daphnids were maintained at a constant temperature of 20 ± 1 °C at 16:8 h light:dark cycle. The acute immobilization test was based on the OECD 202 (31). Increasing concentrations of α-Fe3O4 or γ-Fe3O4 NPs at 0, 50, 100, 250, 500 and 1000 mg/L were prepared in the ISO test medium to determine the sensitivity response of D. magna. A total of 5 daphnids were put in 3 replicates for each concentration tested. Following the 24, 48 and 72 h exposures, daphnids were studied for immobilization effects, with simultaneous comparison with controls.

2.5 Analytical Methods
Cell growth of Chlorella sp. was determined by measuring optic density, dried cell mass and specific growth rate parameters for any set of growth conditions. Optic density was measured at 600 nm with Shimadzu UV 1800 model spectrophotometer. The dried cell mass was calculated by the measurement of pellets, which were dried at 80 °C for overnight (Nach FN 400 model sterilizer) after centrifugation step (3421x g = 5000 rpm for 10', Hettich Universal 320R model centrifuge). The dried cell mass was also determined according to the method developed by (32) at 646.6 nm for chlorophyll a and at 663.6 nm chlorophyll b. The chlorophyll concentrations were expressed in µg of chlorophyll per milliliter.

Specific growth rate (µ) was calculated according to the equation (1) (33);

\[
\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1)
\]

(1)
where $X_i$ and $X_{0i}$: dry cell weight concentrations (g/L) at time $t_i$ and $t_0$, respectively. Maximum biomass productivity was calculated according to the equation (2);

$$P_{max} = \frac{(X - X_0)}{(t - t_0)}$$  \hspace{1cm} (2)$$

where $X$: final and $X_{0i}$ initial biomass concentrations (g/L), $t$: final and $t_0$: initial time of the culture.

Scanning electron microscopy was used to observe the morphology of Chlorella sp. as described previously (30).

All of the experiments were performed in triplicate. The standard error of data was calculated according to the equation (3) formulated by (34) where $\sigma$ represents the square root of the estimated error variance of the quantity.

$$SE = \sqrt{\sigma^2}$$  \hspace{1cm} (3)$$

3. RESULTS AND DISCUSSIONS

3.1 Nanoparticle Characterization

Figure 1 A shows the 20-intensity profiles of $\alpha$-Fe$_2$O$_3$ NPs. The data obtained were matched with lepto program, which confirmed the crystalline structure of Fe$_2$O$_3$. The XRD diffractograms for $\alpha$-Fe$_2$O$_3$ (hematite) NPs showed 8 peaks within 20-80° range with the following hkl values: (012), (104), (110), (113), (024), (116), (214) and (300) and with those given in JCPDS (24-72) data. This confirms the formation of rhomboedral phase and lattice constant $a$: 8.35 Å and $c$: 13.772 Å. The existence of sharp peaks confirmed that $\alpha$-Fe$_2$O$_3$ NPs were highly crystalline and monodisperse (35).

Figure 1 B shows the 20-intensity profiles of $\gamma$-Fe$_2$O$_3$ NPs. The XRD diffractogram for $\gamma$-Fe$_2$O$_3$ (maghemite) NPs showed 7 sharp peaks at hkl (220), (311), (321), (400), (511), (520) and (440). That was consistent with previously published values (36) and also with those given in JCPDS (24-81) data. This confirms the formation of cubic phase and lattice constant $a$: 8.35 Å.

The average length and length distribution of the nanoparticles used in our study was determined by transmission electron microscopy. The results of the evaluation of the photographs were calculated as follows: number average diameter ($D_p$, nm equation (4)) and coefficient of variation for length distributions (CV, % equation (5)). Where $N_i$ determine the number of particles with Di diameter and SD (equation (6)) determines the standard deviation according to the number average diameter value.

$$D_p = \frac{\sum N_i D_i}{\sum N_i}$$  \hspace{1cm} (4)$$

$$CV = \frac{SD}{D_p} * 100$$  \hspace{1cm} (5)$$

$$SD = \sqrt{\frac{\sum N_i (D_i-D_0)^2}{N-1}}$$  \hspace{1cm} (6)$$

Figure 1. XRD spectrum of $\alpha$-Fe$_2$O$_3$ (A) and $\gamma$-Fe$_2$O$_3$ (B) NPs.

A typical TEM image of $\alpha$-Fe$_2$O$_3$ nanoparticle is given in Figure 2 A. The dispersion of $\alpha$-Fe$_2$O$_3$ nanoparticles have a spherical shape, but also contain structures in the form of nanorods, with a lower concentration. Through the TEM photograph, the average diameter of $\alpha$-Fe$_2$O$_3$ nanoparticles was determined as 57.95 nm, the standard deviation value of length distribution was 23.27 nm, and the coefficient of variation for length distribution was 40 %. However, it should be noted that these values are obtained without taking into account the nanorod form. The $\gamma$-Fe$_2$O$_3$ NPs TEM image is given in Figure 2 B. As shown in the figure, the average diameter of the $\gamma$-Fe$_2$O$_3$ nanoparticles was 33.91 nm, the standard deviation value of the length distribution was 8.20 nm, and the coefficient of variation for the length distribution was 24 %. The $\gamma$-Fe$_2$O$_3$ nanoparticles appear to have spherical morphology, generally spherical morphology, compared to $\alpha$-Fe$_2$O$_3$ nanoparticles.

The TEM, DSL and Zeta Potential (positive / negative charge) analysis values of $\alpha$-Fe$_2$O$_3$ and $\gamma$-Fe$_2$O$_3$ NPs in the aquatic environment are shown in Table 1. Immediately after ultrasonication the initial sizes of both $\alpha$-Fe$_2$O$_3$ and $\gamma$-Fe$_2$O$_3$ NPs were found to be 235 nm and 636 nm, respectively. These determined dimensional differences are consistent with zeta potential measurements. In the aquatic environment, an increase in size over time was observed for both NPs. As shown in Table 1, $\alpha$-Fe$_2$O$_3$ and $\gamma$-Fe$_2$O$_3$ NPs have
negatively charged surfaces. Surface loads of suspensions of NPs play an important role in particle stability versus agglomeration in a solvent and in determining interaction with biological systems (37).

**Figure 2.** TEM images of α-Fe₂O₃ (a) and γ-Fe₂O₃ (b) NPs.

The Zeta potential value is also used not only as a positive (+) or negative (-) load change on the surface, but also as an indicator of attractive and repulsive forces on the surface. In a recently published study, it is shown that the surface charge of particles in agglutination in water is not sufficient to prevent aggregation of NPs in water even without counter ions but aggregates were found to be smaller than other NP studies that were compatible with measured surface loads (38).

Table 1. Size distribution and surface load of α-Fe₂O₃ and γ-Fe₂O₃ NPs in aqueous media

<table>
<thead>
<tr>
<th>NPs</th>
<th>TEM* (nm)</th>
<th>DLS* (nm)</th>
<th>Zeta*Potential (mV)</th>
<th>Appearance</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Fe₂O₃</td>
<td>57.95</td>
<td>235</td>
<td>-2.15</td>
<td>Blurred-yellow</td>
<td></td>
</tr>
<tr>
<td>γ-Fe₂O₃</td>
<td>33.91</td>
<td>636</td>
<td>-3.78</td>
<td>Orange</td>
<td></td>
</tr>
</tbody>
</table>

*: Experimental values measured in this study from 10 μg mL⁻¹ suspensions

The statistical distribution of DLS values in the aquatic environment of NPs is given in Figure 3 A and B. When these graphs are examined, it is seen that α-Fe₂O₃ NPs exhibit fairly steady and stable distribution and γ-Fe₂O₃ NPs exhibit more unstable distribution. All of the metal-based NPs are very unstable in the aquatic environment except for the general situation for NPs, the effects of water on physical and chemical properties in the aquatic environment and tend to aggregate together for various reasons. As a direct consequence of this, they lose their nano properties. Although the size of the aggregate particles is only several times larger than the original particle, the properties exhibited by the material differ. Metal or metal oxide NPs exhibited a similar pattern in our previous studies by our research group (38). In this study, it is seen that γ-Fe₂O₃ NPs in aggregate form more aggregates than α-Fe₂O₃ NPs in freshwater environment.

**Figure 3.** Statistical distribution of DLS values of α-Fe₂O₃ (A) and γ-Fe₂O₃ (B) NPs.

3.2 Toxicity Assessment

3.2.1 Microalgal Growth Inhibition

It was found that the growth inhibition of *Chlorella* sp. is concentration dependent following 72 h of exposure to α-Fe₂O₃ and γ-Fe₂O₃. Initially, during the 72 h observation, it was found that microalgal growth decreased with increasing NPs concentrations (Figure 4). The highest growth was obtained at 50 mg/L α-Fe₂O₃ NP concentration as 0.141 g/L. Dry weight of *Chlorella* sp. decreased from 0.094 g/L to 0.065 g/L when γ-Fe₂O₃ NP concentration increased from 100 mg/L to 1000 mg/L. The α-Fe₂O₃ and γ-Fe₂O₃ concentration at 1000 mg/L showed highly toxic effect on *Chlorella* sp. Thus, it was apparent that increasing α-Fe₂O₃ and γ-Fe₂O₃ NPs concentrations caused to
decrease of microalgal growth but did not show completely toxic effect (Table 2).

![Graph](image)

Figure 4. The effect on dry weight (X) of *Chlorella* sp. with α-Fe₂O₃ and γ-Fe₂O₃ NPs during the incubation period.

Chlorophyll (a+b) concentrations of *Chlorella* sp. were also evaluated (Table 2). Following the 72 h exposure, chlorophyll (a+b) concentrations decreased with increasing α-Fe₂O₃ and γ-Fe₂O₃ NPs concentrations. Calculated $P_{\text{max}}$ values are presented in Table 2. As anticipated, 48 h values of productivity at the highest α-Fe₂O₃ and γ-Fe₂O₃ NPs concentrations were lower than the lowest concentrations.

<table>
<thead>
<tr>
<th>Table 2: α-Fe₂O₃ and γ-Fe₂O₃ NP effect on <em>Chlorella</em> sp. Growth parameters during 48 h and 72 h exposures</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPs concentration (mg/L)</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>50 mg/L</td>
</tr>
<tr>
<td>100 mg/L</td>
</tr>
<tr>
<td>250 mg/L</td>
</tr>
<tr>
<td>500 mg/L</td>
</tr>
<tr>
<td>1000 mg/L</td>
</tr>
</tbody>
</table>

In a previous paper, results showed the toxicities of 4 zero-valent iron NPs with different sizes to a green alga *Chlorella pyrenoidosa*. The effects of particle size of iron NPs showed that the algal growth inhibition of increased significantly with decreasing particle size (39). These results are similar in ours with the effect of higher toxicity of smaller particle sizes. In our results the size distribution of α-Fe₂O₃ NPs was detected as 57.95 nm in TEM and size distribution of γ-Fe₂O₃ NPs was detected as 33.91 nm. As shown in Table 2 dry weight, $P_{\text{max}}$, $\mu_{\text{max}}$ and chlorophyll concentrations were lower in the γ-Fe₂O₃ NPs exposed cultures.

*Chlorella* sp. could be demonstrated as a successful bio indicator in this study and also in many other toxicity studies (39, 40). On the other hand other microalgae species have also been tested in toxicity studies such as *Nannochloropsis* sp. and *Isochrysis* sp. (41).

### 3.2.2 Acute Immobilization Tests

The effects of increasing α-Fe₂O₃ and γ-Fe₂O₃ NPs concentrations of *D. magna* were analyzed during 72 h of exposure. The EC₅₀ concentration value was 500 mg/L and LD₅₀ concentration value was 1000 mg/L for α-Fe₂O₃ treated daphnids in 72 h, respectively (Figure 5 A).

![Graph](image)

Figure 5. The effect of α-Fe₂O₃ (A) and γ-Fe₂O₃ (B) NP concentrations on *D. magna*. The no observed effect level (NOEL) and low observed effect level (LOEL) were calculated at 0 mg/L and 50 mg/L for γ-Fe₂O₃ at 24 h, respectively. The LD₅₀ concentration value was 50 mg/L γ-Fe₂O₃ NP treated daphnids in 48 h (Figure 5 B).

Further, the SEM images also confirmed to change in morphology (Figure 6). Interestingly, the images indicate the attachment of α-Fe₂O₃ and γ-Fe₂O₃ NPs on the *Chlorella* sp. caused to aggregation of microalgal cells.
the significant evidence in understanding acute toxicity of iron oxide nanoparticles for environmental protection as part of risk assessment strategies.

ACKNOWLEDGEMENTS
This research is supported by a grant from The Scientific and Technological Research Council of Turkey (TÜBİTAK, Grant No: 114Y087) through TÜBİTAK Center for Department of Bioengineering at Munzur University.

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