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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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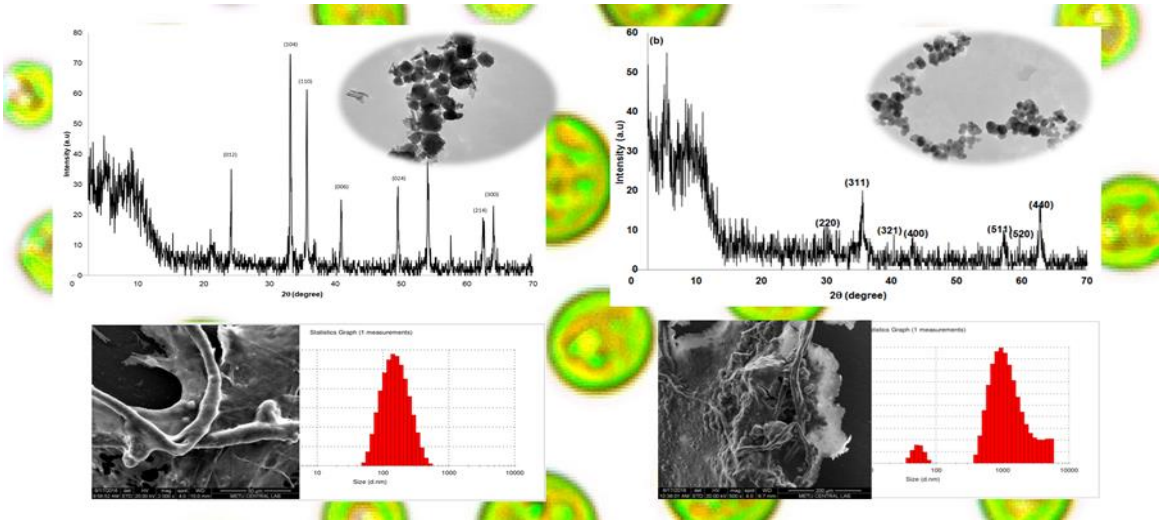
Ecotoxicity Study of Iron Oxide Nanoparticles on *Chlorella* sp. and *Daphnia magna*

Highlights

- *Acute toxicity of α-Fe_2O_3 and ɣ- Fe_2O_3 nanoparticles on two aquatic species were tested.*
- *The EC 50 concentration was 500 mg/L for α-Fe_2O_3 treated Daphnia magna*
- *The LD 50 concentration was 1000 mg/L for α-Fe_2O_3 treated Daphnia magna*
- *Increasing NPs concentrations caused to decrease of Chlorella sp. growth*
- *Increasing NPs concentrations did not show completely toxic effect of Chlorella sp.*

Graphical Abstract

The study investigated the impacts of α-Fe_2O_3 and γ-Fe_2O_3 NPs on behavioral change and ecotoxicity of Chlorella sp. and Daphnia magna and determined the effective concentration (EC_{50}) and lethal dose (LD_{50}) values.



Aim

The aim of the current study was to investigate the effects of α-Fe_2O_3 and γ-Fe_2O_3 NPs on aquatic organisms; Chlorella sp. and D. magna.

Design & Methodology

α-Fe_2O_3 and γ-Fe_2O_3 NPs were analyzed by XRD, TEM, DLTS and Zeta potential

Originality

The effects of particle size and solubility were comparatively examined on the toxicity of α-Fe_2O_3 and γ-Fe_2O_3 NPs to Chlorella sp. and D. magna. in aquaculture.

Findings

Increasing α-Fe_2O_3 and γ-Fe_2O_3 NPs concentrations caused to decrease of microalgal growth but did not show completely toxic effect. The EC_{50} concentration value was 500 mg/L and LD_{50} concentration value was 1000 mg/L for α-Fe_2O_3 treated daphnids in 72 h, respectively.

Conclusion

The paper demonstrates the significant evidence in understanding acute toxicity of iron oxide nanoparticles for environmental protection as part of risk assessment strategies.

Declaration of Ethical Standards

The authors of this article declare that the materials and methods used in this study do not require ethical committee permission and/or legal-special permission.

Chlorella Sp. ve Daphnia Magna Üzerinde Demir Oksit Nanopartiküllerinin Ekotoksisite Çalışması

Araştırma Makalesi / Research Article

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ÖZ

Nanopartiküller, bir çok endüstriyel alanda kullanımları nedeniyle büyük öneme sahiptirler. Ancak, büyük çapta kullanımları neticesinde sucul ortamlar üzerinde toksisiteye neden olmaktadırlar ve bu etkileri detaylı analizlerle henüz açıkça anlaşılamamaktadır. Demir oksit nanoparçacıkları (Fe_2O_3 NP'ler) birçok endüstride yaygın olarak kullanılmaktadırlar ve sularda yaşayan türler için oldukça toksik olarak kabul edilmektedirler. Bu makale iki sucul tür üzerindeki α-Fe_2O_3 ve γ-Fe_2O_3 NP'lerinin akut toksisitesini göstermektedir. α-Fe2O3 ve γ-Fe_2O_3 'ün çeşitli konsantrasyonlarının (0, 50, 100, 250, 500 ve 1000 mg / L) *Chlorella* sp. ve *D. magna* üzerindeki etkileri araştırılmıştır. Çalışmada mikroalgal gelişimin, α-Fe_2O_3 ve γ-Fe_2O_3 NP konsantrasyonlarının artmasıyla azaldığı, ancak önemli bir toksik etki göstermediği belirlenmiştir. Ayrıca 72 saat α-Fe_2O_3 ile muamele edilmiş daphidler için EC50 değeri 500 mg / L ve LD_{50} değeri 1000 mg /L olarak tespit edilmiştir. Çalışma sonucunda, Fe_2O_3 NP'lerin akut toksisitesinin risk değerlendirmesi çevre korumaya yönelik olarak tüm detaylarıyla belirlenmiştir.

Anahtar Kelimeler: α-Fe_2O_3,γ-Fe_2O_3, nanoparçacıklar, nanotoksisite, *Chlorella* sp., *Daphnia magna*.

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 (Fe_2O_3 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-Fe_2O_3 and γ-Fe_2O_3 NPs in two aquatic species. The effects of various concentration (0, 50, 100, 250, 500 and 1000 mg/L) of α-Fe_2O_3 and γ-Fe_2O_3 on the sensitivity response of the *Chlorella* sp. and *D. magna* were investigated. The growth of microalgal decreased with increased concentration of the α-Fe_2O_3 and γ-Fe_2O_3 NPs concentrations but did not show a significant toxic effect. The EC_{50} concentration value was 500 mg/L and LD_{50} concentration value was 1000 mg/L for α-Fe_2O_3 treated daphnids in 72 h, respectively. The findings demonstrate the significant evidence in understanding acute toxicity of Fe_2O_3 NPs for environmental protection as part of risk assessment strategies.

Keywords: α-Fe_2O_3,γ-Fe_2O_3, 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- Fe_2O_3), maghemite (γ-Fe_2O_3), and magnetite (Fe_3O_4) (2). α-Fe_2O_3 is a thermo dynamically 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-Fe_2O_3 is magnetic, which has an isometric crystalline structure. At room temperature, γ-Fe_2O_3 is ferromagnetic, but if smaller than 10 nm, they are considered as super paramagnetic. At high temperature, γ-Fe_2O_3 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 ferric 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

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pollutants. An acute toxicity of citrate coated Fe_3O_4 NP was reported as 57.1 mg/L (48-EC_{50}) and 31.7 mg/L (96-EC_{50}) 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 Fe_3O_4NP were also investigated using pumpkin plants (*Cucurbita maxima*) (21). Additionally, a previous study was reported on the effects on the Fe_2O_3(≥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 Fe_3O_4 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 CO_2 fixation (22). Similarly, the toxicity of super paramagnetic iron oxide nanoparticles was investigated on *Chlorella vulgaris* cells exposed during 72 hours to Fe_3O_4 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-Fe_2O_3 and γ-Fe_2O_3 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-Fe_2O_3 and γ-Fe_2O_3 NPs on behavioral change and ecotoxicity, and determined the effective concentration (EC_{50}) and lethal dose (LD_{50}) values. The attachment and accumulation of α-Fe_2O_3 and γ-Fe_2O_3 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-Fe_2O_3 and γ-Fe_2O_3 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 microalgal cultures in the beginning of the experiments was about 0.2 in 100 mL BG11 media in 250 mL Erlenmayer flasks at 25 ± 2 ºC under continuous illumination at 25 µmol/m^2s (1750 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 Microalgal Growth Inhibition

Exponentially growing algal cells were propagated in Erlenmeyer flasks containing α-Fe_2O_3 and γ-Fe_2O_3 at 50, 100, 250, 500 and 1000 mg/L of the BG11 medium separately. In addition, the control medium consisted of flasks without α-Fe_2O_3 or γ-Fe_2O_3 NPs. All experiments were carried out twice in triplicate.

2.4 Acute Immobilization

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). Increasing concentrations of α-Fe_2O_3 or γ-Fe_2O_3 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 ºC for overnight (Nüve FN 400 model sterilizator) after centrifugation step (3421x g = 5000 rpm for 10', Hettich Universal 320R model centrifuge). The chlorophyll concentrations were determined according 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);

$$\mu = (\ln X_2 - \ln X_1) \div (t_2 - t_1) \quad (1)$$

where X_2 and X_1: dry cell weight concentrations (g/L) at time t_2 and t_1, respectively. Maximum biomass productivity was calculated according to the equation (2);

$$P_{max} = (X - X_0) \div (t - t_0) \quad (2)$$

where X: final and X_0: 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 σ represents the square root of the estimated error variance of the quantity.

$$SE = \sqrt{\sigma^2} \quad (3)$$

3. RESULTS AND DISCUSSIONS

3.1 Nanoparticle Characterization

Figure 1 A shows the 2θ-intensity profiles of α-Fe_2O_3 NPs. The data obtained were matched with leptos program, which confirmed the crystalline structure of Fe_2O_3. The XRD diffractograms for α-Fe_2O_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 rhombohedral phase and lattice constant a: 5.038 Å and c:13.772 Å. The existence of sharp peaks confirmed that α-Fe_2O_3 NPs were highly crystalline and monodisperse (35)

Figure 1 B shows the 2θ-intensity profiles of γ-Fe_2O_3 NPs. The XRD diffractogram for γ-Fe_2O_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 (Dp, nm equation (4)) and coefficient of variation for length distributions (CV, % equation (5)). Where Ni determine the number of particles with Di diameter and SD (equation (6)) determines the standard deviation according to the number average diameter value.

$$Dp = \frac{\sum N_i D_i}{\sum N_i} \quad (4)$$

$$CV = \frac{SD}{D_p} * 100 \quad (5)$$

$$SD = \sqrt{\frac{\sum N_i (D_i - D_p)^2}{(N-1)}} \quad (6)$$

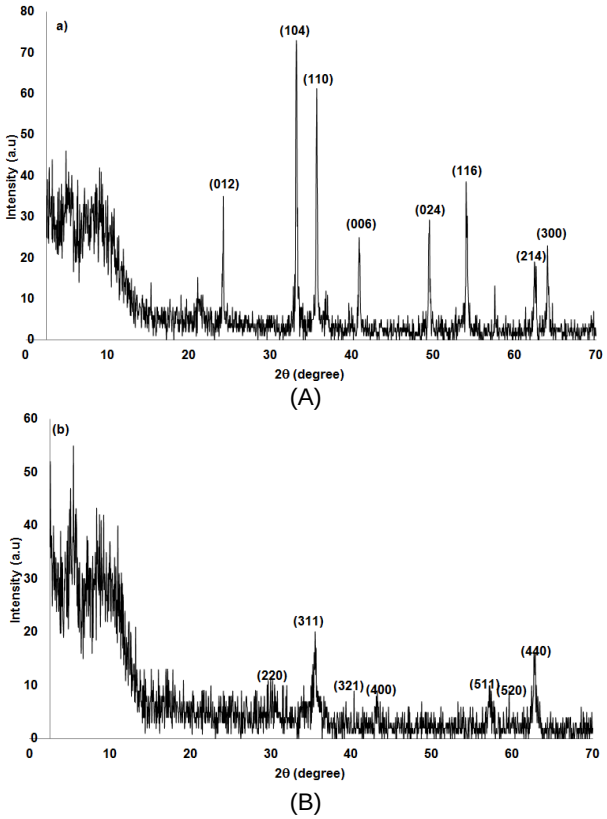


Figure 1. XRD spectrum of α-Fe_2O_3 **(A)** and γ-Fe_2O_3 **(B)** NPs.

A typical TEM image of α-Fe_2O_3 nanoparticle is given in Figure 2 A. The dispersion of α-Fe_2O_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 α-Fe_2O_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 γ-Fe_2O_3 NPs TEM image is given in Figure 2 B. As shown in the figure, the average diameter of the γ-Fe_2O_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 γ-Fe_2O_3 nanoparticles appear to have spherical morphology, generally spherical morphology, compared to α-Fe_2O_3 nanoparticles.

The TEM, DSL and Zeta Potential (positive / negative charge) analysis values of α-Fe_2O_3 and γ-Fe_2O_3 NPs in the aquatic environment are shown in Table 1. Immediately after ultrasonication the initial sizes of both α-Fe_2O_3 and γ-Fe_2O_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, α-Fe_2O_3 and γ-Fe_2O_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).

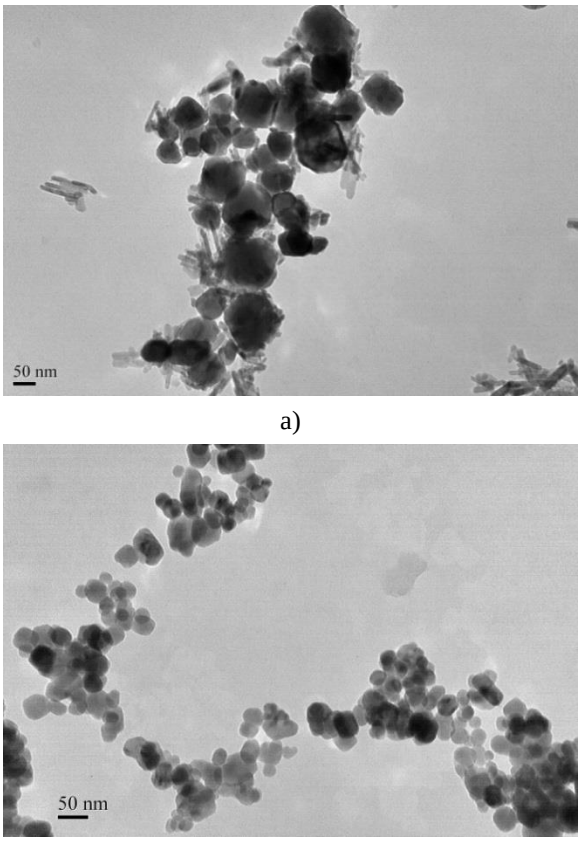


Figure 2. TEM images of α - Fe_2O_3 **(a)** and γ-Fe_2O_3 **(b)** NPs.

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-$Fe_2O_3$and γ-Fe_2O_3NPs in aqueous media

NPs	TEM* (nm)	DLS* (nm)	Zeta*Potential (mV)	Appearance (Color)
α -Fe_2O_3	57.95	235	-2,15	Blurred-yellow
γ - Fe_2O_3	33.91	636	-3.78	Orange

*: Experimentalvaluesmeasured in thisstudyfrom 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-Fe_2O_3 NPs exhibit fairly steady and stable distribution and γ-Fe_2O_3 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-Fe_2O_3 NPs in aggregate form more aggregates than α-Fe_2O_3 NPs in freshwater environment.

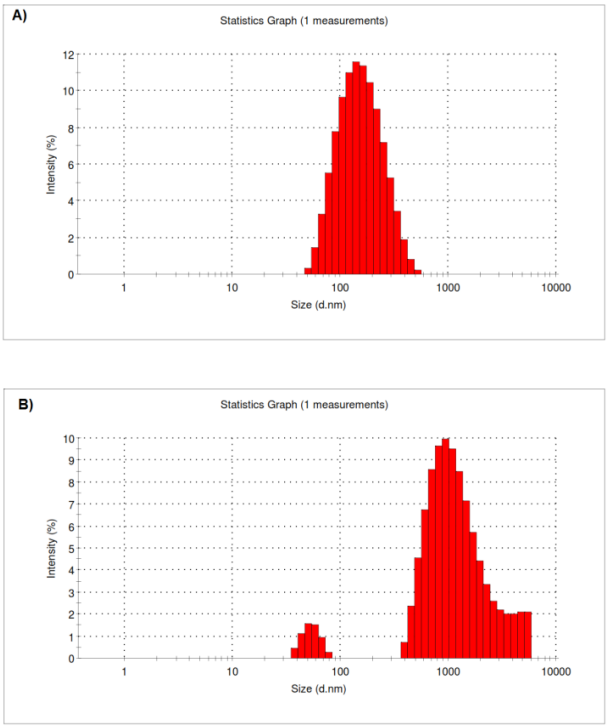


Figure 3. Statistical distribution of DLS values of α - Fe_2O_3 **(A)** and γ - Fe_2O_3 **(B)** NPs.

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-Fe_2O_3 and γ-Fe_2O_3. 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-Fe_2O_3 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-Fe_2O_3 NP concentration increased from 100 mg/L to 1000 mg/L. The α-Fe_2O_3 and γ-Fe_2O_3 concentration at 1000 mg/L showed highly toxic effect on *Chlorella* sp. Thus, it was apparent that increasing α-Fe_2O_3 and γ-Fe_2O_3 NPs concentrations caused to decrease of microalgal growth but did not show completely toxic effect (Table 2).

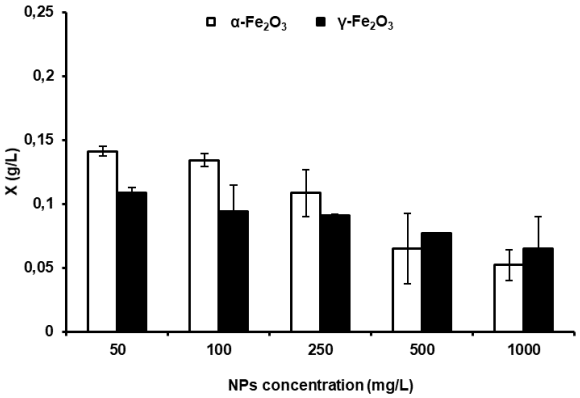


Figure 4. The effect on dry weight (X) of *Chlorella* sp. with α-Fe_2O_3 and γ-Fe_2O_3 NPs during the incubation period.

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-Fe_2O_3 and γ-Fe_2O_3 NPs concentrations.

Calculated P_{max} values are presented in Table 2. As anticipated, 48 h values of productivity at the highest α-Fe_2O_3 and γ-Fe_2O_3 NPs concentrations were lower than the lowest concentrations.

Table 2. α-Fe_2O_3 and γ-Fe_2O_3 NPseffect on *Chlorella* sp. Growth parametersduring 48 h and 72 h exposures

		50 mg/L	100 mg/L	250 mg/L	500 mg/L	1000 mg/L
α-Fe_2O_3	X (g/L)	0.141±0.004	0.134±0.005	0.108±0.018	0.065±0.028	0.052±0.012
	P_{max}^{48h}	0.0022±0.0001	0.0020±0.0001	0.0018±0.0001	0.0007±0.0001	0.0004±0.0001
	μ_{max}^{48h}	0.028±0.0006	0.027±0.0021	0.030±0.0015	0.0151±0.0008	0.010±0.0002
	Chl (a+b) µg/mL	0.076±0.002	0.072±0.002	0.058±0.0001	0.035±0.0031	0.028±0.006
γ- Fe_2O_3	X (g/L)	0.109±0.004	0.094±0.021	0.091±0.001	0.077±0.001	0.065±0.025
	P_{max}^{48h}	0.0014±0.0001	0.0009±0.0001	0.0008±0.0001	0.0008±0.0001	0.0002±0.0001
	μ_{max}^{48h}	0.020±0.0032	0.014±0.005	0.011±0.0017	0.010±0.007	0.003±0.001
	Chl (a+b)	0.059±0.007	0.050±0.002	0.049±0.0003	0.041±0.0003	0.035±0.003

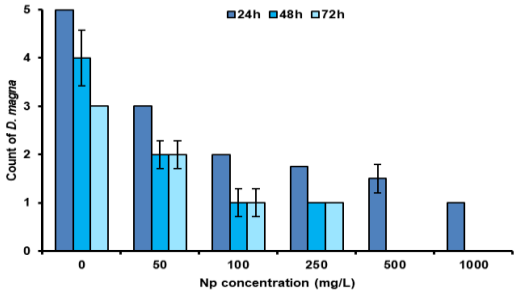
In a previous paper, results showed the toxicities of 4 zero-valent iron NPs with different sizes to a green alga *Chlorella pyrenoidosa*. The effects of particle size of iron NPs showed that the algal growth inhibition of increased significantly with decreasing particle size (39). These results are similar in ours with the effect of higher toxicity of smaller particle sizes. In our results the size distrubition of α-Fe_2O_3 NPs was detected as 57.95 nm in TEM and size distrubition of γ-Fe_2O_3 NPs was detected as 33.91 nm. As shown in Table 2 dry weight, Pmax, µmax and chlorophyll concentrations were lower in the γ-Fe_2O_3 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 Tes

The effects of increasing α-Fe_2O_3 and γ-Fe_2O_3 NPs concentrations of *D. magna* were analyzed during 72 h of exposure. The EC_{50} concentration value was 500 mg/L and LD_{50} concentration value was 1000 mg/L for α-Fe_2O_3 treated daphnids in 72 h, respectively (Figure 5 A).

A)



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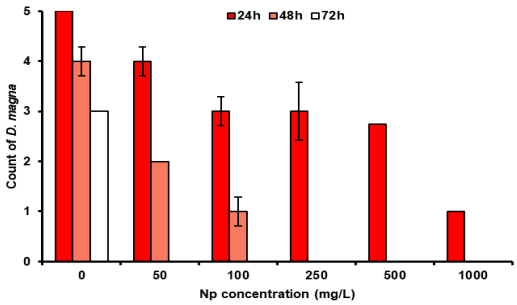
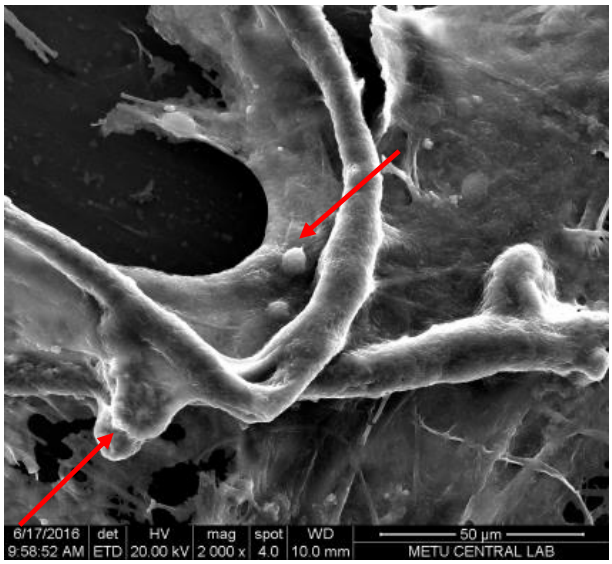


Figure 5. The effect of α-Fe_2O_3 **(A)** and γ-Fe_2O_3 **(B)** NP concentrations on *D. magna*.

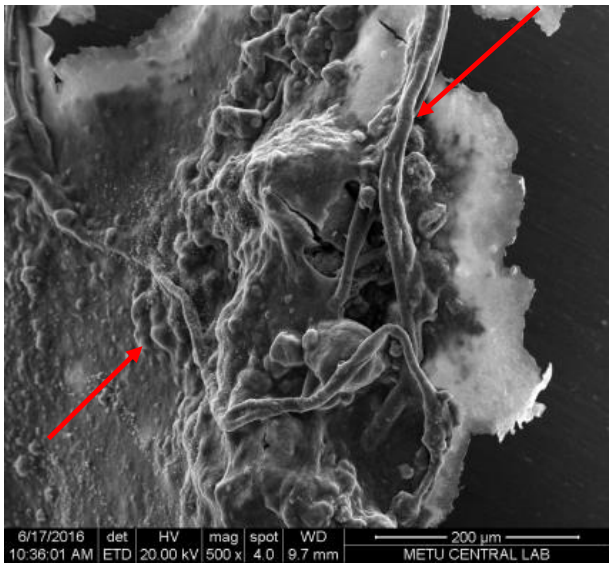
The no observed effect level (NOEL) and low observed effect level (LOEL) were calculated at 0 mg/L and 50 mg/L for γ-Fe_2O_3 at 24 h, respectively. The LD_{50} concentration value was 50 mg/L γ-Fe_2O_3 NP treated daphnids in 48 h (Figure 5 B).

Further, the SEM images also confirmed to change in morphology (Figure 6). Interestingly, the images indicate

the attachment of α-Fe_2O_3 and γ-Fe_2O_3 NPs on the *Chlorella* sp. caused to aggregation of microalgal cells.



A)



B)

Figure 6. SEM image of *Chlorella* sp. following exposure to α-Fe_2O_3 (A) and **γ- Fe_2O_3 (B)** NPs (72 h).

4. CONCLUSIONS

In this study, α-Fe_2O_3 and γ-Fe_2O_3 nanoparticles were analyzed by XRD, TEM, DLTS and Zeta potential before toxicity study. The formation of the particles and crystal structure were determined by XRD. The shape, size, length distribution and standard deviation values of the particles with TEM were determined. With DLTS and Zeta potential measurements, particle size distribution, solubility, aggregations were determined. The paper highlighted the acute toxicity of α-Fe_2O_3 and γ-Fe_2O_3 nanoparticles on *Chlorella* sp. and *Daphnia magna*. Increasingα-Fe_2O_3 andγ-Fe_2O_3 NPs concentrations caused to decrease of microalgal growth but did not show completely toxic effect.The EC_{50} concentration value was 500 mg/L and LD_{50} concentration value was 1000 mg/L for α-Fe_2O_3 treated daphnids in 72 h, respectively. The paper demonstrates the significant evidence in understanding acute toxicity of iron oxide nanoparticles for environmental protection as part of risk assessment strategies.

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DECLARATION OF ETHICAL STANDARDS

The author(s) of this article declare that the materials and methods used in this study do not require ethical committee permission and/or legal-special permission..

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