Effects of Iron-Oxide Nanoparticles (Fe3O4) Released From Synthesized Iron-based Thiourea Catalyst on the Growth, Cell Density, and Pigment Content of Chlorella Vulgaris

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Abstract

This study investigated the effects of Fe$_3$O$_4$ nanoparticles released from synthesized Thiourea catalyst to *Chlorella vulgaris* as an essential primary producer in aquatic systems. A range of Fe$_3$O$_4$ concentrations (0, 10, 100, 250, 500, 750, and 1000 mg L$^{-1}$) was applied for the exposure test. Biological parameters of *C. vulgaris*, including cell density, cell viability, and pigment content were assessed. Bioconcentration factor and bioaccumulation were evaluated for contaminated microalgae. Non-carcinogenic risks were then assessed using target hazard quotient (THQ) for potential human consumptions. Findings showed that *C. vulgaris* cell numbers increased from 0 to 500 mg L$^{-1}$ of Fe$_3$O$_4$. Chlorophyll a represented a time-dependent response, and greatest values were detected in 250 and 500 mg L$^{-1}$ Fe$_3$O$_4$ at 4.2 and 4 mg/g, respectively. Chlorophyll b content showed a time-related manner in exposure to Fe$_3$O$_4$ with the highest values recorded at 250 mg L$^{-1}$ after 96 h. Moreover, bioaccumulation displayed a dose-dependent response as bioaccumulated iron was in the largest amount at 15000 µg/g dw in 1000 mg L$^{-1}$, whereas the lowest one was in the control group at 1700 µg/g dw. The bioconcentration factor showed a concentration-relevant decrease in all iron treatments and 10 mg L$^{-1}$ of Fe$_3$O$_4$ represented the greatest BCF at 327.3611. Non-carcinogenic risks illustrated negligible hazard (THQ < 1) in a dose-response pattern and the largest EDI and THQ were calculated in 1000 mg L$^{-1}$ at 7.4332E-07 (mg kg$^{-1}$ day$^{-1}$) and 1.06189E-09, respectively. In essence, iron is an essential trace element for biological aspects in aquatic systems, but in exceeding concentrations could impose toxicity effects in *C. vulgaris* populations.

1. Introduction

Organocatalysts have received a great deal of attention over the last decade due to their low cost, availability, high yield, short reaction time, simplicity of product isolation, clean reaction profile, environmental benignity, recyclability, and reusability [1]. Having a strong hydrogen bonding capability, organic-based catalysts can significantly promote chemical reactions in environmental studies [2]. Urea has been recently applied as an organic base for organocatalysts used in wastewater management and air pollution to remove heavy metals [3]. Thiourea catalyst is generated from magnetic nanoparticles because of their high surface area, low toxicity and superparamagnetic behavior, and potential applications in many fields [4]. Many metals have been used as the metallic base of Thiourea catalyst, including Al, Fe, Cu, Si, and Zn; however, functionalized iron nanoparticles (Fe$_3$O$_4$) are more interesting due to their easily separable, reusable, non-toxic, low cost, and flexible design [5]. In organic chemistry, Fe$_3$O$_4$ catalyst coated with thiourea is used for the Knoevenagel reaction where formation of C-C double bonds and synthesis of α, β-unsaturated carbonyl compounds from active methylene and carbonyl compounds may occur [7]. Knoevenagel reaction is mainly used in the chemical processes to mitigate the human interference and secure operators from dangerous and carcinogenic solvents [6]. Fe$_3$O$_4$ nanomaterials play an essential role as a functionalized base for Thiourea catalyst, and is greatly used in such reactions in numerous industrial activities [8]. Such iron-based catalyst can release into the aquatic ecosystems and affect the organisms like microalgae as primary producers in these environments [9].
The environmental concentration of iron-based materials has been increased in the biota, including soil, air, and water [10]. Literature have reported the fate, biotransformation, bioaccumulation, and toxicity of such elements in various ecosystems [11, 12]. In particular, aquatic environments are the most important destinations for iron-based compounds through wastewater drainage and landfills in populated areas. Fe₃O₄, in general, possess various behaviors when enter to the water systems, and undergo some processes, including aggregation, dissolution, redox reaction, and interaction with potential macromolecules [13]. These iron-based products can act as the best iron resource for microalgae, and in highly-contaminated systems lead to eutrophication and algal bloom [14]. Furthermore, iron-based materials in overloaded-conditions cause bioaccumulation and bioconcentration in the aquatic species, and thus, oxidative stress could result in through reactive oxygen species (ROS) production [15]. Aquatic flora and fauna are more sensitive to toxic materials compared with terrestrial species, and related compounds of iron can easily pass the cell membrane and cause severe damages to enzymes, proteins, and DNA integrity of organisms[16]. Lei et al. (2016) examined the toxicity of iron-based NPs to green algae in terms of fate, particle size, and oxidation effects [17]. *Chlorella vulgaris* (*C. vulgaris*) was used as an aquatic biological model to investigate iron-based oxidative stress [18]. The toxicity effects of iron oxide nanoparticles were assessed using Zebrafish (*Danio rerio*) in early life stages, that mortality, hatching delay, and malformation occurred in exposing to these nanomaterials [19]. The toxicity of nanoparticles to aquatic species has been investigated in numerous studies as Behzadi Tayemeh et al. (2020) examined the toxic effects of silver nanoparticles and ions on *C. vulgaris* biological responses [20]. Sayadi et al. (2020) used blackfish (*Capoeta fusca*) to study exposure effects of iron oxide nanoparticles and iron salts in causing toxicity, bioaccumulation, and tissue histopathology [21].

Microalga play a vital role in forming the primary production and energy base for all species inhabited in aquatic ecosystems[22, 19]. Such valuable photosynthetic species ranged from microscopic to macro sizes and are the main cause of the food and oxygen production in both freshwater and marine systems. Over the past decade, microalgae have gained significant attention among environmentalists due to their advantageous properties in aquatic ecological balance [23]. Many studies have been conducted concerning the toxic effects of metal contaminants on the biological aspects of microalgae, including growth rate, yield rate, pigment content, reproduction, and nutrient content [24]. Copper nanoparticles caused growth inhibition in *Skeletonema costatum* in exposing to microplastics [23]. Nutritional characteristics of microalgae examined through exposing to metals and metallic NPs, and pigments, biological macromolecules, and phenolic compounds reduced in the presence of these deleterious materials [26]. For this, microalgae have been always considered as ideal candidates and indicators in biological and ecological monitoring of the aquatic biota [27]. Moreover, phytoplanktons are being consumed as functional foods and reliable supplementary ingredients in human diet [26, 27, 29]. Having the richest source of unsaturated fatty acids, antioxidants, proteins, and pigments, microalgae have played a pivotal role in supplying aquatic-based bioactive compounds for consumers [30].

*C. vulgaris* is classified as unicellular green microalgae and known as an essential functional food in the world [19]. *C. vulgaris* is being sold in many countries like Japan, China, Germany, and India as the most
important algal nutrient in human health and aquaculture activities[31]. This freshwater microalga, in addition, is known for its anticancer, anti-inflammatory, antioxidant, and antibacterial merits, which is widely used in pharmaceutical and food industries [32]. *C. vulgaris* has a great potential of biosorption in binding with toxic materials and metals dissolved in the water[33, 34]. Having intracellular metal binding proteins such as metallothioneins, *C. vulgaris* has been identified as a viable microalga in alleviating the adverse effects of xenobiotics in aquatic ecosystems[35]. In addition, *C. vulgaris* has been an ideal biological model for primary producers to investigate the toxicological effects of metals and nanoparticles released into the aquatic environments. Therefore, this study intended to investigate the effect of iron-based nanoparticles released from synthesized Thiourea catalyst to *C. vulgaris*, as a pivotal primary producer in aquatic systems, in terms of bioaccumulation, bioconcentration, cell growth, pigment content, and risk assessment for potential human consumptions.

2. Materials And Method

2.1. Chemicals and instruments

All chemicals and reagents were purchased from Merck Company (Germany). Infrared (IR) spectra is applied to analyze the spectral experiment using spectrometer (Shimadzu FT-IR-8400S). Following the chemicals preparation, all first-made products were examined using $^1$H NMR (500 MHz) spectra and Bruker DRX-500 to identify the chemical characteristics. To solve the chemicals, an advanced spectrophotometer equipped with CDCl$_3$ (as the solvent) and tetramethylsilane (TMS) (as the internal standard) at ambient temperature was applied. Zeiss-Sigma (VP 500) was used to obtain SEM images and EDX spectra was recorded on an Oxford Instrumental® version. A vibrating magnetometer was used to identify the magnetic properties of the synthesized catalyst (MD Co., Iran, www.mdk-magnetic.com). To analyze the thermogravimetric properties, thermal analyzer with a heating rate of 20°C min$^{-1}$ was used over a temperature range of 25 to 1100°C under flowing compressed nitrogen.

2.2. Catalyst preparation

To synthesize the catalyst, 5 mmol FeCl$_3$.6H$_2$O and 2.5 mmol FeCl$_2$.4H$_2$O were dissolved in 100 mL deionized water under vigorous stirring (800 rpm). Then, NH$_4$OH solution (25%, w/w, 30 mL) was added to the prepared mixture at room temperature until adjusting the pH above 11. To form a black suspension, NH$_4$OH solution was continuously added to maintain the pH value between 11 and 12. The resulting black dispersion was continuously stirred for 1 h at room temperature and then refluxed for 6 h. Following the methodology, ethanol (40 mL) was applied at 40°C for 1 h to purify Fe$_3$O$_4$ nanoparticles for coating a layer of silica on the surface of the catalyst. Subsequently, tetraethylorthosilicate (TEOS, 10 mL) was charged to the reaction vessel, and the mixture was continuously stirred for 24 h. The silica-coated nanoparticles were collected by a magnet, followed by washing five times with EtOH, diethylether and drying at 100°C in vacuum for 12 h. A sample of MNPs-SiO$_2$ (1 g) dispersed in a mixture of 50 mL of dry toluene containing (3-chloropropyl) trimethoxy-silane (1ml) as the effective linker with two different electrophile heads. The mixture was refluxed for 48 h. The final product was separated by filtration,
washed with toluene, and dried under vacuum for 24 h at 150°C. The prepared MNPs@SiO$_2$-Si-(CH$_2$)$_3$-Cl (1g) and KI (1.66 g, 0.01 mol) were added to a solution of thiourea (0.76 g, 0.01 mol) and K$_2$CO$_3$ (1.38 g) dissolved in acetonitrile (50 mL) transferred to a round-bottom flask and the mixture was stirred under reflux condition for 8 h. The obtained solid was then magnetically collected from the solution and washed abundantly with water/ethanol followed by drying at 80°C for 12 h.

2.3. Characterization of synthesized Thiourea catalyst

To identify the characteristics of Synthesized catalyst, a range of optico-chemical techniques, including FT-IR, VSM, XRD, SEM, EDX, and TGA were applied. Results relating to the FT-IR spectra of magnetic NPs (Fe$_3$O$_4$@SiO$_2$) and Fe$_3$O$_4$@SiO$_2$@SiO$_3$-(CH$_2$)$_3$-thiourea are demonstrated in figure 1 (IV). The peaks at 568 and 801 cm$^{-1}$ are assigned to the Fe$^{+3}$–O$^-$ and Fe$^{+2}$–O$^-$ symmetrical stretching vibrations, respectively. The covalent bond between silicon and magnetite surface is underlined by the appearance of the band at 1092 cm$^{-1}$ for Si–O groups. Moreover, absorption bands at 800-950 cm$^{-1}$ are associated with vibrating bands for Si–O–Si groups (Fig. 1-IV). These results indicated that the SiO$_2$ layer was formed on the surfaces of Fe$_3$O$_4$. Signals appeared at 3421 cm$^{-1}$ regions can be assigned to the N–H bending, and 1634 cm$^{-1}$ are attributed to (C=S) stretching of thiourea. Magnetic hysteresis measurements were done in an applied magnetic field at r.t, with the field sweeping from −10000 to +10000 Os using a vibrating sample magnetometer (VSM) as a scientific instrument for measuring magnetic properties (Fig. 1-II). The magnetic saturation value of thiourea functionalized MNPs@SiO$_2$@SiO$_3$-(CH$_2$)$_3$NHCSNH$_2$ is 23 emu/g. In addition, the XRD patterns of Fe$_3$O$_4$@SiO$_2$@SiO$_3$ (CH$_2$)$_3$NH(CS)NH$_2$ are demonstrated in figure 1-III. XRD contains seven characteristic peaks ($2\theta = 21.25^\circ$, 37.29°, 43.73°, 52.56°, 65.09°, 69.73°, 76.81°) which match with the peaks of Fe$_3$O$_4$ and this confirms that the crystalline structure of Fe$_3$O$_4$ does not change during functionalization of MNPs (Fig. 1-III).

The morphology of Fe$_3$O$_4$@SiO$_2$@SiO$_3$(CH$_2$)$_3$NH(CS)NH$_2$ before (Fig. 1-VII A), and after (Fig. 1-VII B) reaction was visualized using scanning electron microscopy (SEM) images as depicted in figure 1-VII. Based on the SEM analysis, the catalyst was made up of nanometer-sized iron particles shaping spherical morphology. Energy-dispersive X-ray spectroscopy (EDX) method was used to characterize the elemental composition of Fe$_3$O$_4$@SiO$_2$@SiO$_3$(CH$_2$)$_3$NHCSNH$_2$ (Fig. 1-VI). According to the EDX analysis, the well-dispersion of Fe$_3$O$_4$ nanoparticles was obtained through the preparation process. Thermal Gravimetric Analysis (TGA) and Differential Thermal Analysis (DTA) were applied in three steps (Fig. 1-V). The first rupture is associated with the loss of water 1% wt around 60°C, and the second and main loss in weight was recorded at 5% wt, 0.083 mol/100 g catalyst, associated with the elimination of thiourea and its propyl spacer between 200 to 300°C. The final thermal destruction, approximately 1 % wt, was observed from 350 to 700 °C, corresponding loss of silica portion of nanoparticles.

The doublet peak was observed in the low field relating to the H$^1$, near electron donor group. In addition, the unit singlet peak was related to the H$^3$ and another doublet peak was for H$^2$ that it depended on the
group connecting to the aromatic bond which is the same for electron donor group, but the peak H\textsuperscript{2} had shifted to NO\textsubscript{2} as this group had responded and shifted to the high/low field.

## 2.4. Algal cultivation

*C. vulgaris* was cultivated using the sterilized Bold's Basal Medium (BBM) as prescribed by Behzadi Tayemeh et al. (2020b). The cultivation was performed for 96 h, and an initial density of \(10^7\) cells mL\textsuperscript{-1} in 1.5 L-glass vessels was used as a stock solution.

### 2.5. Toxicity tests and exposure experiments

Exposure tests were conducted in the media using a range of concentrations, including 0, 10, 100, 250, 500, 750, and 1000 mg L\textsuperscript{-1} Fe\textsubscript{3}O\textsubscript{4} NPs. Exposure concentrations were selected according to the coated magnetic Fe\textsubscript{3}O\textsubscript{4} NPs on a synthesized Theurea catalyst. For each treatment, three replicates were considered, and exposure tests were carried out using OECD 201 (OECD, 2011) protocols and guidelines. During the incubation period, cool white fluorescent lights (40W FL T10 230 V G13; 12 h light: 12 h dark regime) were used with constant intensity of 1000 µmol. photons.m\textsuperscript{-2}.s\textsuperscript{-1} on the glass vessels aerated from the bottom and the temperature was adjusted at 26 ± 1°C.

### 2.6. Algal growth

During the exposure time, the density of algal cells was daily evaluated at 24, 48, 72, and 96 h after exposure test. For this, two procedures, including optical density (OD) and visible light microscopy were used to calculate the cell numbers. Spectrophotometric method (PerkinElmer Lambda 25 UV-VIS Spectrometer) at the absorbance of 680 nm was used for OD method, and light microscope equipped with a Neubauer chamber was applied to perform the visual count in triplicates for each treatment (OECD, 2011).

### 2.7. Pigment analysis

Pigments, including Chlorophyll a, Chlorophyll b, and carotenoid were considered as microalgae pigments and evaluated using a standard protocol prescribed by Silkina et al. (2015) with some modifications [36]. Following the pigment analysis, a volume of 13 ml medium was centrifuged for obtaining cultivated algal cells using 20,000 rpm for 5 min at 4°C. The supernatant was rejected and then the maintained cells were preserved at -80°C until experiments. Pigments were extracted according to the method stated by Tsiola et al. (2017) who used 1.5 ml of 90 % ethanol added to the collected cells and then vortexed severely for 2 min and kept for 24 h in a dark condition at room temperature [37]. A microplate reader (BioTek, 800 TS Absorbance Reader; USA) was used to read the pigments in each treatment using 150 µl in triplicates at 470, 666, and 653 nm.

### 2.8. Bioaccumulation and bioconcentration factor

To measure intracellular Fe\textsubscript{3}O\textsubscript{4} concentrations, algal cells were first collected from the water medium and then were thoroughly washed with cultured medium (without heavy metal ion) to remove the potential
extracellular Fe$_3$O$_4$ ions. Digestion was conducted using HNO3:HClO$_4$ at 80°C and 130°C for 1 and 3 h, respectively, based on the method prescribed by Qian et al. (2011) with some modifications [28]. Digested material was diluted with de-ionized water, and Fe$_3$O$_4$ concentrations were detected using flame atomic absorption spectrophotometer (Model 670G, USA). Bioconcentration factor (BCF) was calculated using equation 1 as suggested by Kuppu et al. (2018).

$$BCF \left( \text{Lkg}^{-1} \right) = \frac{\text{CMDT}}{\text{CSMW}}$$

Where CMDT is concentration of metal in dry tissue (mg kg$^{-1}$), and CSMW is the concentration of same metal in water (mg L$^{-1}$).

### 2.9. Health risk assessment of contaminated C. vulgaris

The estimated daily intake (EDI) and target hazard quotient (THQ) were used to evaluate the non-carcinogenic risk of Fe$_3$O$_4$ accumulated in C. vulgaris. Based on the method prescribed by Alipour and Banagar (2018) EDI and THQ were calculated using equations 2 and 3, respectively.

$$\text{EDI} \left( \text{mgkg}^{-1}\text{day}^{-1} \right) = \frac{\text{EF} \times \text{ED} \times \text{AIR} \times \text{C}}{\text{BW} \times \text{ATn}}$$

$$\text{THQ} = \frac{\text{EF} \times \text{ED} \times \text{AIR} \times \text{C}}{\text{RfD} \times \text{BW} \times \text{ATn}} \times 10^{-3}$$

where EF is the exposure frequency (365 days year$^{-1}$); ED is the exposure duration (70 years, average lifetime for adults); AIR is the algal ingestion rate (0.0035 Kg person$^{-1}$ day$^{-1}$); C is the Fe3O4 ions in algae (mg g$^{-1}$); RfD is the oral reference dose (Fe = 0.7 mg kg$^{-1}$ day$^{-1}$); Bw is the average adult body weight (70 kg); and ATn is the average exposure time for non-carcinogenic risk (365 days/year x number of exposure years, assuming 70 years). Non-carcinogenic risk grouping was performed using the method prescribed by Cheng et al. (2018). For target hazard quotient, THQ $\geq$ 1 showed the presence of non-carcinogenic risk, and THQ < 1 represented negligible hazard for people.

### 2.10. Statistical analysis

All of the analyses and comparisons were conducted using SPSS software version 19.0 (SPSS, Chicago IL, USA). Kolmogorov-Smirnov test was performed to assess the normality of data, and one-way ANOVA was used to determine possible differences between treatments. In all analyses, data were presented as mean ± SD, and differences were considered significant at $P < 0.05$. For data management and drawing
graphs, Microsoft Excel (version 2016, windows 7) was applied to define and calculate the risk assessment analysis.

3. Results

3.1. Cell density and algal growth

Cell density analysis is represented in figure 2. Based on the findings, cell density of *C. vulgaris* showed that the number of cells increased with the elevation of iron-based NPs from 0 to 100 mg L\(^{-1}\) while in higher concentrations (i.e. 250, 500, 750, and 1000) demonstrated a decrease, especially after 72 and 96 h exposure period. Results showed that cell density elevated with the increase of nanomaterial concentration from 0 to 500 mg L\(^{-1}\) after 0, 24, and 48 h exposure time (Fig. 2).

3.2. Pigment analysis

Results relating to the chlorophyll a content is depicted in figure 3. It is apparent that the content of chlorophyll a was in the lowest level after 24 h exposure time, whereas in a 96-h period the highest chlorophyll a was recorded in all treatments. Indeed, chlorophyll a content showed a time-dependent response to the concentration of iron nanoparticles. Moreover, in treatments exposed to 250 and 500 mg L\(^{-1}\), chlorophyll a had 4.2 and 4 mg/g, respectively (Fig. 3). Chlorophyll b, in addition, demonstrated a time-relevant response to Fe\(_3\)O\(_4\) NPs as chlorophyll a content increased from 0 to 96 h after exposure period in all concentrations (Fig. 4). The greatest pigment content was detected at the end of the experiment (96 h) while the lowest one was observed in the first day of the test. In terms of Fe\(_3\)O\(_4\) NPs exposure doses, algal populations exposed to 250 and 1000 mg L\(^{-1}\) illuminated the highest and lowest chlorophyll b content, respectively. Findings demonstrated that carotenoid underwent a concentration-dependent decrease in all tested groups (i.e. 0, 10, 100, 250, 500, 750, and 1000 mg L\(^{-1}\)). Furthermore, a time-relevant manner was observed between Fe\(_3\)O\(_4\) NPs concentrations, and carotenoid content increased with the elevation of exposure time from 24 to 96 h in control, 10, and 100 mg L\(^{-1}\) iron nanomaterials. However, *C. vulgaris* populations exposed to 250, 500, 750, and 1000 mg L\(^{-1}\) showed the lowest carotenoid content in comparison with the lower concentrations and control group (Fig. 5).

3.3. Bioaccumulation and bioconcentration factor

Bioaccumulated Fe\(_3\)O\(_4\) in *C. vulgaris* populations exposed to different concentrations is represented in figure 6. Based on the outcomes, bioaccumulation factor exhibited a concentration-related pattern in the tested groups. The highest bioaccumulation recorded at 15072.9 µg/g dw in exposing to 1000 mg L\(^{-1}\) and the lowest one identified at 1592.32 µg/g dw in the control group where algal populations did not expose to Fe\(_3\)O\(_4\) concentration (Fig. 5) and Table 1). Findings regarding the bioconcentration factor (BCF) illustrated that 10 mg L\(^{-1}\) of Fe\(_3\)O\(_4\) had the greatest BCF at 327.3611, whereas other concentrations (0, 100, 250, 500, 750, and 1000) demonstrated BCF no more than 50 (Fig. 7).
Table 1
Detected concentrations of Fe$_3$O$_4$ in contaminated C. Vulgaris

| Fe$_3$O$_4$ concentration (mg L$^{-1}$) | Bioaccumulation (µg/g dw) Mean ± SD |
|--------------------------------------|-------------------------------------|
| Control                              | 1592.32 ± 312.714                   |
| 10                                   | 3273.61 ± 244.298                   |
| 100                                  | 4086.32 ± 1343.91                   |
| 250                                  | 4965.49 ± 1668.21                   |
| 500                                  | 8696.36 ± 514.39                    |
| 750                                  | 11203.9 ± 2152.33                   |
| 1000                                 | 15072.9 ± 1928.64                   |

3.4. Health risk assessment

Risk assessment analysis presented in Table 2. Outcomes suggested that estimated daily intake (EDI) increased with the elevation of Fe$_3$O$_4$ concentration, and the highest EDI was calculated at 7.4332E-07 for C. vulgaris populations who were exposed to 1000 mg L$^{-1}$. Target hazard quotient (THQ) revealed a concentration-dependent response to iron concentrations, and the largest THQ was obtained in exposing to 1000 mg L$^{-1}$ at 1.06189E-09. THQ in all contaminated populations of C. vulgaris to Fe$_3$O$_4$ was below 1 (i.e. THQ < 1), and therefore, cultivated microalgae represented negligible hazard for algal consumers.

Table 2
Estimated daily intake (EDI) and target hazard quotient (THQ) of contaminated C. vulgaris.

| mg kg$^{-1}$ day$^{-1}$ | EDI          | THQ           |
|-------------------------|--------------|---------------|
| 0                       | 7.85253E-08  | 1.12179E-10   |
| 10                      | 1.61438E-07  | 2.30626E-10   |
| 100                     | 2.01517E-07  | 2.87882E-10   |
| 200                     | 2.44873E-07  | 3.49819E-10   |
| 500                     | 4.28861E-07  | 6.12659E-10   |
| 750                     | 5.5252E-07   | 7.89314E-10   |
| 1000                    | 7.4332E-07   | 1.06189E-09   |

4. Discussion
Iron is one of the most abundant metals in the earth's crust (34.6 %) and would be naturally available in the environment [42]. This vital trace element plays a crucial role in producing materials in the photosynthetic organisms as well as cell division processes. There are a variety of iron-based compounds in the environment, but the most common forms are Fe$_2$O$_3$, Fe$_3$O$_4$, FeSO$_4$, FeCl$_3$, and Fe$_2$NO$_3$. Iron in certain concentrations is essential for living-beings in the environment, but at higher concentrations, it can be toxic. In microalgae population, iron involves in metabolism pathways, including photosynthesis, pigment, DNA synthesis, nitrogen fixation, and respiration. Furthermore, iron-based materials are constructed through a wide range of human-made activities which result in iron pollution in the biota. Synthesized catalysts are designed to functionlize chemical reactions via reducing the cost and increasing the speed of processes. For this, many industries have applied organocatalysts containing metals as a reliable and viable base. The ever-increasing application of such catalysts has resulted in environmental pollution and aquatic toxicity for many organisms. Literature reported that wastewater from industrial developments contains 10 to 120 mg L$^{-1}$ iron [43]. Iron content of wastewater and sediment were estimated to be 50 to 3500 µg L$^{-1}$ and 4300 to 7000 mg kg$^{-1}$ in a lake located in Western Australia. Copper smelter companies are one of the most important sources of iron-based materials that cause 882 mg kg$^{-1}$ iron in the sediment. Our findings revealed that the bioaccumulation of Fe$_3$O$_4$ NPs in *C. vulgaris* has a concentration-dependent manner in response to iron-based catalyst used in this study. Fe3O4 accumulated increasingly from 3273.61 ± 244.298 µg/g dw in populations exposed to 10 mg L$^{-1}$ to 15072.9 ± 1928.64 µg/g dw detected in 1000 mg L$^{-1}$ concentration. To support this, *C. vulgaris* has a great potential of absorption and bioaccumulation of environmental metals and is known as an accumulator used in toxicological studies. This bioindicator possesses a hard and thick cell wall containing a considerable amount of fiber that results in absorbing metal ions released into the aquatic biota. Metals such as iron are more desire to absorb and accumulate in the body of aquatic species, rather than being dissolved in the water. Trace elements could be adsorbed by the fish body and its organs in aquatic systems [44]. Another study was conducted using four species of marine algae to evaluate their bioaccumulated iron and its risk for human consumption. Results showed that *Gracilaropsis sp.* and *Sargassum sp.* contained larger amount of iron at 1960 and 1570 µg/g dw, respectively, compared to other species. BCF demonstrated that there is a concentration-relevant decrease among populations exposed to Fe$_3$O$_4$, and the highest value was calculated for 10 mg L$^{-1}$ treatment at 327.3611, whereas algae exposed to 1000 mg L$^{-1}$ showed the lowest BCF at 15.07287 after 96 h of exposure. BCF has indirect relationship with the metal concentration exposed to the water medium, but with the increase of bioaccumulated Fe$_3$O$_4$ in algal body, this factor elevates. In general, microalgae are classified as good metal accumulators in aquatic systems, and BCF > 1000 suggested that the studied species has a great ability to bioconcentrate metals. Researchers claimed that marine diatom *Phaeodactylum tricornutum* could actively adsorb Cd (II) ions to remove such toxic materials from the water. They stated *P. tricornutum* showed BCF is a stronger cadmium bioconcentrator as its BCF was above 1000 in most tested concentrations [45]. In the present study, there was no BCF higher than 327.3611 (10 mg L$^{-1}$) and most tested concentrations displayed BCF below 50. To support this, a complete biosorption can occur by microalgae cells in case they expose to lower Fe$_3$O$_4$ concentrations,
and in turn, higher BCF could be obtained. With the increase of Fe$_3$O$_4$ concentration in the water, binding sites in the cell wall of the *C. vulgaris* are rapidly saturated, which inhibit the binding of more metal ions [45]. Moreover, in highly metal-contaminated environments, intracellular mechanisms in *C. vulgaris* regulate the gradient concentration between the water and organism [46].

According to outcomes emerged to this study, cell density of *C. vulgaris* populations increased in lower concentrations of Fe$_3$O$_4$ at 10, 100, and 250 mg L$^{-1}$ after 72 and 96 h exposure period, and the highest cell growth was observed in populations exposed to 100 mg L$^{-1}$ iron. However, in higher concentrations (i.e. 250, 500, 750, and 1000 mg L$^{-1}$) cell density was inhibited. It is investigated that the cell density in *C. vulgaris* populations exposed to silver nanoparticles (AgNPs) and silver ions (AgNO$_3$) during a 72-h exposure time increased firstly with the elevation of metal nanoparticles and ions, but in higher exposure concentrations and times, it remained stable due to inhibitory effects of such materials [20]. Aquatic microalgae possess limitations to use micronutrients and absorb trace elements in their inhabitants, and in higher concentrations these essential elements act as growth inhibitors. These findings mirrored those reported by Naorbe and Serrano (2018) who stated that microalgae *Tetraselmis tetrathele* showed inhibitory effects in exposing to Hg and Cd ions. They added that Hg and Cd in lower concentrations (i.e. 0, 0.1, 0.3, 0.5, 1, and 2 mg L$^{-1}$) showed no change in the cell density, while in higher doses at 3, 4, and 5 mg L$^{-1}$ after 72 h of exposure time inhibitory effects were observed [48]. Furthermore, our results demonstrated that in the control group as well as after 24 and 48 h of exposure experiment cell density increased even at higher Fe$_3$O$_4$ concentrations (i.e. 500 mg L$^{-1}$); however, cell growth remained stable in facing 750 and 1000 mg L$^{-1}$. This may mean that microalgae such as *C. vulgaris* has their own growth regulatory mechanisms that act as inhibitory factors to adjust their populations based on the environmental conditions. The suppression of *C. vulgaris* growth in exposing to cadmium, lead, and copper stress was studied, and results concerned that the growth and chemical compositions decreased during the first 48 h of exposure time, and copper was more effective inhibitory factor than lead and cadmium to prevent cell growth [49]. Increasing on iron bioavailability above 200 µM could mitigate the growth rate of *C. vulgaris* populations exposed to Fe and increased the amount of lipid radical content in the intracellular space [18].

Pigment analysis, in this study, showed that chlorophyll a content increased over the exposure period (i.e. 0, 24, 48, 72, and 96 h) in all tested concentrations and the highest amount of this pigment was recorded after 96 h of exposure test. Obtained results indicated that *C. vulgaris* could produce chlorophyll a in a concentration-relevant increase up to 250 mg L$^{-1}$, but in further Fe$_3$O$_4$ concentrations it decreased to 1000 mg L$^{-1}$. Metal toxicity can cause a serious reduction in the production of chlorophyll a due to its adverse effects on protein construction. *C. vulgaris* pigment content decreased in exposing to supermagnetic iron oxide nanoparticles (Fe$_3$O$_4$) in a range of concentrations from 12.5 to 400 µg ml$^{-1}$ [39]. Pigment content of microalgae can be used as a reliable biomarker in investigating the health status of the organism, and in whole, community and ecosystem. Literature reported that the most important reason regarding the pigment reduction in microalgae is due to adaptation to unsuitable environmental
conditions through changing the pigments to supply organic nitrogen [51]. According to our results, chlorophyll b content showed a similar pattern as chlorophyll a, and a significant increase was observed during 96-h exposure time. The highest and lowest chlorophyll b were identified at 250 and 1000 mg L$^{-1}$, respectively. Carotenoid content of the studied microalgae showed a concentration-related decrease from 0 to 1000 mg L$^{-1}$. Literatures have proven that the loss of pigment content during the exposure period to metals is mainly because of damage in chloroplast ribosome as well as inhibition in the electron transport chain in the donor center of the \textit{C. vulgaris} cells [52]. In toxic concentration, Fe$_3$O$_4$ interfere in the pigment production process and alter the essential enzymes and proteins accounted for pigment synthesis [41]. It is highly possible that with the ever-increasing applications of iron-based catalysts in chemical reactions used for various industries, the environmental concentration of Fe$_3$O$_4$ increase in the aquatic biota, including freshwater rivers, marine environments, lakes, and wetlands.

The importance of microalgae as functional foods and supplementary ingredients in human diet has been proven in many studies [54–57]. Having a diverse range of unsaturated fatty acids, antioxidants, omega-3, proteins, and pigments, \textit{C. vulgaris} has engrossed many attentions among nutrition scientists and aquaculture companies. However, the health status of the \textit{C. vulgaris}, in terms of bioaccumulated metals, is vital for those who consider such valuable ingredients in their daily diets. Because of this, carcinogenic and non-carcinogenic risk assessment tests are applied to examine the potential risk of edible microalgae for consumers. In this study, estimated daily intake (EDI) and target hazard quotient (THQ) were used to calculate non-carcinogen risk of contaminated \textit{C. vulgaris}. In all contaminated \textit{C. vulgaris} exposure tests, THQ was below 1 (the highest value: 1.06189E-09) and calculated as THQ < 1 meaning that iron-based catalysts had no non-carcinogenic risk for people in exposing to used Fe$_3$O$_4$ concentrations in this study.

**Conclusion**

This study investigated the toxicity of Fe$_3$O$_4$ concentrations used in Thiourea synthesized catalyst to \textit{C. vulgaris} as an essential primary producer in aquatic ecosystems. In response to Fe$_3$O$_4$ concentrations, cell density, pigment analysis, bioaccumulation, and bioconcentartion factor were assessed in \textit{C. vulgaris} populations. Non-carcinogenic risk of contaminated microalgae, moreover, was calculated using EDI and THQ indices. According to the findings, Fe$_3$O$_4$ in lower concentrations (i.e. 10, 100, and 250 mg L$^{-1}$) was pleasant for the cell growth, whereas in higher doses (i.e. 500, 750, and 1000 mg L$^{-1}$) showed inhibitory effects for cell density, especially after 72 and 96 h. Chlorophyll a and b content increased in 10, 100, and 250 concentrations, while carotenoids represented a dose-relevant decrease to Fe$_3$O$_4$ concentrations. Although iron is a vital micronutrient for algal viability and reproduction, inhibitory and toxicity effects may occur in its exceeding concentrations above 500 mg L$^{-1}$. As a strong accumulator in aquatic systems, \textit{C. vulgaris}, could desire to absorb released Fe$_3$O$_4$ ions, that led to increasing bioaccumulation with the elevation of iron concentrations. \textit{C. vulgaris} possesses a great capacity to bioconcentrate iron nanoparticles, and in this regard, this species plays an essential role in biomagnification through food
web in aquatic environments. EDI and THQ revealed that non-carcinogenic risk of Fe$_3$O$_4$ was at negligible risk (THQ < 1) for people. Taken together, although iron-based organocatalysts used in industrial applications are green and safe, in over-used conditions act as inhibitor for primary producers like _C. vulgaris_ and threaten the health status of consumers as aquatic functional food.

**Declarations**

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Author contributions**

_Taherh Ebrahini Yazdanabdad_: Conception and design, investigation and research, drafting of the article, _Ali Forghaniha_: critical revision of the article for important intellectual content, methodology and supervision, _Mozhgan Emtyazjoo_: final approval of the article, collection and assembly of data, _Majid Ramezani_: language revisions and finalizing the manuscript.

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Figures
Figure 1

Schematic of synthesized catalyst Fe3O4@SiO2@SiO3-(CH2)3-thiourea (I), magnetization curve of Fe3O4@SiO2@SiO3-(CH2)3-thiourea (II), XRD pattern of Fe3O4 (III-A) and Fe3O4@SiO2@SiO3-(CH2)3-thiourea (III-B), FT-IR spectra of Fe3O4 (IV-blue curve), Fe3O4@SiO2 (IV-red curve) and Fe3O4@SiO2@SiO3-(CH2)3-thiourea (IV-green curve), thermogravimetric analysis of Fe3O4@SiO2@SiO3-
(CH2)3-thiourea (V), EDX spectrum of Fe3O4@SiO2@SiO3-(CH2)3-thiourea (VI), SEM Analysis of the Fe3O4@SiO2@SiO3-(CH2)3-thiourea (VII-A), Fe3O4@SiO2@SiO3-(CH2)3-thiourea after reaction (VII-B).

**Figure 2**

Cell density of C. vulgaris exposed to different concentrations of Fe3O4 in a 96-h period.
Figure 3

Chlorophyll a content of C. vulgaris exposed to different concentrations of Fe3O4 in a 96-h period.
Figure 4

Chlorophyll b content of C. vulgaris exposed to different concentrations of Fe3O4 in a 96-h period.

Figure 5

Carotenoid content of C. vulgaris exposed to different concentrations of Fe3O4 NPs in a 96-h period.
Figure 6

Bioaccumulation of Fe3O4 in C. vulgaris during a 96-h exposure period (* indicates significant differences).
Figure 7

Bioconcentration factor of Fe3O4 in C. vulgaris during a 96-h exposure period.