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Accumulation and elimination of iron oxide nanomaterials in zebrafish (*Danio rerio*) upon chronic aqueous exposure

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ABSTRACT

A 52-day continuous semi-static waterborne exposure (test media renewed daily) regimen was employed to investigate the accumulation and elimination profiles of two iron oxide nanomaterials (nano-Fe₂O₃ and nano-Fe₃O₄) in zebrafish (*Danio rerio*). Adult zebrafish were exposed to nanomaterial suspensions with initial concentrations of 4.0 and 10.0 mg/L for 28 days and then were moved to clean water for 24 days to perform the elimination experiment. Fe content was measured in fish body and feces to provide data on accumulation and elimination of the two iron oxide nanomaterials in zebrafish. The experiment revealed that: (1) high accumulation of nano-Fe₂O₃ and nano-Fe₃O₄ were found in zebrafish, with maximum Fe contents, respectively, of 1.32 and 1.25 mg/g for 4.0 mg/L treatment groups and 1.15 and 0.90 mg/g for 10.0 mg/L treatment groups; (2) accumulated nanoparticles in zebrafish can be eliminated efficiently (the decrease of body burden of Fe conforms to a first-order decay equation) when fish were moved to nanoparticle-free water, and the elimination rates ranged from 86% to 100% by 24 days post-exposure; and (3) according to analysis of Fe content in fish excrement in the elimination phase, iron oxide nanomaterials may be adsorbed via the gastrointestinal tract, and stored for more than 12 days.

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Introduction

Nanotechnology and the production of nanoparticles (NPs) are exponentially growing, but research into the toxicological impact of nanoparticles on human health and the environment is still in its infancy (Elsaesser and Howard, 2012). Iron oxide nanomaterials are widely used in remediation and biomedical applications such as drug delivery (Gupta and Gupta, 2005), cell labeling (Chen et al., 2011), and magnetic resonance imaging (Puppi et al., 2011; Rümenapp et al., 2012). The research on health risks and ecological impacts of iron oxide nanomaterials is very limited because iron oxide nanomaterials are generally regarded as non- or low-toxic materials (Karlsson et al., 2008, 2009; Soenen and De Cuyper, 2010). However, recent studies have

revealed that iron oxide nanomaterials pose a potential health risk. Superparamagnetic iron oxide nanoparticles show cytotoxicity (Mahmoudi et al., 2010). Zhu et al. (2009a, 2011) also found that ferric oxide nanoparticles had potential lung and systemic cumulative toxicity in rats, and intravascular iron oxide nanoparticles may induce human endothelial inflammation and dysfunction. Moreover, iron oxide nanomaterials could serve as significant carriers of toxic chemicals (Guan et al., 2008; Tang et al., 2009) and increase exposures to adsorbed pollutants.

In the last decade, a wealth of acute toxicity studies focused on the short-term effects of engineering nanomaterials (ENMs). Works that have been done on chronic endpoints, such as biological and ecological accumulation, only began to increase in the last 3–4 years (Hou et al., 2013). As a result of the extensive

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application of metal oxide nanomaterials, the aquatic environment as a sink of most contaminants is particularly at risk of exposure to ENMs. Recently, studies on the accumulation of metal oxide nanomaterials in aquatic organisms have been carried out. TiO_2 was a relatively common research nanomaterial for aquatic organisms including daphnia, carp and zebrafish (Zhang et al., 2007; Sun et al., 2009; Zhu et al., 2010a, 2010b). Meanwhile, the exposure time is crucial for the toxic effects of metal oxide nanomaterials on aquatic organisms. Compared with 24 hr exposure, the accumulated content and mortality of brine shrimp (*Artemia salina*) exposed to nano- TiO_2 and nano- ZnO increased remarkably at 96 hr (Ates et al., 2013a, 2013b). In addition, the cubic and octahedral Cu_2O micro/nanocrystals both showed Cu accumulation toward *Daphnia magna* after a 3-day exposure (Fan et al., 2012). Montes et al. (2012) demonstrated the accumulation of Ce and Zn in soft tissues of a marine suspension-feeder, *Mytilus galloprovincialis*, exposed to nano- CeO_2 and nano- ZnO for 96 hr. Similarly, Gomes et al. (2012) found the accumulation and toxicity of nano- CuO in digestive gland of *M. galloprovincialis* exposed to nano- CuO for 15 days. Given that the exposure of aquatic organisms to NPs is probably long-term, the uptake and accumulation of NPs by aquatic organisms could impact the overall effects of nanomaterials and background toxins. Therefore, the chronic exposure of aquatic organisms to metal oxide nanomaterials as well as their clearance processes are worthy of examination.

As a common model organism, zebrafish (*Danio rerio*) is widely used in ecological toxicity research for ENMs. The objective of this study was to investigate the accumulation and elimination behavior of iron oxide nanomaterials (nano- Fe_2O_3 and nano- Fe_3O_4) in zebrafish under chronic exposure according to the total Fe content in fish body. Meanwhile, the elimination routes of NPs were examined through the total Fe content in their excrement. Little data have been found on NPs in the excrement of aquatic organisms. Therefore, this aspect is important for understanding the process of accumulation and elimination of iron oxide nanomaterials in aquatic vertebrates under long-term exposure.

1. Materials and methods

1.1. Materials

Experimental subjects were commercially available adult blue zebrafish (mixed gender) with 29.7 ± 1.8 mm body length and 0.29 ± 0.09 g mass. Prior to nanomaterial exposure, zebrafish were acclimated to the experimental condition. They were maintained for more than two weeks in a semiautomatic circulating water system with tap water, and fed by dry food from the market once per day. The total mortality of zebrafish was less than 3% during cultivation. The powders of nano- Fe_2O_3 and nano- Fe_3O_4 were uncoated at >99% purity. The size distributions were measured by nanoparticle size and zeta potential analyzer (Zetasizer Nano ZS90, Malvern, UK).

1.2. Experimental design

Semi-static experiments (designed according to OECD Guideline 305, 2011) including a 28-day uptake period and a 24-day elimination period were performed to measure the profile of accumulation and elimination in zebrafish exposed to nano- Fe_2O_3 and nano- Fe_3O_4 . The results of preliminary experiments showed that visible precipitation occurred within

a few hours for suspensions >10.0 mg/L and that operating errors in preparation were bigger for suspensions <4.0 mg/L, so the concentrations of the experimental suspensions were set to 4.0 and 10.0 mg/L. Four NP treatment groups were expressed as Fe_2O_3 -4.0, Fe_2O_3 -10.0, Fe_3O_4 -4.0, and Fe_3O_4 -10.0, respectively. The zebrafish (initially 55 individuals per exposure) were exposed to NP suspensions for 28 days, and then were removed to clean water (tap water pre-aerated for 24 hr) until day 52. Samples were taken with increasing intervals to optimally capture the accumulation and excretion profiles: days 0, 2, 5, 9, 14, 19, 24, 28, 30, 34, 40, 46, and 52. At each time point, four zebrafish were randomly sampled, pooled, and the total Fe amounts measured. On days 30, 34, 40, 46, and 52, fish excrement was also sampled and total Fe content measured. In addition, a control group (NP-free tap water) was set up, and four fish were sampled on days 0, 28 and 52 to determine the background values of total Fe content in the fish body and excrement. The exposure solution was refreshed every day with a new suspension of NPs to maintain the exposure at relatively consistent level at accumulation phase, and clean water renewal was also performed daily during the elimination phase. During the whole test period, in order to simulate real environmental conditions where food was available, zebrafish were allowed to be fed daily with dry food (Fe concentration of about 2 mg/g) after test medium changes, and the supplied diet was based on the amount of zebrafish contained in test tank (~2 mg dry food per fish). Sampling and feeding were conducted just before and just after test medium renewal, respectively. Furthermore, the mortality and behavior immobilization were also recorded. The experiments were performed at room temperature ($23 \pm 2^\circ\text{C}$) with a natural light–dark cycle.

1.3. Preparation and measurement of NP suspensions

Nano- Fe_2O_3 and nano- Fe_3O_4 powders were dispersed in glass beakers containing 5 L of tap water (pre-aerated for 24 hr, pH 7.7 and dissolved oxygen at 8.54 ± 0.18 mg/L) to a concentration of 4.0 and 10.0 mg/L. No dispersing agent was added to the suspension of nanomaterials in order to avoid any confounding toxicity. The test medium was sonicated (250 W, 40 kHz, 24°C) in a water bath using ultrasonic cleaners (KQ-250DE, Kunshan Ultrasonic Instrument Co., Ltd., Kunshan, Jiangsu Province, China) for 10 min and then hand-stirred for 1 min by three times. The suspensions were generated immediately prior to use in each experiment.

The 25 mL of NP suspension was sampled from the middle part of the test tank into 50 mL glass beakers and evaporated. Dried iron oxide NPs adsorbed onto beakers were decomposed into Fe ions by heating with HNO_3 – H_2O_2 (6:1, V/V) until the pellets were completely dissolved into a colorless solution. After cooling, the solutions were moved to 25 mL volumetric flasks. Released total Fe content was determined using an inductively coupled plasma mass spectrometer (ICP-MS, ELAN DRC-e, PerkinElmer SCIEX, Waltham, Massachusetts, USA). The tank NP concentration was indicated by measured total Fe content.

1.4. Quantification of NPs in fish tissue and excrement

At each sampling time point during the experiment, four zebrafish from each exposure were rinsed with cool physiological

saline, sacrificed by freezing, and blotted dry. The samples were fully dried in an oven at 80°C overnight, and were accurately weighed to 0.1 mg accuracy. Dried tissue was digested using a microwave digestion system (MDS-6G, Sineo Microwave Chemistry Technology Co., Ltd., Shanghai, China) with $\text{HNO}_3\text{--H}_2\text{O}_2$ (8:2, V/V), and then was heated continuously until the digestion solution was nearly dry (2 mL). After appropriate dilution and filtration, the total Fe content was determined using ICP-MS. The measured value of total Fe in fish body in control group was 0.56 ± 0.04 mg/g.

At each sampling time point during the elimination phase, the medium containing almost all of the fish excrement was siphoned into 500 mL glass breaker from the bottom of the tank. After filtration and drying, the fish excrement samples were scraped gently and accurately weighed (at 0.1 mg precision). The samples were digested and measured by the same method as with dried fish tissues. The total Fe level in excrement in the control group was determined to be 1.65 ± 0.12 mg/g.

1.5. Calculation and statistical analysis

The concentration of nano- Fe_2O_3 and nano- Fe_3O_4 can decline during the period between medium renewals. To characterize the decline of NP concentration in test water, we calculated the average sedimentation rate (S) (represented by S at the period of day 11–day 17) in accumulation phase by Eq. (1):

$$S = \frac{C_0 - C_1}{C_0} \times 100\% \quad (1)$$

where, C_0 (mg/L) and C_1 (mg/L) are the initial and final Fe content at every renewal period, respectively.

During the elimination phase, accumulated NPs in fish body can be purged by zebrafish, so the body burden of Fe decreases with time. The elimination rate (E_j) of NPs on day j after accumulation phase is given by Eq. (2):

$$E_j = \frac{C_{\max} - C_j}{C_{\max}} \times 100\% \quad (2)$$

where, C_{\max} (mg/g) is the measured maximum amount of Fe in the fish body, and C_j (mg/g) indicates the Fe content in fish body by j days post-exposure.

In order to further quantify and understand the discharge of NPs from zebrafish, a first-order decay equation (Eq. (3)) was used to model the Fe elimination process:

$$C(t) = C_a + C_b \cdot e^{-k_d \cdot t} \quad (3)$$

where, t (day) is the number of days post-exposure, $C(t)$ (mg/g) is the body burden of Fe at the time t , C_a (mg/g) is the residual amount of Fe in fish body which could not be excreted, C_b (mg/g) is the part of accumulated amount of Fe in fish body which could be totally excreted, k_d (day^{-1}) is the depuration rate coefficient, and the sum of C_a and C_b is the total accumulated amount of Fe at the beginning of elimination phase.

The half-life of Fe in fish body was determined by Eq. (4) based on k_d values:

$$t_{1/2} = \frac{\ln 2}{k_d} \quad (4)$$

where, $t_{1/2}$ (day) is the time needed to eliminate 50% of Fe amount on day 28.

Quantitative data are reported as a mean \pm standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) with LSD test using SPSS 19. A p -value of <0.05 was considered statistically significant. The fitting of the decay equation was performed with Origin 8.0.

2. Results

2.1. Characterization of NPs and suspensions

The diameter distributions of two kinds of nanomaterials are shown in Fig. 1. The average sizes of nano- Fe_2O_3 and nano- Fe_3O_4 were 80–90 nm and 140–160 nm, respectively. During the experiment, nano- Fe_2O_3 and nano- Fe_3O_4 aggregated and settled to the bottom of experimental tank. As shown in Fig. 2, the initial Fe content in Fe_2O_3 -4.0 and Fe_2O_3 -10.0 treatment groups decreased by $30.0\% \pm 4.2\%$ and $29.1\% \pm 5.0\%$, respectively. The larger reduction occurred in Fe_3O_4 -4.0 and Fe_3O_4 -10.0 treatment groups (S values were $61.7\% \pm 4.6\%$ and $78.5\% \pm 6.2\%$, respectively). It is apparent that the actual exposure of nano- Fe_2O_3 to zebrafish was greater than that of nano- Fe_3O_4 . The decreasing amplitude of the initial concentration might associate with the density, aggregation of the two kinds of nanomaterials, and disturbance by fish activities.

2.2. Accumulation and elimination of NPs in zebrafish

We set up a long-term waterborne exposure of nano- Fe_2O_3 and nano- Fe_3O_4 to examine the uptake and efflux of NPs by zebrafish. The daily monitoring of mortality and general health of zebrafish revealed one dead individual and seven individuals with extravasated blood in all four treatment groups during the experiment, and the skin color of fish exposed to nano- Fe_2O_3 changed from blue to reddish yellow at the first time point and maintained this appearance during the accumulation phase. These observed adverse effects may be related to the tissue accumulation of NPs or a direct contact effect on the fish surface. But precisely how zebrafish skin exposed to NPs mediated a systemic effect on extravasated blood and color changes will need subsequent studies.

Fig. 3a shows the profile of accumulation and elimination of nano- Fe_2O_3 in zebrafish. In the accumulation phase, nano- Fe_2O_3 was readily observed in fish body. In addition to

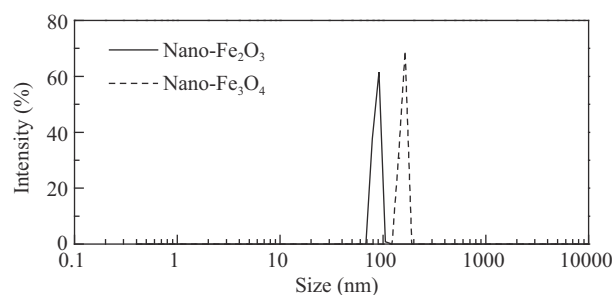


Fig. 1 – Size distributions of nano- Fe_2O_3 and nano- Fe_3O_4 .

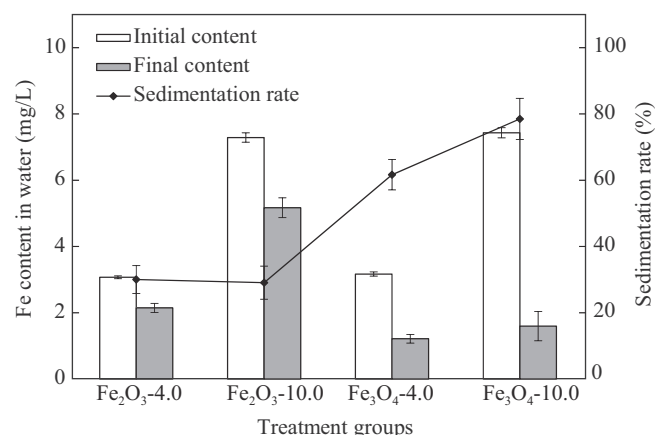


Fig. 2 – Mean initial/final Fe contents in water during seven renewal periods (days 11–17). Fe₂O₃-4.0: the treatment group of 4.0 mg/L nano-Fe₂O₃; Fe₂O₃-10.0: the treatment group of 10.0 mg/L nano-Fe₂O₃; Fe₃O₄-4.0: the treatment group of 4.0 mg/L nano-Fe₃O₄; Fe₃O₄-10.0: the treatment group of 10.0 mg/L nano-Fe₃O₄.

days 5 and 9, the body burden of Fe was significantly higher ($p < 0.05$) than that before exposure (day 0). A rapid increase of Fe content was observed in the initial 2 days, followed by a small amplitude decrease in days 5–9 for both of concentrations groups. The body burden of Fe kept increasing and reached the maximum value (C_{\max}) at day 14 for the 4.0 mg/L treatment group, and day 19 for the 10.0 mg/L treatment group. Fe content was readily lost in the later exposure period. There were 51.9% and 50.9% reductions of C_{\max} at the longest exposure time (day 28) for 4.0 and 10.0 mg/L treatment groups, respectively. Overall, the trend of Fe content over time was consistent and there was no significant difference ($p > 0.05$) between 4.0 and 10.0 mg/L treatment groups.

In the elimination phase, the Fe content in fish body were gradually reduced over time, and tended to be relatively stable after 12 days post-exposure (day 40). This result

revealed that clearance of nano-Fe₂O₃ from zebrafish was a relatively fast process before day 40 and entered a slow mode after that. The elimination rates of nano-Fe₂O₃ for 4.0 and 10.0 mg/L treatment groups reached 96.4% and 100% at 24 days post-exposure (day 52), reverting to the original content level.

Fig. 3b shows the relationship between body burden of Fe and time under nano-Fe₃O₄ treatment groups. During the accumulation phase, zebrafish could accumulate significantly nano-Fe₃O₄ in the body. Similar to the nano-Fe₂O₃, there were modest decreases at days 5–9, with consistent trend and no significant differences ($p > 0.05$) for Fe content in the two concentration groups. Compared with nano-Fe₂O₃, the time to reach C_{\max} was longer (24 and 19 days, respectively) and the elimination of nano-Fe₃O₄ in 10.0 mg/L treatment group was incomplete.

Table 1 lists the accumulation and elimination parameters for the four treatment groups. Because the actual exposure concentrations of nano-Fe₂O₃ were higher than nano-Fe₃O₄, the C_{\max} values in nano-Fe₂O₃ treatment groups were higher than nano-Fe₃O₄ treatment groups ($p > 0.05$). Interestingly, the C_{\max} for the 4.0 mg/L treatment groups was slightly higher than for the 10.0 mg/L treatment groups ($p > 0.05$). This suggests that accumulated NP content in zebrafish body did not increase as the exposure concentration increased in the experimental concentration range. The elimination rates showed the vast majority of ingested NPs can be cleared by zebrafish, while the Fe₃O₄-10.0 treatment group still had approximately 14% of C_{\max} retained in fish body at the end of the experiment.

The curve-fitting results on loss of Fe content from fish body during the elimination phase are shown in Fig. 4, and parameters of the decay models are listed in Table 2. A first-order decay model fits the test data, with R^2 in the range of 0.829–0.933. The results showed that k_d of 4.0 mg/L treatment groups were smaller than 10.0 mg/L treatment groups for two iron oxide nanomaterials. In other words, $t_{1/2}$ of 4.0 mg/L treatment groups was longer. This corresponds with the results in Table 1, in which C_{\max} in 4.0 mg/L treatments were higher than that in 10.0 mg/L treatments for two iron oxide nanomaterials.

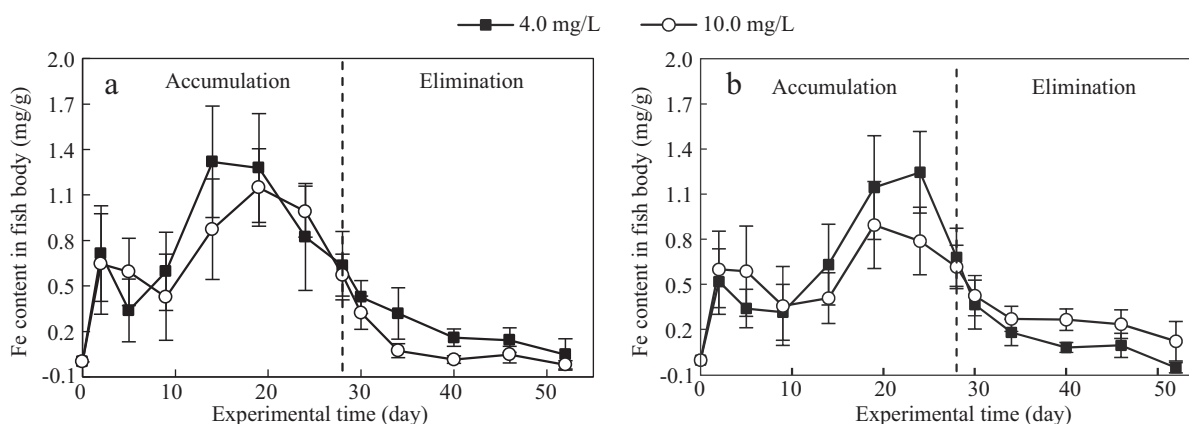


Fig. 3 – The profile of accumulation and elimination of nano-Fe₂O₃ (a) and nano-Fe₃O₄ (b).

Table 1 – Maximum amount of Fe in fish body (C_{\max}) and elimination rates of nanoparticles by 24 days post-exposure (E_{24}) in different treatment groups.

Items	Fe ₂ O ₃ -4.0	Fe ₂ O ₃ -10.0	Fe ₃ O ₄ -4.0	Fe ₃ O ₄ -10.0
C_{\max} (mg/g)	1.32 ± 0.37	1.15 ± 0.26	1.25 ± 0.27	0.90 ± 0.29
E_{24}	96.4%	100%	100%	86.0%
Fe ₂ O ₃ -4.0: the treatment group of 4.0 mg/L nano-Fe ₂ O ₃ ; Fe ₂ O ₃ -10.0: the treatment group of 10.0 mg/L nano-Fe ₂ O ₃ ; Fe ₃ O ₄ -4.0: the treatment group of 4.0 mg/L nano-Fe ₃ O ₄ ; Fe ₃ O ₄ -10.0: the treatment group of 10.0 mg/L nano-Fe ₃ O ₄ .				

Table 2 – Parameters of the first-order decay models for Fe content in fish body.

Treatment groups	C_a (mg/g)	C_b (mg/g)	k_d (day ⁻¹)	R^2	$t_{1/2}$ (day)
Fe ₂ O ₃ -4.0	0.064 ± 0.053	0.508 ± 0.061	0.135	0.933	5.13
Fe ₂ O ₃ -10.0	-0.004 ± 0.014	0.581 ± 0.082	0.314	0.921	2.21
Fe ₃ O ₄ -4.0	-0.075 ± 0.061	0.633 ± 0.124	0.117	0.845	5.92
Fe ₃ O ₄ -10.0	0.227 ± 0.033	0.387 ± 0.076	0.316	0.829	2.19

C_a : the residual amount of Fe in fish body which could not be excreted; C_b : the part of accumulated amount of Fe in fish body which could be totally excreted; k_d : the depuration rate coefficient; $t_{1/2}$: the half-life of Fe in fish body.

2.3. Quantification of NPs in fish excrement

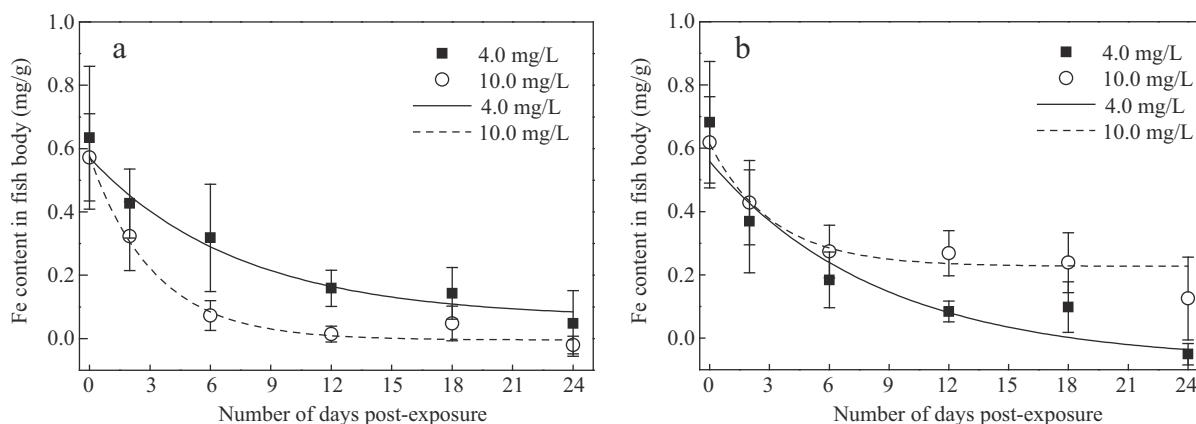
The decreases of body burden of Fe when zebrafish were moved to clean water suggested that zebrafish might excrete most ingested NPs via some pathway. On the other hand, some red and black granules were observed in fish feces on the first two days of the elimination phase for nano-Fe₂O₃ and nano-Fe₃O₄, respectively. These granules may be attributable to the direct efflux of unbiodegradable NPs ingested in fish body. Therefore, it was thought that ingested NPs may be located in the gastrointestinal tract (GI tract), and can be eliminated through the excretion process. As shown in Fig. 5, on day 30 (2 days post-exposure), the Fe content in fish feces in the four treatment groups were 7.0–7.6 times as high as that (1.65 ± 0.12 mg/g) in the control group, then decreased to varying degrees. Finally they were reduced to the same level as Fe content in the control group until day 40 (12 days post-exposure). This finding corresponds to results of body burden of Fe in the elimination phase. Both showed that the residence time of NPs in fish body was more than 12 days.

3. Discussion

During the entire experiment period, a semi-static exposure regimen was employed to ensure that exposure

concentrations were maintained at a relatively constant level and that the clean water met the general water quality requirements for zebrafish. However, the concentration drops (29.1%–78.5%) of prepared suspensions were observed in all four treatment groups. This indicated that the real exposure concentrations of NPs to zebrafish were less than the initial concentration during the most of exposure interval. Similar results were reported in other studies, in which the nano-TiO₂ and nano-ZnO (initial concentration of 1.0 mg/L) decreased by approximately 40% and 80% over the first hour, and then remained steady or fell slowly for the subsequent 23 hr (Zhu et al., 2009b; Wang et al., 2011). So far, most aquatic and terrestrial experiments with ENMs have been conducted using either a static or semi-static exposure regimen. However, maintenance of steady exposure concentrations is still challenging (Handy et al., 2012).

Overall, the profiles and trends of accumulation for four treatment groups were consistent. The brief drops of body burden of Fe might result from stress responses of zebrafish in the early period (days 5–9) of accumulation phase in an attempt to remove NPs from the body. During this drop period, the excreted amount of NPs was greater than the amount absorbed by zebrafish, leading to a net release of NPs and a slight reduction of Fe content. Over time, the zebrafish adaptation to the exposure environment made stress

**Fig. 4 – Fitting curves for elimination of nano-Fe₂O₃ (a) and nano-Fe₃O₄ (b).**

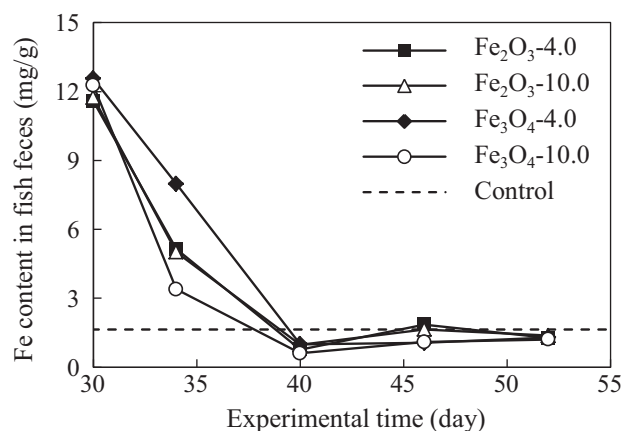


Fig. 5 – Changes of Fe content in fish feces in the elimination phase.

responses weaken or even disappear, so the body burden of Fe increased gradually after 9 days. Nevertheless, the amounts of Fe did not reach a steady state in the exposure phase, while a decline of Fe content occurred after reaching C_{\max} (on days 14, 19 and 24, respectively). This suggests that the accumulation processes of the two iron nanomaterials in zebrafish are different from classic bioaccumulation which assumes membrane passage. According to Zhu et al. (2010a), chronic exposure to nano-TiO₂ produced a negative impact on *D. magna*'s feeding behavior, reducing NP intake. Since oral ingestion is the major pathway of NPs into the fish body (Hou et al., 2013), we infer that the decline of Fe content after reaching C_{\max} is attributable to the NP-mediated chronic toxicity to the gut, causing zebrafish to reduce food ingestion and thus reduce NP uptake. Similar results were observed in 28-day chronic exposure of zebrafish to silver NPs, in which the biggest tissue burdens were measured on day 21 and day 7 after exposure for eviscerated carcass and gill tissue, respectively (Griffitt et al., 2013). However, the time to reach the maximum accumulation was different. This may be related to NP properties, concentration of NPs used in accumulation experiments, the intestinal tolerance of NP-mediated chronic toxicity, and the experiment conditions.

In the elimination period, the clearance of nano-Fe₂O₃ and nano-Fe₃O₄ was evident from the reduction of Fe content in fish body. Most or all of accumulated NPs could be depurated by zebrafish at the end of the elimination phase. The high elimination rate (86%–100%) might be not related only to body reaction to NPs, but also associated with the feeding behavior of zebrafish during the experiment. Some studies have shown that the presence of food may improve the clearance process of NPs from fish (Zhu et al., 2010a; Hu et al., 2012). The decay curves for Fe content in fish body showed that the 4.0 mg/L treatment groups for two iron oxide nanomaterials had a smaller k_d and a longer $t_{1/2}$ than 10.0 mg/L treatment groups. These findings correspond with C_{\max} . The effective elimination, however, was established on the basis of a long elimination period (24 days). Comparatively speaking, the declining trend of Fe content in fish body and feces was consistent along with experiment time. Both showed a fast decay phase (days 28–40) and a stable period (days 40–52).

Based on these observations, we speculate that accumulated NPs are adsorbed within the GI tract of zebrafish, and can leave the fish via an excretion process. This inference is supported by previous study (Sun et al., 2007), in which accumulated amounts of arsenic and TiO₂ in intestine and stomach (>90%) were much higher than other parts (gills, liver, scales, and muscle) under a 25-day exposure to carp. The observed nanomaterial granules in fish feces over the first two days of the elimination phase also indicated that removal of iron oxide nanomaterials from zebrafish was mainly a physical clearance process. Johnston et al. (2010) found that the bioavailability is low for unmodified metal oxide NPs in an aquatic environment. But adverse effects might occur in aquatic environments when particles are not biodegraded or readily excreted from the body (Kunzmann et al., 2011). By comparing the Fe content in fish body and feces under Fe₃O₄-10.0 treatment group, we found that the elimination of nano-Fe₃O₄ from the body was incomplete when Fe content in excrement returned to normal level (Fe content in control group). This suggests that the small amount of nanoparticles or dissolved iron ions may cross gut mucosal barriers and distribute to other organs. Some study results have shown that nano-TiO₂ can be absorbed through the gill and gut and enter the circulatory system to various organs of carp (Sun et al., 2007, 2009; Zhang et al., 2007) or trout (Ramsden et al., 2009). This may be one of the reasons why the body burden of Fe decreased fast in the early period and slow in last period of elimination phase.

Comparatively speaking, the amounts of nano-Fe₂O₃ and nano-Fe₃O₄ found in the zebrafish were very similar in this study. This might be largely because uptake of NPs by aquatic organisms is a fast process, so the initial NP concentration plays a more important role there than for other time points (Hu et al., 2012). Though the sedimentation rate of nano-Fe₃O₄ was much higher than nano-Fe₂O₃, the uptake of NPs by zebrafish had been completed essentially before they settled out. Therefore, the same initial concentration of NPs in water caused no significant differences ($p > 0.05$) in intake amounts between nano-Fe₂O₃ and nano-Fe₃O₄. On the other hand, as oral ingestion is the major pathway of NPs into fish, food ingestion could affect the NP uptake. Therefore, the same weight of food might also lead to the similar accumulation amount of two nanomaterials in fish body. There was no significant difference ($p > 0.05$) in body burden of Fe between 4.0 and 10.0 mg/L treatment groups during the whole experiment time. However, some research showed that accumulation of NPs in aquatic organism tissue was dose-dependent (Montes et al., 2012; Griffitt et al., 2013). This may be determined by the material properties and biological species. Moreover, Hu et al. (2012) also discovered that the dose-dependent relationship occurred only in a certain dose range when *Ceriodaphnia dubia* were exposed to nano-Fe₂O₃. The amount of nano-Fe₂O₃ found in *C. dubia* did not increase when exposure concentrations were higher than a limit concentration (20 mg/L). This might be caused by the limited gut capacity of *C. dubia*, which suggests that gut capacity of aquatic organisms is one of the factors that influence the body burden of NPs. The fact that the two exposure levels used within this study induced the similar body levels of Fe supports the viewpoint that saturation of sorption sites for NPs with the GI tract was taking place.

4. Conclusions

In summary, our data derived from the Fe content in fish body and excrement indicated that the two kinds of iron oxide nanomaterials can be accumulated in zebrafish, and can be cleared with a high elimination rate by 24 days post-exposure. The decrease of bodily levels of nanomaterials conforms to a first-order decay model. The iron oxide nanomaterials entering zebrafish may be adsorbed within the gastrointestinal tract, and reserved for more than 12 days.

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