



ORIGINAL ARTICLE

# Iron oxide nanoparticles to an Indian major carp, *Labeo rohita*: Impacts on hematology, iono regulation and gill $\text{Na}^+/\text{K}^+$ ATPase activity



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Hematology;  
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Gill  $\text{Na}^+/\text{K}^+$ -ATPase

**Abstract** In this study, the chronic toxicity effects of iron oxide ( $\text{Fe}_2\text{O}_3$ ) nanoparticles (NPs) ( $500 \text{ mg l}^{-1}$ ) on certain hematological, ionoregulatory and gill  $\text{Na}^+/\text{K}^+$  ATPase activity of an Indian major carp, *Labeo rohita* were estimated for a period of 25 days under static bioassay. A significant increase in hemoglobin (Hb) content, red blood cell (RBC) count and hematocrit (Ht) value was noticed throughout the study period when compared to control groups. In contrast, mean cellular volume (MCV), mean cellular hemoglobin (MCH) (except on 5th day) and mean cellular hemoglobin concentration (MCHC) levels and white blood cell (WBC) counts were found to be decreased during the above study period.  $\text{Fe}_2\text{O}_3$  NPs also caused alterations in iono regulation resulting in hyponatremia ( $\text{Na}^+$ ), hypochloremia ( $\text{Cl}^-$ ) (except on 5th day) and hypokalemia ( $\text{K}^+$ ) (except up to 15th day). A biphasic trend in gill  $\text{Na}^+/\text{K}^+$ -ATPase activity was noticed during the above treatment period. Our results demonstrate that high  $\text{Fe}_2\text{O}_3$  NP concentrations in the aquatic environment may have adverse physiological effects on fish. These data may be useful to

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assess the environmental risk posed by NPs. However the toxicity of various sizes of the nanoparticle could be evaluated using different aquatic organisms.

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## 1. Introduction

The production of nanomaterials is growing exponentially (millions of tonnes of NPs yearly) throughout the world (Simonet and Valcarcel, 2009; Klaine et al., 2012) due to their unique physicochemical properties such as high surface area and an enhanced reactivity (Farkas et al., 2011; Petersen and Henry, 2012). The increasing number and quantity of manufactured NPs in various fields like chemical industry, electronics, biomedicine, food additives, and semiconductors and others may pave a way for the discharge of these particles in the various segments of the environment (Zhang and Elliott, 2006; Blaise et al., 2008; Klaine et al., 2012). The properties of NPs differ remarkably from small molecules which may cause a lot of hazardous effects on environment and also human health (Colvin, 2003; Moore, 2006; Gaiser et al., 2012). The aquatic environment may act as a sink for the entry of these NPs (Farre et al., 2009; Scown et al., 2010; Gaiser et al., 2012) that are easily taken by aquatic organisms such as mollusks, crustaceans and fish (Ward and Kach, 2009; Johnston et al., 2010; George et al., 2014). The uptake and effects of NPs in the aquatic biota may be a major concern (Moore, 2006) which leads to extensive toxicological studies (Griffitt et al., 2008).

Metallic nanoparticles (NPs) which include particles made from Au, Ag, Pt, Fe, and Cu and metal oxides such as ZnO and TiO<sub>2</sub> particles (Glenn et al., 2012) are widely used due to their unique properties such as diverse surface chemistries and can be prepared into a variety of shapes and sizes (Murphy et al., 2005). Among the metal oxide nanoparticles, Iron oxide NPs (particularly magnetite (Fe<sub>2</sub>O<sub>3</sub>) and hematite forms ( $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> and  $\beta$ -Fe<sub>2</sub>O<sub>3</sub>) are widely used in biomedical, bioengineering and clinical applications due to their unique magnetic properties and high catalytic abilities (Weissleder et al., 1990; Huber, 2005; Smith et al., 2007; Kadar et al., 2011; Naqvi et al., 2010; Singh et al., 2010; Chen et al., 2012). Furthermore, iron-based nanoparticles (NPs) are also used for soil and groundwater remediation and water treatment processes (Yavuz et al., 2006; Chen et al., 2012). Iron NPs may enter the surface of water via effluent discharge or waterborne transportation, underground and surface aquifers and may pose a risk to aquatic organisms and humans (Chen et al., 2012; Shen et al., 2012; Zhu et al., 2012). Hence studies on the fate and ecotoxicological risk due to iron NPs in aquatic environment are urgently needed (Chen et al., 2012).

Fish species have been extensively used for assessing the effects of NPs on aquatic ecosystem. For example, fluorescent silica nanoparticles (10 and 50 mg l<sup>-1</sup>) affect the early development of *Oryzias latipes* embryos (Lee et al., 2011). Rainbow trout (*Oncorhynchus mykiss*) exposed to dietary titanium dioxide nanoparticles (10 or 100 mg kg<sup>-1</sup>) shows biochemical disturbances in the brain (Ramsden et al., 2009). Smith et al. (2007) observed a dose dependant rise in ventilation rate, gill pathologies (edema, altered mucocytes, hyperplasia) and

mucus secretion in juvenile rainbow trout exposed to single walled carbon nanotubes (SWCNT). Wise et al. (2010) reported that silver nanoparticles are cytotoxic and genotoxic to fish cells. Likewise nano zinc oxide (nZnO) was found to be toxic to crustaceans and fish (Wong et al., 2010). Previous literature indicates that nanometals can be lethal to fish in mg to  $\mu\text{g l}^{-1}$  range, depending on the type of material (Shaw and Handy, 2011).

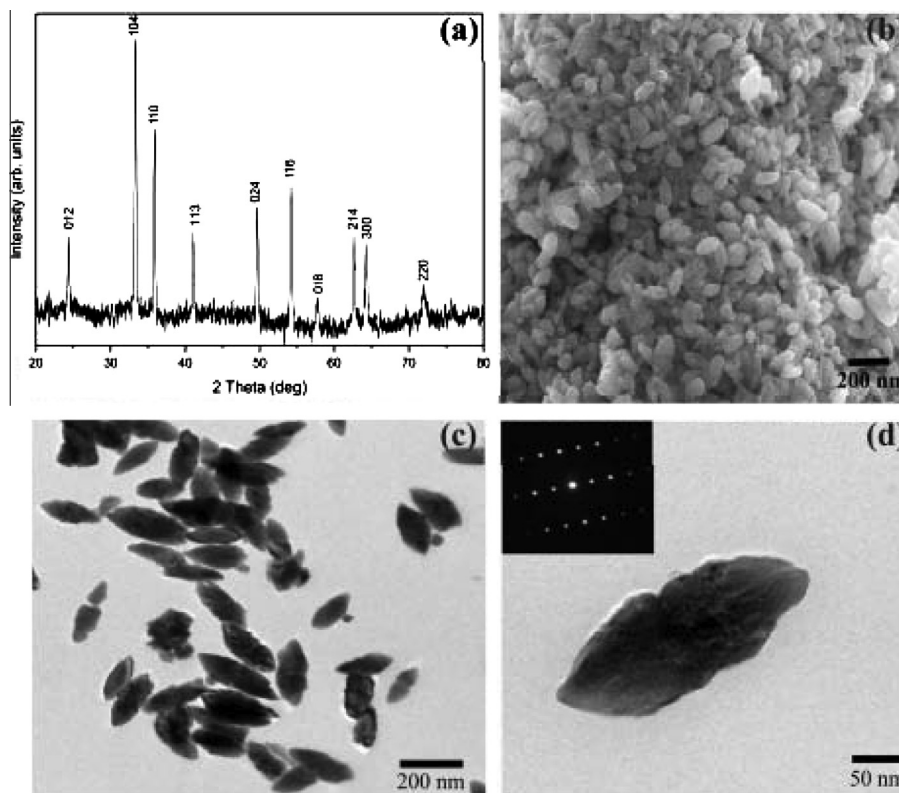
However, the information on the harmful effects of metal oxide NPs particularly iron oxide nanoparticles on aquatic animals is very limited (Bystrzejewska-Piotrowska et al., 2009; Zhu et al., 2012; Castro-Bugallo et al., 2014). Moore (2006) suggested that uptake, bioavailability and harmful effects of nanoscale materials in the aquatic environment can be evaluated by suitable biomarkers. Biological end points or biomarkers such as hormonal, hematological, iono regulation, biochemical parameters and histopathology which are used in the field of aquatic toxicology can be also used to evaluate the impact of engineered nanoparticles on aquatic organisms (Klaine et al., 2008; Handy et al., 2012). Recently, Shaw and Handy (2011) suggested that the risk of nanomaterials on fish hematology, plasma biochemistry and biochemical alterations in internal organs needs to be investigated. This paper critically evaluated the toxic effect of iron oxide nanoparticles at higher concentration on the physiology of an Indian major carp *Labeo rohita* using certain hematological, ionoregulatory and gill Na<sup>+</sup>/K<sup>+</sup>-ATPase biomarkers. The alterations of these parameters may help to generate data on the possible mechanism of Fe<sub>2</sub>O<sub>3</sub> NPs toxicity at an unrealistic dose in the aquatic environment.

## 2. Materials and methods

The Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore 46, Tamil Nadu, India, has been registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The experiments and the handling of the organisms were carried out as per the guidelines of CPCSEA.

### 2.1. Fish

Healthy specimens of *L. rohita* fingerlings were procured from Tamil Nadu Fisheries Development Corporation Limited, Aliyar Fish Farm, Aliyar, Tamil Nadu, India and acclimatized to laboratory conditions for about 20 days before the commencement of the experiment. During acclimatization, fish were fed with rice bran and ground nut oil cake (*ad libitum*) once a day. Feeding was given at least one hour prior to replacement of water. Water (one-third) was changed frequently to remove the excretory wastes. Feeding was withheld for 24 h before the commencement of the experiment to keep the experimental animals more or less in the same metabolic



**Figure 1** Structure and morphological analysis of  $\text{Fe}_2\text{O}_3$  NPs (a) XRD pattern of spindle like hematite nanostructure (b) FESEM image of spindle-like nanostructure (c) TEM Image of spindle-like nanostructure (d) TEM Image of a single spindle-like nanostructure (inset shows the corresponding SAED pattern).

state. During acclimatization, the fish stock was maintained at natural photoperiod and ambient temperature. This ensures sufficient oxygen for the fish and the environment is devoid of any accumulated metabolic wastes. In the present study, tap water free from chlorine was used with the following physicochemical characteristics (APHA, 1998); temperature:  $27.0 \pm 2.0$  °C; pH:  $7.0 \pm 1$ , dissolved oxygen:  $6.8 \pm 0.02$  mg  $\text{l}^{-1}$ ; total alkalinity:  $32.0 \pm 5$  mg  $\text{l}^{-1}$ ; salinity:  $1.3 \pm 0.1$  ppt; total hardness:  $18.0 \pm 0.2$  mg  $\text{l}^{-1}$ ; calcium:  $3.4 \pm 0.4$  mg  $\text{l}^{-1}$  and magnesium:  $2.05 \pm 0.2$  mg  $\text{l}^{-1}$ .

### 2.2. Preparation of $\text{Fe}_2\text{O}_3$ NPs

Iron oxide nanoparticles ( $\text{Fe}_2\text{O}_3$ , spindle shaped) were prepared in the Thin Films and Nanomaterials Laboratory, School of Physical Sciences, Bharathiar University. The particles were prepared by using forced hydrolysis or the reflux condensation method. The structure and morphological analysis are shown in Fig. 1. The prepared sample was characterized using X-ray diffraction (XRD), scanning electron microscope (SEM) and Fourier transforms infra red spectroscopy. The structural analysis of the prepared sample made using XRD shows the pure hematite phase of iron oxide. The average size of the spindle shaped particle is about 200 nm in length and 100 nm in diameter.

### 2.3. Preparation of stock solution

Powder form of ultra fine  $\text{Fe}_2\text{O}_3$  NPs was disposed in distilled water. A stock solution of  $15 \text{ g l}^{-1}$   $\text{Fe}_2\text{O}_3$  NPs was prepared by

dispersing nanoparticle in distilled water with sonication 6 h bath type sonicator (40 kHz frequency Vibronics-250 wts) and subsequently for a further 30 min sonication immediately prior to dosing each day. NPs were kept in suspension in the water using aeration or a peristaltic pump for minimizing the settling of NPs. The dispersion was very good at final working concentration. In addition, and despite extensive sonication, a few aggregates of NPs were also observed in stock solution.

### 2.4. Iron oxide ( $\text{Fe}_2\text{O}_3$ ) NP exposure

For the assessment of  $\text{Fe}_2\text{O}_3$  NP toxicity, aquarium with 10 L of water was taken. Then to each aquarium, different concentrations of the  $\text{Fe}_2\text{O}_3$  NPs (i.e. 2, 10, 100, 250, 500, 750, 1000 ppm) were added. A control aquarium with 10 L of water was also taken. Ten healthy fish, with an average length of 7.0 cm and average weight of 5.0 g were selected and introduced into each aquarium. The manifestation and survival time of fish was observed in each concentration after 24. There was no mortality of fish exposed to 2, 10, 100 and 250 ppm of  $\text{Fe}_2\text{O}_3$  NPs. However, mortality was observed at 500, 750, 1000 ppm. Based on the above observation, 500 ppm was selected for the present study. For the chronic assay 300 fingerlings were selected from the stock and divided into three groups (one control and two experiments) with 100 fish in aquarium filled with water. Simultaneously, three replicates were also maintained. Then 500 ppm of  $\text{Fe}_2\text{O}_3$  NPs was added into two experimental aquariums after removal of the same volume of water. Experiments were conducted for a period of 25 days with 5 day sampling frequency.

Upon completion of the stipulated exposure period of 5, 10, 15, 20 and 25 days, 20 fish were taken out and sacrificed without anesthetizing for further analysis. After removal of fish at various intervals of time, the volume of the experimental and control media was adjusted to maintain a constant density of fish per unit volume of water.

### 2.5. Hematological studies

Fish from control and iron oxide treated aquarium were sacrificed and blood was drawn by cardiac puncture using a plastic disposable syringe. The collected blood sample was transferred into small vials, which were previously rinsed with heparin. The whole blood sample was used for the estimation of hematological parameters like Hb, RBC and WBC counts. The remainder of the blood sample was centrifuged in a cooling centrifuge at 10,000 rpm for 20 min to separate the plasma, which was used for the estimation of electrolytes (sodium, potassium and chloride). Hb was estimated by the Cyanmethemoglobin method. Hct was estimated by the microhematocrit (capillary) method (Nelson and Morris, 1989). RBC and WBC were counted by the method of Rusia and Sood (1992) using hemocytometer. Erythrocyte indices of fish viz., MCV (Mean cell volume), MCH (Mean cell hemoglobin) and MCHC (Mean cell hemoglobin concentration) were also calculated according to standard formulas.

$$\text{MCV}(\text{fl}) = \frac{\text{Hct}(\%) \times 10}{\text{RBC count in millions}/\text{mm}^3}$$

$$\text{MCH}(\text{pg}) = \frac{\text{Hb}(\text{g}/\text{dl}) \times 10}{\text{RBC count in millions}/\text{mm}^3}$$

$$\text{MCHC}(\text{g}/\text{dl}) = \frac{\text{Hb}(\text{g}/\text{dl})}{\text{Hct}(\%) \times 100}$$

### 2.6. Ionic regulation

Sodium and potassium were estimated by the method of Maruna (1958) and chloride was estimated by the modified method of Tietz (1990), and Young et al. (1975).

### 2.7. Gill $\text{Na}^+/\text{K}^+$ -ATPase assay

After drawing the blood fish were washed with double distilled water and blotted dry with absorbent paper. Then the gills were separated from the control and iron oxide treated fish and 100 mg tissue from each was weighed and homogenized with 1.0 ml of 0.1 M Tris-HCl buffer (pH 7.5) using a Teflon homogenizer. Then the contents were centrifuged at 1000 rpm at 4 °C for 15 min and the clear supernatant was used for the sodium potassium ATPase activity. The specific activities of gill  $\text{Na}^+/\text{K}^+$ -ATPase were estimated following the method of Shiosaka et al. (1971).

0.3 ml of Tri-HCl buffer (pH7.5), 0.1 ml of 0.02 M ATP, 0.1 ml of 100 mM NaCl and 0.1 ml of KCl were taken in test tubes; 0.1 ml of distilled water was added to 'Blank' and 0.1 ml of tissue extract (gill) of control and  $\text{Fe}_2\text{O}_3$  NP treated fish was added to respective tubes. The reaction mixture was mixed and incubated in water bath at 37 °C for 15 min and the reaction was terminated with 2.00 ml of 5% TCA. Tubes

were kept at 4° for 30 min and centrifuged for 5 min at 500 rpm. To the supernatant, 1 ml of ammonium molybdate and 0.4 ml of ANSA reagent were added and allowed to stand for 10 min at room temperature and the intensity of the blue color developed was read at 680 nm against reagent. Suitable standards were also run through each batch of assays. The enzyme activity was expressed in terms of micrograms of inorganic phosphorous formed per gram of tissue.

### 2.8. Statistical analysis

The significance ( $p < 0.05$ ) between control and  $\text{Fe}_2\text{O}_3$  NP treated fish was analyzed by using Student's  $t$  test.

## 3. Results

### 3.1. Hematological responses

Hematological parameters of *L. rohita* exposed to  $\text{Fe}_2\text{O}_3$  NPs for a period of 25 days are presented in Table 1. During the above treatment period, both Hb and Hct contents were increased up to 10th day in  $\text{Fe}_2\text{O}_3$  NP treated fish and then declined from that of the respective control groups (Table 1). RBC count was increased in  $\text{Fe}_2\text{O}_3$  NP treated fish throughout the exposure period registering a direct relationship with the exposure period (10–237%) (Table 1). WBC count was decreased significantly ( $P < 0.05$ ) in  $\text{Fe}_2\text{O}_3$  NP treated fish when compared with their respective controls (Table 1). Hematological indices such as MCV and MCH were decreased (except at the end of 5th day) significantly ( $p < 0.05$ ) when compared to their respective control groups (Table 1). However, a non significant decrease in MCHC value was observed in  $\text{Fe}_2\text{O}_3$  NP treated fish throughout the study period (except at the end of 15 and 20th day).

### 3.2. Ionoregulation

Fish exposed to  $\text{Fe}_2\text{O}_3$  NPs showed a significant increase ( $p < 0.05$ ) in plasma sodium level (except at the end of 5th day) throughout the study period when compared with the control groups (Fig. 2). Plasma potassium level in  $\text{Fe}_2\text{O}_3$  NP treated fish was found to be increased up to 15th day and underwent a significant ( $p < 0.05$ ) decrease during subsequent exposure period (Fig. 3). However, the decrease in plasma chloride level was not significant on 5th day when compared to control groups (Fig. 4).

### 3.3. Gill $\text{Na}^+/\text{K}^+$ -ATPase activity

Gill  $\text{Na}^+/\text{K}^+$ -ATPase in  $\text{Fe}_2\text{O}_3$  NP treated fish was significantly ( $p < 0.05$ ) increased up to 20th day and then slightly decreased at the end of the study period when compared to that in the control groups (Fig. 5).

## 4. Discussion

Scown et al. (2009) reported that aquatic environment is particularly vulnerable to contamination from ENMs (engineered nanomaterials) and the knowledge on the behavior; entry and toxicity of these ENMs are very limited. However, nanoparti-

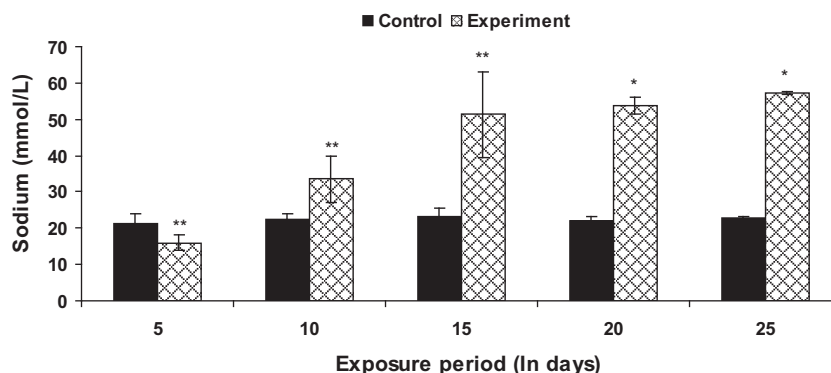
**Table 1** Changes in the hematological parameters of fish *L. rohita* treated with Fe<sub>2</sub>O<sub>3</sub> NPs (500 mg l<sup>-1</sup>).

Hematological parameter	Exposure time (d)	Control	Experiment	Percent change
Hb (g/dl)	5	5.550 ± 0.158	7.014 ± 0.026**	(+26.37)
	10	4.362 ± 0.389	6.057 ± 0.005*	(+38.86)
	15	5.710 ± 0.013	3.710 ± 0.110**	(-35.02)
	20	5.900 ± 0.027	4.269 ± 0.182*	(-27.63)
	25	5.360 ± 0.017	4.104 ± 0.048**	(-23.40)
Hct (%)	5	12.80 ± 1.173	17.91 ± 0.003*	(+39.92)
	10	16.60 ± 0.510	20.68 ± 0.052*	(+24.58)
	15	16.39 ± 0.295	11.38 ± 0.337*	(-30.57)
	20	15.03 ± 0.005	12.13 ± 0.045*	(-19.32)
	25	17.53 ± 0.094	12.84 ± 0.580*	(-26.76)
RBC (million/cu mm)	5	0.22 ± 0.007	0.24 ± 0.011**	(+10.00)
	10	0.25 ± 0.005	0.42 ± 0.032*	(+67.46)
	15	0.22 ± 0.022	0.51 ± 0.165**	(+131.91)
	20	0.24 ± 0.016	0.72 ± 0.048**	(+198.33)
	25	0.23 ± 0.009	0.78 ± 0.027*	(+237.39)
WBC (1000/cu mm)	5	83.51 ± 0.757	40.86 ± 0.991*	(-51.07)
	10	84.22 ± 0.213	48.00 ± 1.122*	(-43.01)
	15	84.15 ± 0.375	50.25 ± 1.117*	(-40.28)
	20	84.33 ± 0.373	57.85 ± 0.859*	(-36.83)
	25	83.95 ± 0.678	53.27 ± 0.815*	(-31.08)
MCV (fl)	5	568.00 ± 47.67	746.13 ± 32.70*	(+31.38)
	10	659.72 ± 24.16	502.17 ± 40.67*	(-23.88)
	15	767.01 ± 26.24	147.08 ± 17.04*	(-80.71)
	20	666.35 ± 54.18	172.91 ± 13.39*	(-74.05)
	25	780.91 ± 34.35	165.48 ± 4.71*	(-78.81)
MCH (picograms)	5	192.93 ± 15.96	252.31 ± 11.11*	(+30.77)
	10	220.75 ± 11.56	170.35 ± 13.89*	(-22.83)
	15	259.18 ± 16.58	48.04 ± 5.10*	(-81.46)
	20	243.96 ± 11.37	58.44 ± 4.20*	(-76.05)
	25	263.08 ± 11.10	55.05 ± 1.820*	(-79.08)
MCHC (g/dl)	5	33.92 ± 0.159	33.82 ± 0.028**	(-0.29)
	10	33.46 ± 0.195	33.01 ± 0.069*	(-1.34)
	15	33.59 ± 0.477	32.61 ± 0.056**	(-2.91)
	20	35.64 ± 0.113	33.83 ± 0.284*	(-5.07)
	25	33.69 ± 0.081	33.25 ± 0.335**	(-1.30)

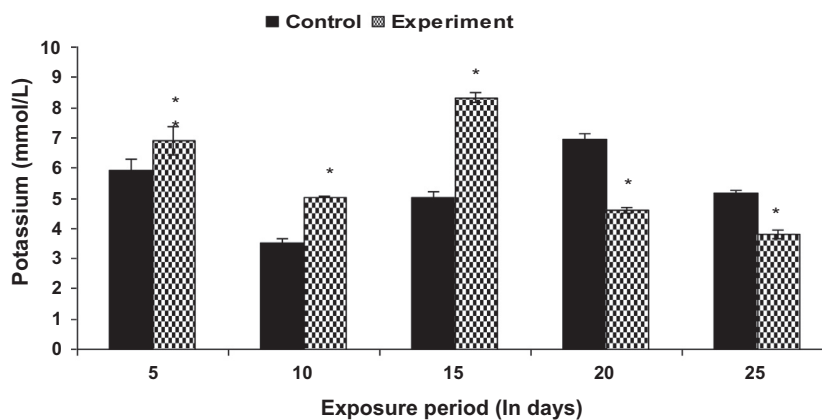
Values are means ± S.E. of five individual observations, (+) denotes percent increase over control, (-) denotes percent decrease over control.

\* Significant,  $p < 0.05$ .

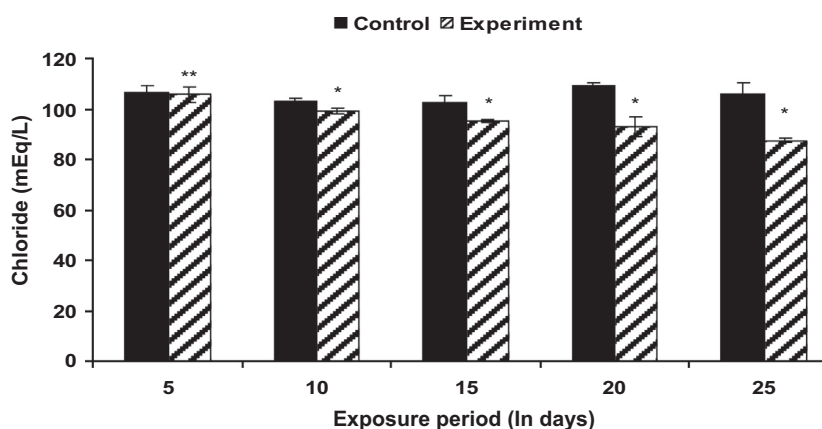
\*\* Not significant,  $p > 0.05$  (based on  $t$ -test).



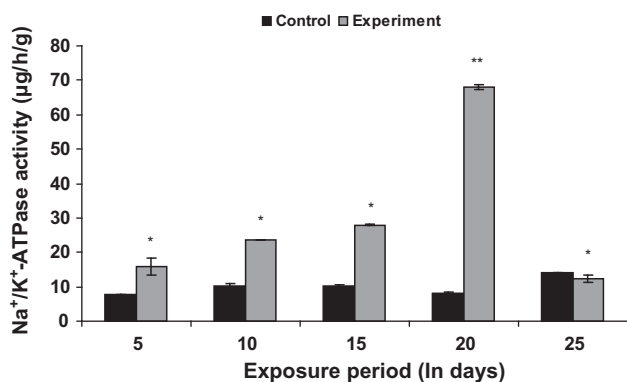
**Figure 2** Plasma Na<sup>+</sup> level in control and Fe<sub>2</sub>O<sub>3</sub> NPs treated *L. rohita* (500 mg l<sup>-1</sup>; 25 days). Bars represent means of the SE of five individual observations with (\*) significant and \*\* not significant at  $p < 0.05$  (based on  $t$  test).



**Figure 3** Plasma K<sup>+</sup> level in control and Fe<sub>2</sub>O<sub>3</sub> NP treated *L. rohita* (500 mg l<sup>-1</sup>; 25 days). Bars represent means of the SE of five individual observations with (\*) significant and \*\* not significant at  $p < 0.05$  (based on  $t$  test).



**Figure 4** Plasma Cl<sup>-</sup> level in control and Fe<sub>2</sub>O<sub>3</sub> NP treated *L. rohita* (500 mg l<sup>-1</sup>; 25 days). Bars represent means of the SE of five individual observations with (\*) significant and \*\* not significant at  $p < 0.05$  (based on  $t$  test).



**Figure 5** Gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activities in control and Fe<sub>2</sub>O<sub>3</sub> NP treated *L. rohita* (500 mg l<sup>-1</sup>; 25 days). Bars represent means of the SE of five individual observations with (\*) significant and \*\* not significant at  $p < 0.05$  (based on  $t$  test).

cles may produce reactive oxygen species (ROS) upon their interaction with organisms or other agents present in the environment (Castro-Bugallo et al., 2014). Manufactured NPs upon their release in the aquatic environment may conjugate

with biological molecules and gain soluble properties which may affect the aquatic organisms through oxidative stress resulting damages in lipids, carbohydrates, proteins and DNA (Kohen and Nyska, 2002; Niazi and Gu, 2009). In this line, Li et al. (2009) and Chen et al. (2012) reported that attachment of nano-iron particles with the gill region may lead to damage in the epithelial cell resulting in the entry of these particles into the fish body and alter the antioxidants and antioxidant enzymatic activity. Furthermore incorporation or adsorption of dissolved cations may be the mechanism of NP toxicity in aquatic organisms (Franklin et al., 2007; Arojo et al., 2009). Previous studies reported direct adherence/adsorption of nFe<sub>2</sub>O<sub>3</sub> aggregates on the surface of the exposed organisms and their effects such as delay of hatching, damage in cell wall and outer membranes, depletion of oxygen exchange and hypoxia (Cheng et al., 2007; He et al., 2011).

Furthermore, direct adherence/adsorption of nFe<sub>2</sub>O<sub>3</sub> aggregates on the surface of the exposed organisms leads to a high level of free ions resulting in the accumulation of nFe<sub>2</sub>O<sub>3</sub> (Zhu et al., 2012). In the present study the observed mortality of fish in higher concentrations of Fe<sub>2</sub>O<sub>3</sub> NP treated groups might have resulted from the excessive accumulation of Fe<sub>2</sub>O<sub>3</sub> NPs in the body of fish. Imbalance in homeostasis and aberrant cellular responses, DNA damage, oxidative stress and

inflammatory processes has been reported at cellular level (Singh et al., 2010). Iron nanoparticles may penetrate the body of animals, accumulate and induce toxic effects (Chen et al., 2011). Excessive accumulation of free ion can cause toxicity via generation of reactive oxygen species (ROS) (Dixon and Stockwell, 2014). A similar mechanism may be operated in the present study also. Furthermore release of free ion can potentially cross the nuclear or mitochondrial membrane and cause cytotoxic effect due to its catalytic function in the production of ROS in the Fenton reaction ( $\text{H}_2\text{O}_2$  is converted into highly reactive hydroxyl or superoxide radicals catalyzed by  $\text{Fe}^{2+}/\text{Fe}^{3+}$  ions) (Muller et al., 2007; Singh et al., 2010; Huang et al., 2013).

Hematological parameters such as Hb, Hct, RBCs and WBCs are frequently used as indicators of metal pollution in the aquatic environment and also to determine the sublethal toxicity of pollutants (Nussey et al., 1995). In the present study exposure of fish *L. rohita* to  $\text{Fe}_2\text{O}_3$  NPs showed significant alterations in the hematological parameters. Similar to our findings, Smith et al. (2007) reported a significant decrease in the hematocrit and blood hemoglobin in rainbow trout exposed to SWCNT. In contrast to above findings,  $\text{TiO}_2$  NPs did not cause any major disturbances in hematology of rainbow trout (Federici et al., 2007; Handy and Shaw, 2007). The observed reduction in Hb and Hct contents might have resulted from structural changes in gill structure due to  $\text{Fe}_2\text{O}_3$  NP accumulation and toxicity. The observed increase in Hb and Hct level may reflect the increased demand for oxygen under  $\text{Fe}_2\text{O}_3$  NP toxicity.

It has been already reported that nanoparticles may accumulate in gills and damage the organ resulting in respiratory disturbances (Griffitt et al., 2007; Handy et al., 2008; Li et al., 2009; Ates et al., 2013). Further, excess amount of iron can result in iron flocs on the gills which may also lead to respiratory disturbances (Dalzell and MacFarlane, 1999). In the present study the observed increase in RBC count might have resulted from oxygen deficiency due to gill damage caused by  $\text{Fe}_2\text{O}_3$  NP toxicity. A significant decrease in WBC count during  $\text{Fe}_2\text{O}_3$  NP exposure may indicate a decrease in nonspecific immunity of the fish due to  $\text{Fe}_2\text{O}_3$  NP stress. Moreover, attachment of nanoparticles to the membranes or storage of these particles inside the cells may impair cellular functions (Bystrzyewska-Piotrowska et al., 2009). In contrast to our findings no significant changes in WBC count were observed in fish exposed to SWCNT (Smith et al., 2007). The observed alterations in erythrocyte indices may be a response to the compensation for the impaired oxygen uptake caused by the toxicant ( $\text{Fe}_2\text{O}_3$  NPs). A significant decrease in MCH was noted in fish exposed to SWCNT at high concentration (Smith et al., 2007). Furthermore  $\text{Fe}_2\text{O}_3$  NPs may affect the immune system of fish resulting in alterations in the hematological parameters.

Plasma electrolytes such as sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ), and chloride ( $\text{Cl}^-$ ) are highly sensitive to environmental changes and their measurement can be used as potential biomarkers of chemical exposure in aquatic organisms (Mayer et al., 1992). The gills of fish due to their large surface area and intimate contact with water are likely to be the important target organ for aquatic pollutants such as metals, pesticides and nanoparticles (Griffitt et al., 2007; Farkas et al., 2011). Similar to our study, depletion of plasma  $\text{Na}^+$  and  $\text{Cl}^-$  and

$\text{Na}^+/\text{K}^+$ -ATPase has been reported in fish exposed to silver nanoparticles (Farmen et al., 2012). A decrease in plasma electrolytes has been reported in zebra fish (*Danio rerio*) exposed to silver nanoparticles indicating that AgNPs might have inhibited the  $\text{Na}^+/\text{K}^+$ -ATPase activity (Katuli et al., 2014) because in teleost fish gill  $\text{Na}^+/\text{K}^+$ -ATPase plays an important role in the maintenance of electrolytes between extra and intra cellular milieu (McCormick, 1993). Likewise significant alterations in plasma  $\text{Na}^+$  and  $\text{K}^+$  were noted in fish exposed to SWCNT (Smith et al., 2007). In the present study the decrease in plasma electrolytes during  $\text{Fe}_2\text{O}_3$  NP exposure indicates that the  $\text{Fe}_2\text{O}_3$  NPs may act as a stressor and inhibit the  $\text{Na}^+/\text{K}^+$ -ATPase activity resulting in alterations in ionoregulation of fish. Furthermore alteration of electrolytes may be due to accumulation and toxic effect of  $\text{Fe}_2\text{O}_3$  NPs in the gill surface. A histological alteration in gill such as edema was noted in rainbow trout exposed to  $\text{TiO}_2$  NPs (Federici et al., 2007). Similarly, histological alterations such as cell swelling and hyperplasia were also noticed in gill of fish exposed to Fe-NPs (Li et al., 2009). Osmoregulatory failure may be another possible reason for the observed decreased levels of major plasma ions. In this line impaired osmoregulation has been reported in fish exposed to nanoparticles (Farmen et al., 2012).

In freshwater fish the enzyme  $\text{Na}^+/\text{K}^+$ -ATPase plays an important role in active transport mechanisms for ions. Inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase activity in gills of  $\text{Fe}_2\text{O}_3$  NPs treated fish *L. rohita* may be due to a change in the physical properties of the membrane or alterations in the lipid content of the membrane due to the accumulation of iron oxide nanoparticles. Furthermore release of free ions from  $\text{Fe}_2\text{O}_3$  NPs might have affected the gill structure. Inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase activity was also reported in fish exposed to various nanoparticles (Griffitt et al., 2007; Ramsden et al., 2009; Shaw and Handy, 2011; Farmen et al., 2012). Farmen et al. (2012) reported that silver nanoparticles may impair the osmoregulatory capacity by inhibiting the  $\text{Na}^+/\text{K}^+$ -ATPase activity. In the present study the accumulation of dissolved iron particles in the gill region may impair the osmoregulation. However at the end of the study period gill  $\text{Na}^+/\text{K}^+$ -ATPase activity was found to be increased. Similar to our findings a significant increase in  $\text{Na}^+/\text{K}^+$ -ATPase activity was noted in fish exposed to carbon nanotubes (Smith et al., 2007; Fent et al., 2010). Towle (1981) suggested that high  $\text{Na}^+/\text{K}^+$ -ATPase activity in gills of the teleosts appeared to provide an adaptive mechanism to support the increased  $\text{Na}^+$  uptake, required in the dilute freshwater environments.

In the present study the alterations in hematological, ionoregulatory and enzymological parameters might have resulted from the release of metals into solution (Keenan et al., 2009; Phenrat et al., 2009; Chen et al., 2012) or the specific physico-chemical properties of the iron NPs (Chen et al., 2012). They also reported that  $\text{nFe}_3\text{O}_4$  aggregates may be loosely formed and can easily penetrate the cell membrane. Lee et al. (2007) reported that AgNPs upon exposure cross the chorion of zebrafish embryos by Brownian diffusion indicating that early life stages of fish are sensitive to nanometals. Moreover, the toxicity of nanoparticles also depends upon their surface properties, size, ionic strength, redox state, organic matter (Illés and Tombácz, 2005; Zhang et al., 2007; Klaine et al., 2008; Farkas et al., 2011; McShane et al., 2012).

## 5. Conclusion

The concentration used in this study has a profound influence on the hematological, ionoregulatory and gill  $\text{Na}^+/\text{K}^+-\text{ATPase}$  activity of an Indian major carp, *L. rohita*. These parameters could be effectively used as potential biomarkers or biological end points in assessing the toxicity of engineered nanoparticles on aquatic organisms. The results of the present study highlight the need for safe disposal and protocols for these metal oxides. However, further research is needed on the direct effect of metal ion and/or release of metal ions from nanoparticles.

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