Ecotoxicology and Environmental Safety 186 (2019) 109743

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Effects of iron oxide nanoparticles (Fe_3O_4) on life history and metabolism of the Neotropical cladoceran *Ceriodaphnia silvestrii*

Renan Castelhano Gebara[a,b,*], Jaqueline Pérola Souza[c], Adrislaine da Silva Mansano[c], Hugo Sarmento[a,b], Maria da Graça Gama Melão[a,b]

[a] Department of Hydrobiology, Universidade Federal de São Carlos (UFSCar), Rodovia Washington Luís, Km 235, 13565-905, São Carlos, SP, Brazil
[b] Post-Graduate Program in Ecology and Natural Resources (PPGERN), Universidade Federal de São Carlos (UFSCar), Rodovia Washington Luís, Km 235, 13565-905, São Carlos, SP, Brazil
[c] Nanomedicine and Nanotoxicology Group, Physics Institute of São Carlos (IFSC), Universidade de São Paulo (USP), Avenida Trabalhador Sãocarlense, 400, 13560-970, São Carlos, SP, Brazil

ARTICLE INFO

Keywords:
Cladoceran
Magnetite
Microcrustacean
Nanotoxicity
Respiration
Species sensitivity distribution

ABSTRACT

Nanoparticles (NPs) production is increasing worldwide. These products are likely to end up in aquatic environments. However, few studies evaluated the chronic toxicity of iron-based NPs (Fe-NPs) to cladocerans and their potential ecotoxicological hazards. In this study we aimed to investigate the effects of iron oxide nanoparticles (Fe_3O_4-NPs) to *Ceriodaphnia silvestrii* Daday, 1902, assessing acute (48 h) and chronic toxicity (up to 14 d). Besides traditional endpoints (immobility and lethality), we also evaluated physiological responses (respiration rates) in a 48 h-exposure. No immobility was observed (EC_{50} > 100 mg L^{-1}) after 48 h, whereas respiration rates at the highest concentration were 400% of that in control, indicating that this endpoint was more sensitive during acute toxicity. In chronic assays, Fe_3O_4-NPs affected body length (8.24% growth inhibition in 7 d-exposure) and number of eggs (7-d IC_{10}: 3.53, IC_{20}: 6.69 mg Fe L^{-1}) and neonates (7-d IC_{10}: 1.25, IC_{20}: 3.75 mg Fe L^{-1}). Based on species sensitivity distribution (SSD), *C. silvestrii* was a sensitive organism, suggesting Fe-NPs as a possible threat for this species. Our results also indicate that the NPs caused a physical barrier, impairing food absorption, since we observed NPs agglomerations into cladocerans' gut. We demonstrate that Fe_3O_4-NPs affects *C. silvestrii* metabolism and reproduction and our results support the use of sublethal endpoints to assess environmental safety. The release of these NPs into freshwater environments should be carefully evaluated, since disturbances on cladoceran population dynamics could cause strong impacts on the entire food web structure and ultimately on ecosystem functioning.

1. Introduction

The environmental fate of nanoparticles (NPs) is of great concern since their production has been progressively increasing mainly due to their great potential for technological and biomedical applications (Krishna et al., 2018; Zhu and Liao, 2015). Despite the increased production of NPs, there are few specific recommendations for their disposal into aquatic environments. The European Union and Switzerland are the only regions that have specific legislation for nanomaterials (Amenta et al., 2015). In the United States of America, the Food and Drug Administration (FDA) regulated nanomaterials with the same parameters used for bulk particles, although FDA provides some specific guidance documents (USEPA, 2014). In Brazil, for example, there is no specific legislation for nanomaterials (Amenta et al., 2015), despite being a leading country in nanotechnology research in Latin America (Kay and Shapira, 2009).

The iron-based NPs (Fe-NPs) are widely studied for biomedical proposals (Zhu and Liao, 2015). Fe-NPs are also widespread in technology acting as photocatalytic and nanoadsorbents of organic and inorganic compounds in water and wastewater treatment (Dave and Chopda, 2014; Santhosh et al., 2019), furthermore Fe-NPs also have potential for soil and groundwater remediation (Grieger et al., 2010). Nonetheless, a study demonstrated that Fe-NPs used in soil remediation could be transported by the groundwater flow for more than 20 m of distance (Zhang, 2003). The Fe-NPs transported into aquatic environments could be a threat and may cause important consequences to the

* Corresponding author. Department of Hydrobiology, Universidade Federal de São Carlos (UFSCar), Rodovia Washington Luís, Km 235, 13565-905, São Carlos, SP, Brazil.
E-mail address: nangebara@gmail.com (R.C. Gebara).

https://doi.org/10.1016/j.ecoenv.2019.109743
Received 13 April 2019; Received in revised form 27 August 2019; Accepted 28 September 2019

biota, since there is evidence that Fe-NPs can be bioaccumulated by cladoceran (Hu et al., 2012) and fish (Zhang et al., 2015). Regarding the transfer to higher levels of food chain, biomagnification from algae to cladoceran was not yet observed even at high Fe-NPs concentrations (Bhuvaneshwari et al., 2017), although studies with other NPs suggests a potentially trophic transfer, *e.g.* from: algae to daphnia (Bouldin et al., 2008); algae to amphipod (Jackson et al., 2012); and cladoceran to fish (Zhu et al., 2010).

Previous studies have assessed the Fe-NPs toxicity on various aquatic species, such as bacteria (*e.g.* Wang et al., 2014), algae (*e.g.* Magro et al., 2018), bryophyte (*e.g.* Canivet et al., 2015), macrophyte (*e.g.* Barhoumi et al., 2015), rotifer (*e.g.* Mashjoor et al., 2018), cladocerans (*e.g.* Magro et al., 2018) and fish (*e.g.* Zhu et al., 2012). However, there is no study dealing with the chronic toxicity of Fe_3O_4-NPs on tropical cladocerans.

We investigated the effects of iron oxide nanoparticles (Fe_3O_4-NPs) on the Neotropical cladoceran *Ceriodaphnia silvestrii* by performing acute and chronic toxicity tests and respirometric analysis. Oxygen consumption measurements have been used to characterize the toxicity of metals (Massarin et al., 2010), pharmaceuticals (Lamichhane et al., 2013), herbicides and other organic compounds (Martins et al., 2007) on cladocerans. Additionally, respiration of fish has been used to assess toxicity of NPs (Cowart et al., 2011). Under unfavorable conditions, energy for growth and reproduction can be reallocated to activate repair mechanisms or reallocated to neutralize or remove chemicals from the body which results in increased metabolic rates (Calow, 1991; Zeman et al., 2008). Thus, respirometric analysis is highly relevant to assess alterations of metabolic rates. In addition, as far as we know, this is the first time that respiration rates are used as endpoint to determine NPs toxicity to cladocerans.

We aimed to investigate the toxicity of Fe_3O_4-NPs on Neotropical cladoceran *C. silvestrii*, in order to measure which NPs' concentrations could affect these organisms. During acute toxicity tests, we evaluated immobility and respiration rates after 48 h of exposure; while reproduction, growth and survival parameters were assessed in chronic toxicity during 14 d. As far as we know, this is the first study of chronic toxicity of Fe_3O_4-NPs to tropical microcrustaceans. It is important to test the effects of contaminants on tropical organisms, since they could be more sensitive to substances than temperate species (Mansano et al., 2018). We also compared the Fe-NPs' toxicity observed in *C. silvestrii vs.* data for other aquatic organisms, plotting an SSD (species sensitivity distribution) curve.

2. Material and methods

2.1. Nanoparticles: source, characterization and metal determination

Iron oxide nanoparticles (Fe_3O_4, magnetite, purity ≥ 98%, powder, particle size < 50 nm) were purchased from Sigma-Aldrich (CAS: 1317-61-9). The Fe_3O_4-NPs were characterized in ultrapure water and in the exposure media during the chronic toxicity tests (0.25; 8.61 and 44.23 mg L^{-1} of Fe_3O_4-NPs, at 0 h and 72 h from the beginning of the test). Characterization of NPs consisted of determining the zeta-potential (mV), polydispersity index (PdI) and hydrodynamic size (d.nm) by dynamic light scattering in Malvern Zetasizer Nano ZS90 (England). Analyses were performed with triplicates for each concentration.

Test solutions for metal determination were sampled in the water column of the vessels after their preparation, but immediately before the animals were placed in it. The vessels were not shaken during sampling to avoid an overestimation of NPs' concentrations. The test solutions from chronic and respirometric assays were prepared by filtering samples through pre-washed (1M HCl immersion for 24 h followed by vigorous rinse with ultrapure water) cellulose acetate filters (0.45 μm pore size, Sartorius, Germany), in order to quantify the particulate and dissolved iron. After that, the filters were acid digested (HNO_3 ultrapure, JT Baker) for 48 h at 92 ± 1 °C. Samples for particulate and dissolved analysis were acidified to a final concentration of 5% HNO_3. The iron concentrations in Fe_3O_4-NPs suspensions were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 7000 Series, Thermo Scientific).

2.2. Zooplankton cultures

The cladoceran *C. silvestrii* was obtained from laboratory cultures of the NEEA/CRHEA (São Carlos School of Engineering - EESC, University of São Paulo - USP, Brazil). Animals were maintained in 2 L beakers filled with 1.5 L of soft synthetic water (ASTM, 2002) (46–48 mg $CaCO_3$ L^{-1}, pH 7.0–7.6). Synthetic water was made by adding the following analytical grade chemicals in deionized water: $CaSO_4 \cdot 2H_2O$ (1.74×10^{-4} M), KCl (2.68×10^{-5} M), $NaHCO_3$ (5.71×10^{-4} M) and $MgSO_4 \cdot 7H_2O$ (2.47×10^{-4} M). Microcrustaceans were maintained in an incubator under controlled conditions of temperature (25 ± 1 °C) and photoperiod (12:12 h, light: dark).

Zooplankton was fed with 2×10^5 cells mL^{-1} of the Chlorophyceae algae *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, J. Kristiansen & O.M. Skulberg, 1987, which was grown in a modified L.C. Oligo medium (AFNOR, 1980), with the following composition: Ca $(NO_3)_2 \cdot 4H_2O$ (1.7×10^{-4} M), $NaNO_3$ (1.0×10^{-3} M), $MgSO_4 \cdot 7H_2O$ (1.2×10^{-4} M), K_2HPO_4 ($2.3\ 10^{-4}$ M), $CuSO_4 \cdot 5H_2O$ (6.0×10^{-8} M), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (2.4×10^{-8} M), $ZnSO_4 \cdot 7H_2O$ (1.0×10^{-7} M), $CoCl_2 \cdot 6H_2O$ (1.3×10^{-7} M), $Mn(NO_3)_2 \cdot H_2O$ ($1.5\ 10^{-7}$ M), $C_6H_8O_7 \cdot H_2O$ (1.4×10^{-7} M), H_3BO_3 (4.9×10^{-7} M), $C_6H_5FeO_7 \cdot H_2O$ (3.1×10^{-6} M), $FeCl_3 \cdot 6H_2O$ (1.9×10^{-6} M), $FeSO_4 \cdot 7H_2O$ (1.1×10^{-6} M) and $NaHCO_3$ (1.8×10^{-4} M). Erlenmeyer flaks were gently aerated in aseptic conditions and kept in controlled conditions of temperature (25 ± 2 °C) and light intensity (12:12 h light:dark cycle; 150 μmol $m^{-2}s^{-1}$) until the exponential growth phase. After that, cultures were centrifuged at 2000 rpm for 15 min (Eppendorf 5702 R, Germany), the pellet was re-suspended in synthetic water, counted in a Neubauer chamber under an optical microscope (Zeiss Axiostar Plus, Germany) and then supplied as food to the cladocerans. In addition, cladocerans were fed with a complementary compound (0.5 mL L^{-1}) which consisted of a solution of dried yeast dissolved in deionized water (5 g L^{-1}) added with a filtered solution of fermented fish ration (5 g L^{-1}), in a 1:1 ratio. Cladoceran sensitivity had been tested periodically with a reference substance (sodium chloride - NaCl) in order to check their viability for toxicity tests.

2.3. Toxicity tests

The toxicity tests followed standard procedures (ABNT, 2016, 2017). During the tests, the NPs stock solutions were dispersed using an ultrasonic bath (Unique USC-1400, Brazil) for 30 min (135 Watts RMS/40 kHz) before use. New stock solutions from the powder NPs were made once at the beginning of each test. The test solutions were made by dilutions of the stock solution in the synthetic water. The deionized water used in the experiments was obtained by reverse osmosis. During the toxicity tests, the following physical and chemical variables were measured: pH (Analion PM 608, Brazil), conductivity (MS Tecnopon mCA-150P, Brazil), dissolved oxygen concentration (Hanna Instruments, Brazil) and water hardness. It is important to note that Fe_3O_4-NPs were settled to the base of experimental flasks during the toxicity tests, therefore the NPs were manually resuspended every 24 h during the tests. Treatments were assigned by complete randomization.

Firstly, acute toxicity tests were performed with a wide range of Fe_3O_4-NPs nominal concentrations of 0.00; 0.01; 0.10; 1.00; 10.00 and 100.00 mg L^{-1}. For each treatment, 20 neonates (< 24 h-old) of *C. silvestrii* were distributed in 4 replicates, thus 5 individuals per replicate. Each replicate consisted in a polystyrene flask with 50 mL capacity and filled with 20 mL of test solution (without renewal, static test). The flasks were kept in incubator at a controlled temperature (25 ± 1 °C) in the dark, without food and immobile organisms were

Table 1
Mean values (x) and standard deviation (SD) for characterization parameters of Fe_3O_4-NPs (mg L^{-1}) during chronic exposure: hydrodynamic size (diameter in nm), polidispersivity index (PdI) and zeta-potential (mV). Samples (n = 3 replicates) were collected during 2 periods (Tc): 0 and 72 h after beginning the chronic toxicity test. Fe_3O_4-NPs were also characterized dispersed in ultrapure water. Different letters in each column indicate that there was statistical difference ($p < 0.05$), comparing separately parameters from 0 to 72 h. Lowercase letters indicate one-way ANOVA and Tukey's post hoc test; while uppercase letters correspond to Kruskal-Wallis analysis, followed by Tukey's post hoc test.

Treatments (mg L^{-1})	Tc (h)	Hydrodynamic size (nm)	PdI	Zeta-potential (mV)	Hydrodynamic size (nm)	PdI	Zeta-potential (mV)
		Soft synthetic water (test solutions)			Ultrapure water		
		x ± SD	x ± SD	x ± SD	x ± SD	x ± SD	x ± SD
0.25	0	729.6 ± 29.40^{A}	0.834 ± 0.08^{a}	- 25.1 ± 0.66^{a}	128.1 ± 22.8^{a}	0.780 ± 0.05^{a}	−28.2 ± 0.9^{a}
8.61	0	710.6 ± 59.62^{A}	0.540 ± 0.13^{a}	- 21.7 ± 1.55^{a}	237.4 ± 30.2^{b}	0.794 ± 0.04^{a}	−22.9 ± 0.4^{b}
44.23	0	1451.3 ± 299.09^{B}	0.723 ± 0.24^{a}	- 22.4 ± 0.32^{a}	243.8 ± 34.43^{b}	0.802 ± 0.06^{a}	−20.3 ± 0.4^{c}
0.25	72	1110.0 ± 262.51^{a}	0.952 ± 0.08^{a}	- 24.7 ± 2.37^{a}	–	–	–
8.61	72	1277.3 ± 244.30^{a,b}	0.904 ± 0.16^{a}	- 23.9 ± 0.32^{a}	–	–	–
44.23	72	1712.7 ± 165.54^{b}	0.481 ± 0.13^{b}	- 19.9 ± 0.23^{b}	–	–	–

quantified after 48 h.

Based on the results of the acute toxicity test, chronic toxicity tests were performed with Fe_3O_4-NPs measured concentrations of 0.00 (control); 0.25; 2.49; 8.61; 13.32 and 44.23 mg L^{-1}. The test had 10 replicates per treatment with 1 organism (< 24 h-old) each. Organisms were placed in 50 mL polystyrene flasks containing 20 mL of test solution and maintained under controlled conditions of temperature (25 ± 1 °C) and photoperiod (12:12 h, light:dark) in a static renewal exposure system (test solutions were renewed 100%, three times a week). Organisms were fed daily with 2×10^5 cells mL^{-1} of *R. subcapitata* and three times per week with 0.02 mL ind^{-1} of a suspension of dried baker's yeast (described in 2.2 section). During chronic toxicity testes, animals were exposed to the Fe_3O_4-NPs only by contaminated medium (direct exposure). The endpoints observed were age of primipara, number of eggs and neonates per female, daily length, maximum length, hatching rate and longevity. The cladocerans were measured daily using a stereomicroscope fitted with a micrometer eyepiece (Leica MZ6, Germany). Organisms from the 2.49 mg L^{-1} treatment level were photographed to show the internalizations of the NPs.

Regarding respirometric experiment, neonates (< 24 h-old) were initially pre-exposed to Fe_3O_4-NPs measured concentrations of 0.00 (control); 3.93; 15.92 and 58.80 mg L^{-1} and maintained at 25 ± 1 °C, in the dark and without food similarly to the immobility acute toxicity test (without test solution renewal, static test). After 48 h, 12 animals of each treatment (4 replicates, 3 animals each) were gently placed in 2 mL respirometry chambers. The chambers contained test solutions made from autoclaved synthetic water in order to avoid the presence of other microorganisms that could affect respiration measurements (*e.g.* bacteria). It is important to note that before the test, the autoclaved water was vigorously aerated in aseptic conditions using a sterile pipette, inside a laminar flow with UV light, to avoid oxygen depletion. Oxygen concentration continuous measurements in incubation chambers were carried out with a microrespiration system (Unisense, sensor OPMR-8573, Denmark). The sensor signal was calibrated using aerated water (100% of oxygen saturation) and a solution of 10 g L^{-1} of Na_2SO_3 containing cobalt nitrate ($Co(NO_3)_2$) as a catalyst (0% of oxygen saturation). The decrease of oxygen in the experimental chambers was monitored and 10–15 min were used in the analysis of oxygen consumption rates (μmol O_2 ind^{-1} h^{-1}), through the following equation (1) described by Massarin et al. (2010):

$$R = \frac{[O_2]_0 \, x \, (1 - exp^{-k \cdot \Delta t}) x V}{\Delta t} \quad (1)$$

where $[O_2]_0$ is the initial O_2 concentration (μmol L^{-1}) at t = 0, V is the incubation volume (in liters), Δt is the incubation time, and *k* is the incubation slope.

2.4. Data analysis

Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene median test). Normal distributed data were compared with one-way ANOVA, followed by Dunnett's and Tukey post-hoc tests; variables with non-normal distribution were analyzed using one-way ANOVA on ranks (Kruskal-Wallis test) followed by Dunn's and Tukey post-hoc tests. Significant differences were considered with $\alpha = 0.05$. The IC (inhibitory concentration) values of reproductive parameters from chronic toxicity tests were determined by ICPIN 2.0 program, using linear interpolation method with 80 resamples.

Species sensitivity distribution (SSD) curve was fitted to compare the sensitivity of *C. silvestrii* (production of neonates as endpoint) and other aquatic organisms to Fe-NPs, with data compiled from scientific literature, using endpoints related to reproduction. The SSD was plotted using Log-normal values of toxicity data with ETX 2.0 software (Van Vlaardingen et al., 2004), assuming a 5% log-normality (Anderson-Darling test). In addition, it was calculated the 5th (HC_5, hazardous concentrations for 5% of the species) and 50th percentile (HC_{50}, hazardous concentrations for 50% of the species) according to Aldenberg and Jaworska (2000) method.

We emphasize that some of the results are both expressed as mg Fe_3O_4-NPs L^{-1} and mg Fe L^{-1}, the former corresponding to mass of Fe and O per volume of water, and the latter to mass of Fe per volume of water.

3. Results and discussion

3.1. Nanoparticles: characterization and metal determination

The results of hydrodynamic size indicated that Fe_3O_4-NPs significantly ($p < 0.05$) aggregated right after preparing the test solutions and this aggregation tended to increase ($p < 0.05$) from 0.25 to 44.23 mg NPs L^{-1}, after a period of 72 h in all concentrations tested (Table 1). The initial average diameters were higher than 700 nm, and after 72 h they reached mean values higher than 1000 nm (Table 1). There was probably aggregation and agglomeration due to interaction of NPs with the chemical components of the exposure medium (synthetic water) and products originated from biological activity (*e.g.* cladocerans excretion products). A transmission electron microscopy (TEM) of this uncoated NP in deionized water is available in Blinova et al. (2017). Our results of characterization is in accordance with literature, stating that Fe-NPs tend to aggregate in aqueous solutions and the aggregation level tends to enhance with increasing NPs concentrations (Baalousha, 2009). In another study with Fe_3O_4-NPs, the level of NPs agglomeration and sedimentation was so rapid that the authors stated that it was not even possible to measure zeta potential and particles size (Blinova et al., 2017). According to Handy et al.

(2008), that should be more flexibility in the definition of NPs for ecotoxicology, so that particles aggregates in the micrometric scale could still be considered a nanomaterial, provided that aggregates come from particles initially smaller than 100 nm (in this study, NPs in their powder form were initially < 50 nm). PdI values, that indicate the level of dispersion of the samples, were higher than 0.480 in all treatments (Table 1). This indicates that NPs were aggregated and agglomerated in the exposure medium. Zeta potentials of Fe_3O_4-NPs were between −19 mV and −25 mV (Table 1) indicating that although Fe-NPs were aggregated they were stable in the exposure medium during 72 h.

Regarding metal determination, the results of ICP-OES are available in Fig. S1, supplementary material. The limits of detection and quantification were 1.1 and 3.3 μg L^{-1}, respectively. Dissolved iron corresponded to 1.48 and 0.26% (mean values) of total measured in chronic and respirometric tests, respectively, indicating low free iron ions in test solutions.

3.2. Acute toxicity tests

Acute toxicity tests were validated since less than 10% of the control group died or remained immobile after 48 h in the acute toxicity tests (ABNT, 2016). During the experiments, temperature and pH have not varied more than 1 °C and 1 unit, respectively, and dissolved oxygen concentrations were higher than 7.0 mg O_2 L^{-1} (Table S1, supplementary material).

The results of the acute toxicity test (concentrations ranging from 0.01 to 100.00 mg L^{-1}) with immobility as endpoint suggested that Fe_3O_4-NPs were non-toxic for *C. silvestrii*. The number of immobile and dead organisms was ≤10% in all treatments after 48 h (EC_{50} > 100 mg L^{-1}). A previous study also implied low toxicity for uncoated Fe_3O_4-NPs on *Daphnia magna* Straus, 1820, after 48 h of exposure and no significant difference between the effects of bulk and Fe_3O_4-NPs in acute toxicity tests (Blinova et al., 2017). However, studies indicate that acute toxicity effects of Fe_3O_4-NPs to cladocerans may vary according to presence or absence of NPs coating or with their type of coating (Baumann et al., 2014).

On the other hand, Fe_3O_4-NPs induced significant changes ($p < 0.05$, Dunnett's test) in *C. silvestrii* metabolic rates (Fig. 1). At 58.8 mg L^{-1} the mean respiration rate was higher (0.04 μmol O_2 ind^{-1} h^{-1}) when compared to control (0.01 μmol O_2 ind^{-1} h^{-1}). The oxygen consumption proved to be a more sensitive endpoint than classical acute toxicity test, since it detected toxicity in the concentration of 58.8 mg L^{-1}, in which no immobility or lethality was observed. The respiration rates increased as the Fe_3O_4-NPs concentrations increased. It indicates that *C. silvestrii* probably altered their metabolism in adverse conditions. Respiration has a high energy cost (80–90%) for aquatic invertebrates and biomass can be transformed in energy for organism's survival under stress conditions (Leidy and Ploskey, 1980; Moshiri et al., 1969). Therefore, the increase in respiration rates probably led to consequences on animals' fitness: high respiration rates may lead to lower growth efficiency, lower body size and lower offspring. The mechanism behind these changes in oxygen consumption rates may be related to oxidative stress induced by Fe_3O_4-NPs. NPs have been pointed as a possible cause for formation of reactive oxygen species (ROS). It is known, for example, that NPs can induce ROS formation and oxidative stress to algae (Sousa et al., 2018), cladocerans (Mansano et al., 2018) and fishes (Govindasamy and Rahuman, 2012).

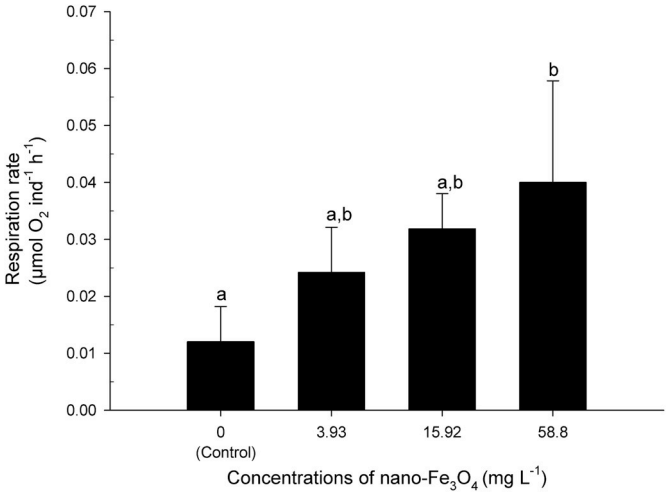

Fig. 1. Respiration rates (μmol O_2 ind^{-1} h^{-1}) of *Ceriodaphnia silvestrii* pre-exposed for 48 h to Fe_3O_4-NPs (0.00 – control; 3.93; 15.92 and 58.80 mg L^{-1}). Bars and lines represent the mean values and standard deviation, respectively (n = 4 replicates). Different letters indicate that there was statistical difference (one-way ANOVA followed by Dunnett's test to compare control *vs* treatments; and one-way ANOVA, followed by Tukey test to compare treatments with NPs to each other, with $p < 0.05$).

3.3. Chronic toxicity tests and species sensitivity distribution (SSD)

Chronic toxicity tests were validated according to ABNT (2017), since in controls less than 20% of the organisms died, and the mean number of live neonates was ≥15, after 7 d. Regarding physical and chemical variables, temperature and pH have not varied more than 1 °C and 1 unit, respectively, and dissolved oxygen concentrations were higher than 7.7 mg O_2 L^{-1} (Table S1, supplementary material).

At 44.23 mg L^{-1}, *C. silvestrii* had growth inhibition in respect to body length of 8.24 and 12.00% on 7th and 14th d, respectively (Fig. 2-a). It is important to note that the growth inhibition may have important consequences in food web structure since smaller cladocerans could suffer more predation (*e.g.* Gliwicz and Umana, 1994), as well as other ecological consequences probably related to fecundity, body reserves and survival.

We observed a reduction in the number of accumulated eggs per female at 44.23 mg L^{-1} starting from day 6 (Fig. 2-b) and reaching significant decrease (Dunnett's test, $p < 0.05$) of 51.99% on day 14 in respect to control. Neonates production on day 7 (Fig. 2-c) was significant (Dunn's test, $p < 0.05$) lower at 13.32 mg L^{-1} in respect to control group, thus it is important to emphasize that if the test had only 7 d, 13.32 mg L^{-1} would be the LOEC, but we observed that cladocerans were able to recovery in that concentration from day 8 onwards. We also observed a 61.27% reduction in the number of neonates at 44.23 mg L^{-1}. From Table 2, the number of neonates per female had in general lower IC values than the number of eggs per female, indicating that the production of neonates was the most sensitive endpoint for *C. silvestrii* in the 7-d exposure. As mentioned before, we hypothesize that energy intended for growth and reproduction was probably reallocated to respiration as a trade-off since metabolic rates increased during Fe_3O_4-NPs exposure. Moreover, the energy may also have been allocated to defense mechanisms by detoxification and antioxidant processes.

During chronic exposure we observed an accumulation of Fe_3O_4-NPs in the gut of *C. silvestrii* in all treatments with NPs (*e.g.* daphnids exposed to 2.49 mg L^{-1}, Fig. 3-a and b); moreover, bioaccumulation of Fe-NPs was already observed in *Ceriodaphnia dubia* Richard, 1894, (Hu et al., 2012). Besides that, we also observed NPs adhered to eggs (Fig. 3-a), giving them a grey color that indicates iron transfer (probably via stored nutrients). The accumulation of NPs in the gut of *C. silvestrii* may have blocked the absorption of food, leading to a decrease in number of eggs and neonates. According to Mendonça et al. (2011), NPs accumulated in the gastrointestinal tract may change the gut wall cell's typical shape and affect the food absorption. We deduce that the toxicity observed in our chronic experiments was not caused by free iron ions in the medium, since most iron quantified was in its particulate form (Fig. S1, supplementary material). It seems possible that large agglomerated

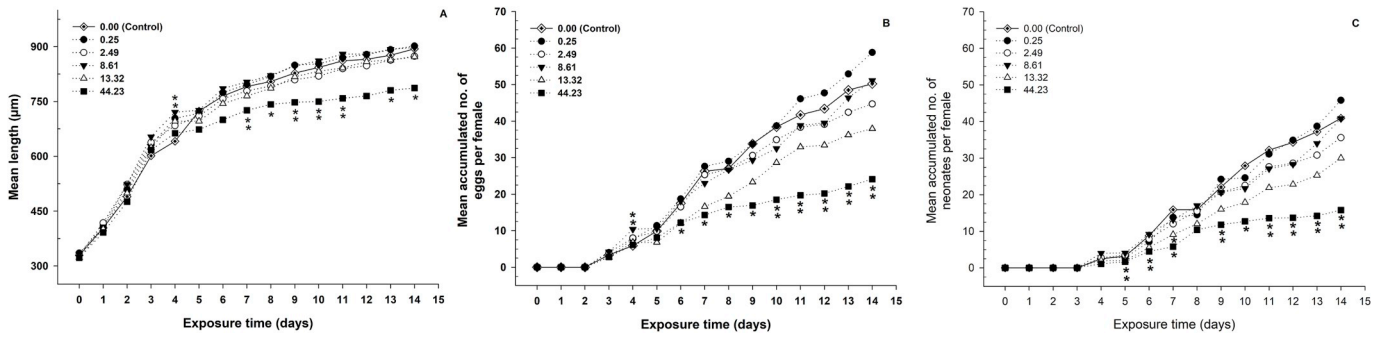

Fig. 2. Mean body length (A) and mean number of accumulated eggs (B) and neonates (C) per female of *Ceriodaphnia silvestrii* (n = 10 replicates) exposed for 14 d to Fe_3O_4-NPs (0.00 - control; 0.25; 2.49; 8.61; 13.32 and 44.23 mg L^{-1}). Values are means and asterisks represent significant differences between treatments and control group ($p < 0.05$). One asterisk represents Kruskal-Wallis and Dunn's test; and two asterisks represent one-way ANOVA and Dunnett's test.

or aggregated particles were accumulated into organisms' guts, acting as a physical barrier, causing physiological changes and ultimately affecting the organism homeostasis.

Regarding the life parameters of *C. silvestrii* (Table 3), the endpoints age of primipara, size of primipara and longevity did not differ significantly compared to control. Hatching rates also did not differ between control (83.05%) and at 44.23 mg L^{-1} (66.47%). The maximum length at 44.23 mg L^{-1} was significantly inhibited (Dunnett's test, $p < 0.05$), with length 16.56% smaller than control. It is important to note that during daily measurements, we observed (on day 4, at 0.25 mg L^{-1}) that an animal expelled eggs few seconds after their production. Aborted eggs are not usually reported mostly due to their reduced size, which make it difficult to observe them (Sobral et al., 2001). This behavior was observed only once during the entire chronic exposure; however, it could have also happened in other concentrations, which may have led to an overestimation of the percentage of hatching and/or an underestimation of the number of eggs.

According to data from literature regarding reproductive endpoints (Table S2, supplementary material), *D. magna* was the most sensitive organism to Fe-NPs, followed by *C. silvestrii*, the second most sensitive organism. When *D. magna* was added on SSD curve, the analysis did not pass on Anderson-Darling normality test, due to the extremely sensitivity of this species compared to the other organisms, thus *D. magna* could not be included in the curve. From SSD curve (Fig. 4), HC_5 and HC_{50} values were 1.65 mg Fe L^{-1} (0.49–3.26) and 8.99 mg Fe L^{-1} (5.04–16.02) respectively, suggesting Fe-NPs as a possible threat to *C. silvestrii*. In general, cladocerans are the most sensitive aquatic organism group to Fe-NPs when compared to the other species.

Our study emphasizes the importance of chronic toxicity tests since acute toxicity tests, in most cases, could not lead to a conclusion about the environmental safety of some compounds that are not toxic in short periods. Chronic exposures generate results that can be used to create or adjust environmental policies, as the endpoints analyzed here (growth and reproductive) are of great importance to evaluate the harmful effects of contaminants to cladoceran life cycle. We also found that respiration rates are a more sensitive endpoint than those commonly used in acute tests with mortality or immobility as endpoint. We believe that further studies of Fe-NPs toxicity on cladocerans approaching the use of different biomarkers such as lipidic classes, fatty acids, protein and carbohydrate analysis would be highly relevant to nanotoxicology field.

4. Conclusions

Our results indicate no immobility *C. silvestrii* after 48 h (EC_{50} > 100 mg L^{-1}). However, in acute experiments of O_2 consumption, we observed a significant effect on organisms' metabolism, demonstrating that respiration was a more sensitive endpoint than classical immobility or lethality parameters. In the chronic toxicity tests, Fe_3O_4-NPs caused significant inhibition on growth and reproduction of *C. silvestrii*, which are essential aspects of species fitness in natural environments. We believe that NPs disturbed the food absorption, exerting a physical toxicity, since we observed NPs' agglomerates or aggregates into the digestive tract of *C. silvestrii*. Therefore, we conclude that anthropogenic actions that could lead to Fe_3O_4-NPs release into aquatic environments should be regulated since Fe_3O_4-NP could affect reproduction, leading to disturbances of cladoceran population dynamics in natural environments, with consequences in food web structure. We find *C. silvestrii* to be a useful test species when rapidly triaging toxicity of NPs or assessing endpoints at lower levels of biological organization, being indeed a sensitive species according to our SSD analysis. It should be pointed out that the findings of this study refer to controlled laboratory conditions. In natural ecosystems there are several factors that may change the toxicity thresholds such as species interactions, oscillations in physical and chemical variables of the water, dietary exposure and presence of organic matter. This study intends to be helpful in the elaboration of NPs regulation guidelines and establishment of threshold levels of NPs in aquatic ecosystems.

Table 2
Median inhibitory concentration (IC) values for reproductive life parameters of *Ceriodaphnia silvestrii* (n = 10 replicates) exposed to Fe_3O_4-NPs in chronic test during 7 and 14 d. The values in parentheses represent 95% confidence intervals. Note that for IC_{50} values ICPIN 2.0 did not calculate the confidence interval, thus standard deviations were provided. The acronym "N.C." means "non-calculable".

Parameters		Mean accumulated no. of eggs per female		Mean accumulated no. of neonates per female	
Time of exposure		7 d	14 d	7 d	14 d
mg Fe_3O_4-NPs L^{-1}	IC_{10}	4.88 (0.19–10.02)	4.79 (0.20–12.89)	1.73 (0.08–9.73)	5.09 (0.19–14.78)
	IC_{20}	9.25 (2.33–19.44)	10.78 (1.76–23.69)	5.18 (0.15–15.72)	8.78 (1.50–20.49)
	IC_{50}	N.C.	N.C.	25.19 ± 8.89	27.97 ± 8.49
mg Fe L^{-1}	IC_{10}	3.53 (0.13–7.25)	3.47 (0.14–9.33)	1.25 (0.06–7.04)	3.68 (0.14–10.70)
	IC_{20}	6.69 (1.69–14.07)	7.80 (1.27–17.15)	3.75 (0.11–11.37)	6.35 (1.09–14.82)
	IC_{50}	N.C.	N.C.	18.23 ± 6.42	20.24 ± 6.14

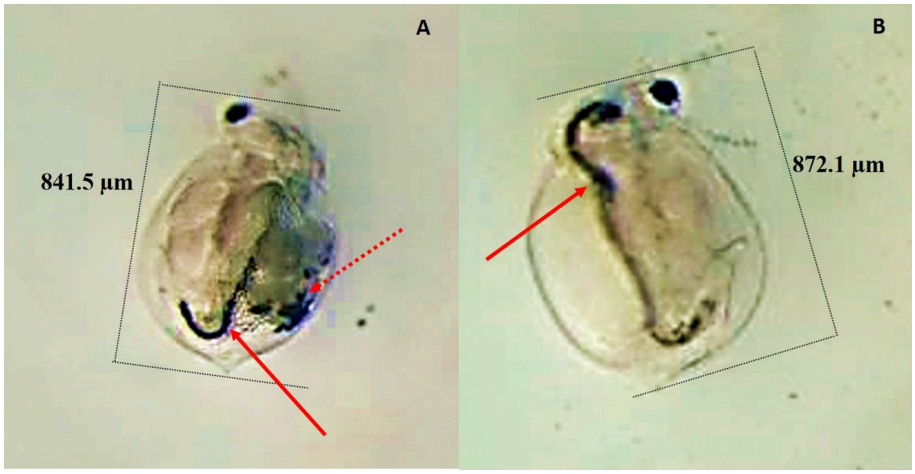

Fig. 3. *Ceriodaphnia silvestrii* exposed to 2.49 mg L^{-1} Fe_3O_4-NPs for 9 (A) and 11 (B) d during chronic toxicity tests. The figure indicates the NPs cladocerans' internalization in their digestive system (solid arrows) and eggs (dotted arrow).

Table 3
Life history parameters of *Ceriodaphnia silvestrii* exposed to Fe_3O_4-NPs (mg L^{-1}) along 14 d. Mean values are presented with standard deviations (n = 10 replicates). Lines with different superscript letter are statistically significant: Kruskal Wallis and Dunn's test to compare control *vs* treatments ($p < 0.05$), while Kruskal-Wallis followed by Tukey test compared treatments with NPs to each other ($p < 0.05$).

Parameters	Control	Treatments with Fe_3O_4-NPs (mg L^{-1})				
	0.00	0.25	2.49	8.61	13.32	44.23
Age of primipara (d)	5.00 ± 0.97	4.70 ± 0.63	4.70 ± 0.42	4.50 ± 0.00	5.37 ± 1.80	4.83 ± 0.50
Length of primipara (µm)	636.48 ± 75.97	673.96 ± 43.49	669.37 ± 28.68	686.97 ± 30.12	692.32 ± 33.96	649.40 ± 28.22
Maximum length (µm)	876.69 ± 41.46^{A}	880.51 ± 41.34^{A}	845.32 ± 60.26^{A,B}	861.39 ± 60.68^{A}	761.94 ± 199.33^{A,B}	731.49 ± 122.88^{B}
Hatching (%)	83.05 ± 11.07	76.64 ± 11.20	78.01 ± 17.07	75.99 ± 14.15	80.61 ± 14.74	66.47 ± 18.42
Longevity (d)	13.85 ± 1.87	13.55 ± 2.39	13.25 ± 2.17	12.70 ± 3.33	11.90 ± 5.23	11.64 ± 4.27

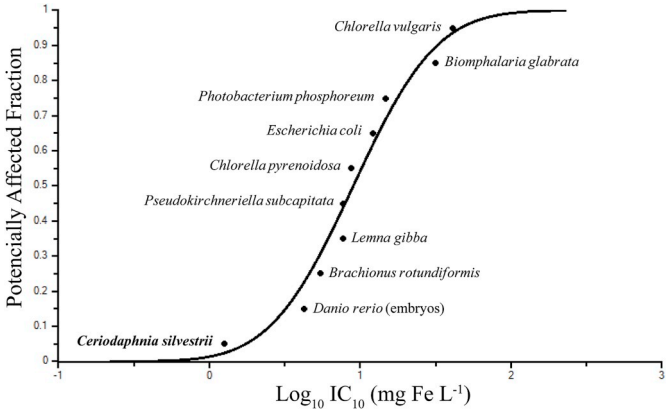

Fig. 4. Species Sensitivity Distribution (SSD) constructed based on 7-d IC_{10} (mg Fe L^{-1}) for number of neonates per female of *Ceriodaphnia silvestrii* (n = 10 replicates) exposed to Fe_3O_4-NPs (present study, in bold). Other literature values for aquatic organisms (Table S2, supplementary materials) were obtained from specific jounals, in order to supplement the data.

Funding

This study was funded by the São Paulo Research Foundation - FAPESP, Brazil [process numbers 2014/14139-3 and 2016/00753-7]; and scholarships were granted by the National Council for Scientific and Technological Development - CNPq, Brazil [grant numbers 132379/2015-5, 305229/2016-8 and 309514/2017-7].

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors are thankful to Dr Valtencir Zucolotto and Nanomedicine and Nanotoxicology Group for nanoparticles characterization.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.109743.

References

ABNT - Brazilian National Standards Organization (Associação Brasileira de Normas Técnicas), 2016. Aquatic Ecotoxicology - Acute Toxicity - Test with *Daphnia* Spp (Cladocera, Crustacea). pp. 27 NBR 12713. Rio de Janeiro, RJ.

ABNT - Brazilian National Standards Organization (Associação Brasileira de Normas Técnicas), 2017. Aquatic Ecotoxicology - Chronic Toxicity - Test Method with Ceriodaphnia Spp (Crustacea, Cladocera). NBR 13373. Rio de Janeiro, RJ. pp. 20.

AFNOR - Association Française de Normalisation, 1980. Norme experimentale. Essais deseaux Determination de l'nhibition de *Scenedesmus subspicatus* par une substance. Paris, T90-304.

Aldenberg, T., Jaworska, J.S., 2000. Uncertainty of the hazardous concentration and fraction affected for normal species sensitivity distributions. Ecotoxicol. Environ. Saf. 46, 1–18.

Amenta, V., Aschberger, K., Arena, M., Bouwmeester, H., Botelho Moniz, F., Brandhoff, P., Gottardo, S., Marvin, H.J., Mech, A., Quiros Pesudo, L., Rauscher, H., Schoonjans, R., Vettori, M.V., Weigel, S., Peters, R.J., 2015. Regulatory aspects of nanotechnology in the agri/feed/food sector in EU and non-EU countries. Regul. Toxicol. Pharmacol. 73, 463–476.

ASTM - American Society for Testing Materials, 2002. Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians. E 729 – 96. pp. 22 United States.

Baalousha, M., 2009. Aggregation and disaggregation of iron oxide nanoparticles: influence of particle concentration, pH and natural organic matter. Sci. Total Environ. 407, 2093–2101.

Barhoumi, L., Oukarroum, A., Taher, L.B., Smiri, L.S., Abdelmelek, H., Dewez, D., 2015.

Effects of superparamagnetic iron oxide nanoparticles on photosynthesis and growth of the aquatic plant *Lemna gibba*. Arch. Environ. Contam. Toxicol. 68, 510–520.
Baumann, J., Koser, J., Arndt, D., Filser, J., 2014. The coating makes the difference: acute effects of iron oxide nanoparticles on *Daphnia magna*. Sci. Total Environ. 484, 176–184.
Bhuvaneshwari, M., Kumar, D., Roy, R., Chakraborty, S., Parashar, A., Mukherjee, A., Chandrasekaran, N., Mukherjee, A., 2017. Toxicity, accumulation, and trophic transfer of chemically and biologically synthesized nano zero valent iron in a two species freshwater food chain. Aquat. Toxicol. 183, 63–75.
Blinova, I., Kanarbik, L., Irha, N., Kahru, A., 2017. Ecotoxicity of nanosized magnetite to crustacean *Daphnia magna* and duckweed *Lemna minor*. Hydrobiologia 798, 141–149.
Bouldin, J.L., Ingle, T.M., Sengupta, A., Alexander, R., Hannigan, R.E., Buchanan, R.A., 2008. Aqueous toxicity and food chain transfer of quantum dots in freshwater algae and *Ceriodaphnia dubia*. Environ. Toxicol. Chem. 27, 958–1963.
Calow, P., 1991. Physiological costs of combating chemical toxicants: ecological implications. Comp. Biochem. Physiol., C 100, 3–6.
Canivet, L., Dubot, P., Garcon, G., Denayer, F.O., 2015. Effects of engineered iron nanoparticles on the bryophyte, *Physcomitrella patens* (Hedw.) Bruch & Schimp, after foliar exposure. Ecotoxicol. Environ. Saf. 113, 499–505.
Cowart, D.A., Guida, S.M., Shah, S.I., Marsh, A.G., 2011. Effects of Ag nanoparticles on survival and oxygen consumption of zebra fish embryos, *Danio rerio*. J. Environ. Sci. Health A Toxic/Hazard. Subst. Environ. Eng. 46, 1122–1128.
Dave, P.N., Chopda, L.V., 2014. Application of iron oxide nanomaterials for the removal of heavy metals. J. Nanotechnol. 2014, 1–14.
Gliwicz, Z.M., Umana, G., 1994. Cladoceran body size and vulnerability to copepod predation. Limnol. Oceanogr. 39, 419–424.
Govindasamy, R., Rahuman, A.A., 2012. Histopathological studies and oxidative stress of synthesized silver nanoparticles in Mozambique tilapia (*Oreochromis mossambicus*). J. Environ. Sci. 24, 1091–1098.
Grieger, K.D., Fjordboge, A., Hartmann, N.B., Eriksson, E., Bjerg, P.L., Baun, A., 2010. Environmental benefits and risks of zero-valent iron nanoparticles (nZVI) for in situ remediation: risk mitigation or trade-off? J. Contam. Hydrol. 118, 165–183.
Handy, R.D., von der Kammer, F., Lead, J.R., Hassellov, M., Owen, R., Crane, M., 2008. The ecotoxicology and chemistry of manufactured nanoparticles. Ecotoxicology 17, 287–314.
Hu, J., Wang, D., Wang, J., Wang, J., 2012. Bioaccumulation of Fe_2O_3(magnetic) nanoparticles in *Ceriodaphnia dubia*. Environ. Pollut. 162, 216–222.
Jackson, B.P., Bugge, D., Ranville, J.F., Chen, C.Y., 2012. Bioavailability, toxicity, and bioaccumulation of quantum dot nanoparticles to the amphipod *Leptocheirus plumulosus*. Environ. Sci. Technol. 46, 5550–5556.
Kay, L., Shapira, P., 2009. Developing nanotechnology in Latin America. J. Nanoparticle Res. 11, 259–278.
Krishna, V.D., Wu, K., Su, D., Cheeran, M.C.J., Wang, J.P., Perez, A., 2018. Nanotechnology: review of concepts and potential application of sensing platforms in food safety. Food Microbiol. 75, 47–54.
Lamichhane, K., Garcia, S.N., Huggett, D.B., DeAngelis, D.L., La Point, T.W., 2013. Chronic effects of carbamazepine on life-history strategies of *Ceriodaphnia dubia* in three successive generations. Arch. Environ. Contam. Toxicol. 64, 427–438.
Leidy, G.R., Ploskey, G.R., 1980. Simulation Modeling of Zooplankton and Benthos in Reservoirs: Documentation and Development of Model Constructs. pp. 311 Washington D.C., USA.
Magro, M., De Liguoro, M., Franzago, E., Baratella, D., Vianello, F., 2018. The surface reactivity of iron oxide nanoparticles as a potential hazard for aquatic environments: a study on *Daphnia magna* adults and embryos. Sci. Rep. 8, 13017.
Mansano, A.S., Souza, J.P., Cancino-Bernardi, J., Venturini, F.P., Marangoni, V.S., Zucolotto, V., 2018. Toxicity of copper oxide nanoparticles to Neotropical species *Ceriodaphnia silvestrii* and *Hyphessobrycon eques*. Environ. Pollut. 243, 723–733.
Martins, J.C., Saker, M.L., Teles, L.F., Vasconcelos, V.M., 2007. Oxygen consumption by *Daphnia magna* Straus as a marker of chemical stress in the aquatic environment. Environ. Toxicol. Chem. 26, 1987–1991.
Mashjoor, S., Yousefzadi, M., Zolgharnain, H., Kamrani, E., Alishahi, M., 2018. Organic and inorganic nano-Fe_3O_4: alga Ulva flexuosa-based synthesis, antimicrobial effects and acute toxicity to briny water rotifer *Brachionus rotundiformis*. Environ. Pollut. 237, 50–64.
Massarin, S., Alonzo, F., Garcia-Sanchez, L., Gilbin, R., Garnier-Laplace, J., Poggiale, J.C., 2010. Effects of chronic uranium exposure on life history and physiology of *Daphnia magna* over three successive generations. Aquat. Toxicol. 99, 309–319.
Mendonça, E., Diniz, M., Silva, L., Peres, I., Castro, L., Correia, J.B., Picado, A., 2011. Effects of diamond nanoparticle exposure on the internal structure and reproduction of *Daphnia magna*. J. Hazard Mater. 186, 265–271.
Moshiri, G.A., Cummins, K.W., Costa, R.R., 1969. Respiratory energy expenditure by the predaceous zooplankter *Leptodora kindtii* (focke) (Crustacea: cladocera). Limnol. Oceanogr. 14, 475–484.
Santhosh, C., Malathi, A., Dhaneshvar, E., Bhatnagar, A., Grace, A.N., Madhavan, J., 2019. Iron oxide nanomaterials for water purification. In: Thomas, S. (Ed.), Nanoscale Materials in Water Purification, pp. 431–446.
Sobral, O., Chastinet, C., Nogueira, A., Soares, A.M.V.M., Gonçalves, F., Ribeiro, R., 2001. In vitro development of parthenogenetic eggs: a fast ecotoxicity test with *Daphnia magna*? Ecotoxicol. Environ. Saf. 50, 174–179.
Sousa, C.A., Soares, H.M.V.M., Soares, E.V., 2018. Toxic effects of nickel oxide (NiO) nanoparticles on the freshwater alga *Pseudokirchneriella subcapitata*. Aquat. Toxicol. 204, 80–90.
USEPA - U. S. Environmental Protection Agency, 2014. Technical fact sheet – Nanomaterials. Available in: https://www.epa.gov/sites/production/files/2014-03/documents/ffrrofactsheet_emergingcontaminant_nanomaterials_jan2014_final.pdf.
Van Vlaardingen, P., Traas, T.P., Wintersen, A.M., Aldenberg, T., 2004. ETX 2.0. A Program to Calculate Hazardous Concentrations and Fraction Affected, Based on Normally Distributed Toxicity Data. RIVM Report No. 601501028/2004. Available in: https://rivm.openrepository.com/handle/10029/256981.
Wang, D., Gao, Y., Lin, Z., Yao, Z., Zhang, W., 2014. The joint effects on *Photobacterium phosphoreum* of metal oxide nanoparticles and their most likely coexisting chemicals in the environment. Aquat. Toxicol. 154, 200–206.
Zeman, F.A., Gilbin, R., Alonzo, F., Lecomte-Pradines, C., Garnier-Laplace, J., Aliaume, C., 2008. Effects of waterborne uranium on survival, growth, reproduction and physiological processes of the freshwater cladoceran *Daphnia magna*. Aquat. Toxicol. 86, 370–378.
Zhang, W.-x., 2003. Nanoscale iron particles for environmental remediation: an overview. J. Nanoparticle Res. 5, 323–332.
Zhang, Y., Zhu, L., Zhou, Y., Chen, J., 2015. Accumulation and elimination of iron oxide nanomaterials in zebrafish (*Danio rerio*) upon chronic aqueous exposure. J. Environ. Sci. (China) 30, 223–230.
Zhu, X., Tian, S., Cai, Z., 2012. Toxicity assessment of iron oxide nanoparticles in zebrafish (*Danio rerio*) early life stages. PLoS One 7, e46286.
Zhu, X., Wang, J., Zhang, X., Chang, Y., Chen, Y., 2010. Trophic transfer of TiO(2) nanoparticles from daphnia to zebrafish in a simplified freshwater food chain. Chemosphere 79, 928–933.
Zhu, Y., Liao, L., 2015. Applications of nanoparticles for anticancer Drug delivery: a review. J. Nanosci. Nanotechnol. 15, 4753–4773.