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RESEARCH ARTICLE

Effects of γ-Fe_2O_3 nanoparticles on the survival and reproduction of *Biomphalaria glabrata* (Say, 1818) and their elimination from this benthic aquatic snail

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Abstract This study aims to evaluate the effects of maghemite nanoparticles (γ-Fe_2O_3) coated with meso-2, 3-dimercaptosuccinic acid (DMSA) stabilizer on the survival and reproduction of the aquatic snail *Biomphalaria glabrata*. The cumulative means of egg masses and eggs per individual in the control group at the end of 4 weeks were 18.8 and 326.7, respectively. These values at the concentration of 1 mg/L were 17.2 and 291.6; at 10 mg/L, they were 19.6 and 334.4 ,and at 100 mg/L, they were 14.3 and 311.1. Results showed no significant differences between the tested and the control groups at the level of $p < 0.05$. Exposure of embryos for 10 days showed absence of mortality, malformation, or hatching delay. X-ray microtomography confirmed the presence of nanoparticles in exposed individuals and showed the complete elimination of the nanoparticles after 30 days in clean water. In the studied conditions, it is clear that γ-Fe_2O_3 coated with stabilizing DMSA did not alter the fecundity or the fertility of the snail *B. glabrata* after 4 weeks of exposure, and accumulation was not present after 30 days in clean water.

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José Sousa Filho is deceased.

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Introduction

In general, nanoscale particles formed by metals are known as magnetic nanoparticles (MNPs) (Gupta and Gupta 2005). In the last decade, studies of magnetic nanoparticles have led to a better understanding of their properties and behavior, allowing them to be used in medicine for the development of new, more specific and effective treatments as an alternative to early diagnosis of various pathologies such as cancer. However, there is a lack of knowledge about the risks of these nanoscale materials to biological systems, since their behavior and mode of interaction are completely unknown, due to their unusual characteristics (Stern and Mcneil 2008).

Nanomaterials exhibit physicochemical characteristics and behavior that are completely different from other water pollutants such as metals and pesticides. In general, they do not dissolve easily, and exhibit colloidal forms, which can remain in suspension or join together. Their surface and shape are relevant to understanding their behavior and fate in the environment, and consequently, how they will interact with living organisms. The conditions of the aqueous medium, such as salinity, pH, and quantity of organic matter, will directly influence the physical state and toxicity of nanoparticles (Handy et al. 2008).

Magnetic nanoparticles have been widely studied in aquatic toxicology, and their toxic effect has been more evident in the gills, digestive tract, liver, and brain of fish species (Farré et al. 2009). Regarding MNPs, Kahru and Dubourguier (2010) showed a lack of available data on the environmental effects of iron oxide-based particles (Fe_2O_3). To evaluate the effects of these materials in aquatic environments, mainly fish

species, followed by crustaceans, have been extensively used in bioassays (Farré et al. 2009). Aquatic gastropods are benthic mollusks, and have great potential to accumulate material deposited in the sediment of aquatic ecosystems. Therefore, the aims of this study were to investigate the effects of γ-Fe_2O_3 magnetic nanoparticles on the survival, reproduction, and embryonic development of the aquatic snail *Biomphalaria glabrata*, as well as to understand the fate of this nanomaterial within the snail's body.

Material and methods

Characterization of tested NPs

As test material, a magnetic fluid (MF) containing maghemite (γ-Fe_2O_3) NPs stabilized by meso-2, 3-dimercaptosuccinic acid (DMSA) coating was used. The fluid was produced in the Institute of Chemistry at the University of Brasilia (UNB). The characteristics of MF-DMSA used herein are described in Table 1.

Zeta potential and the hydrodynamic diameter of the maghemite NPs were obtained by light dispersion phase using ZetaSizer Nano ZS equipment (Malvern Instruments, Malvern, UK). For this characterization, the samples were diluted in a proportion of 1:1000 with distilled water, and the analysis was immediately carried out.

Maghemite NPs were prepared as described by Fauconnier and co-workers (Fauconnier et al. 1997; Van Ewijk et al. 1999). To characterize the samples of NPs, they were diluted at 1:100 and 1:1000, respectively, in distilled water and placed on transmission electronic microscope (TEM) screens that had been previously covered with Formvar®. After the screens had been dried for 3 hours, the electromicrographs were obtained by TEM, JEOL 1011 (Jeol, Tokyo, Japan) (Fig. 1).

Test organisms

Biomphalaria glabrata (Say, 1818) is a freshwater pulmonate snail (Molluska, Gastropoda) found in Brazilian water bodies. It has been widely studied because it is one of the intermediate hosts of *Schistosoma mansoni*. Indeed, owing to their importance in the transmission of schistosomiasis, the biology and ecology of all snails belonging to the *Biomphalaria* genus have been intensively studied. Since *Biomphalaria* snails are also easily bred and kept under laboratory conditions, their use in ecotoxicity assays has been suggested by several authors (Ravera 1977; Bellavere and Gorbi 1981; Münzinger 1987; Oliveira-Filho et al. 2005; Oliveira-Filho et al. 2009a; Oliveira-Filho et al. 2009b; Oliveira-Filho et al. 2010). All snails used in the experiments were from the breeding stock of *B. glabrata* maintained at the Laboratory of Ecotoxicology of Embrapa Cerrados, Planaltina, Federal District, Brazil.

Table 1 Physicochemical characteristics of maghemite NPs tested

Nanoparticles	Maghemite (γ-Fe_2O_3)
Solvent	Water
Size	5.7 nm
Hydrodynamic diameter	46.4 ± 8.8 nm
Zeta potential	−30.5 ± 0.5 mV
Iron concentration	1.500 mg/L
Particle concentration	$225.8x10^{16}$ particles/mL
pH	7.2
Color	Brown

Reproduction assays

Adult snails with shell diameter of 12 ± 3 mm were individually exposed, in glass vessels of 300 mL (ten replicates), to γ-Fe_2O_3 MNPs at the concentrations of 0 (control), 1, 10, and 100 mg/L diluted in synthetic soft water, with hardness of 40–42 mg/L as $CaCO_3$.They were kept under controlled environmental conditions (25 ± 1 °C and light/dark cycle 16/8 h) for 4 weeks. The assay solution was renewed twice a week, and snails were fed with fresh lettuce leaves (a piece of approximately 1 cm^2) plus 1 mg of fish chow per snail. The chow is commercially available in aquarium stores. To recover egg masses laid by the snails, glass vessels were internally covered with cellophane sheets, which were changed twice a week,

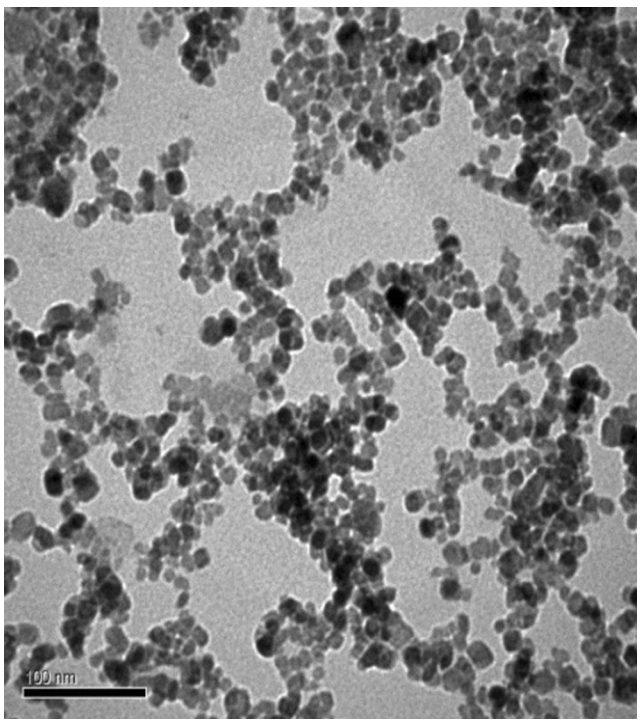

Fig. 1 Characterization of maghemite NPs. TEM electromicrograph of the maghemite-(γ-Fe_2O_3), (×250000)

and the numbers of eggs and egg masses per snail were recorded. The evaluation was performed by the observation of mortality and production of eggs and egg masses in four consecutive weeks. After the 4 weeks of exposure, three snails were removed from the control and from 100-mg/L treatments, and the remaining snails were kept in clean water until 15 and 30 days after the end of exposure, for the observation of iron nanoparticles in the snails' body using X-ray microtomography.

Embryonic development assays

Snail embryos were exposed within the egg masses to the same concentrations of magnetic iron nanoparticles, as previously described, for 10 days, based on a method proposed by Oliveira-Filho et al. (2010). Tests were carried out with egg masses laid on small pieces of cellophane sheet that had been left floating on the aquarium water. Within 15 h of spawning, when embryos were still in the blastula stage of development (Camey and Verdonk 1968; Kawano et al. 1992), pieces of cellophane sheet with adhered egg masses were carefully transferred to Petri dishes where they were further exposed to test concentrations. The evaluation was performed with the exposure of four to five egg masses (approximately 100 embryos) in each concentration group. Control (unexposed) egg masses were similarly treated, except that they were not exposed to any material tested. Except for the time needed for examination of embryo development and viability, Petri dishes containing egg masses were kept within climatic chambers under controlled photoperiod (12 h light/12 h dark), illumination (provided by fluorescent lamps), and environmental temperature (25 ± 1 °C). All egg masses were examined daily under a stereomicroscope up to the 10th day of exposure. Mortality, anomalies in embryo development (presence of malformations), and the day of hatching were recorded.

X-ray microtomography of the snails

The presence of metallic nanoparticles within the snails' body was evaluated immediately after the end of exposure, on the 15th day after the end of exposure, and on the 30th day after the end of exposure. For this, three, three and four snails, respectively, from the control and 100 mg/L treatments, were removed from the solutions, fixed in Davidson fixative for 2 h and preserved in 70 % alcohol, for subsequent analysis under CT Skyscan MicroCT 1076. The images were obtained through X-ray microtomograph Skyscan 1076, Aartselaar, Belgium. The X-ray microtomography was performed with isotropic voxel size of 18 μm 50 KV, 0.5 mm in nickel-time scanning filter for approximately 26 min for each animal. Two-dimensional reconstructions were performed with the NRecon software (V 1.6.9, 64 bit version with GPU acceleration, Skyscan, Kontich, Belgium), which uses a number of cone beam reconstructions, resulting in two-dimensional images in grayscale. Three-dimensional images were made with CTvox software (V 1.5.0, 64 bit version, Skyscan, Kontich, Belgium) and CTvol (2.2 V, 64 bit version, Skyscan, Kontich, Belgium). This technique was proposed by Jenneson et al. (2004) as an important tool in the environmental analysis of nanoparticles. All data presented were confirmed with a three-dimensional analysis of the snails.

Statistical analysis

Lethal concentrations were determined by the Trimmed Spearman Karber program (Hamilton et al. 1977). Differences between the tested groups and the control group in the number of produced eggs and egg masses as well as proportions of dead and malformed embryos, and embryos that did not hatch, were evaluated by one-way ANOVA followed by the Dunnett Procedure (Dunnett 1955; USEPA 2002) and Tukey test.

Results and discussion

As well as in the present study, aggregate formation and sedimentation of the materials in aqueous solution (Cheng et al. 2007; Musee et al. 2010; Nations et al. 2011; Hu et al. 2012; Zhu et al. 2012) were observed in other ecotoxicological studies with metallic nanoparticles. In this context, as suggested by Zhu et al. (2012), assays should be interesting with a benthic aquatic species, as well as snails, because they could present results with a potential target organism of nanoparticles released into the environment.

Reproduction and embryonic development assays

After 4 weeks of exposure, there were no deaths among the tested groups. The effects of the exposure to γ-Fe_2O_3 magnetic nanoparticles on the fecundity of adult *B. glabrata* in this exposure time are shown in Figs. 2 and 3.

The cumulative mean of eggs per individual (mean ± SE) in the control group after 4 weeks was 326.7 ± 38.7, and at the concentrations of 1 mg/L 291.6 ± 53.3, 10 mg/L 334.4 ± 80.5, and at 100 mg/L 311.1 ± 81.2, respectively (Fig. 2). In the case of egg masses per individual, the cumulative mean (mean ± SE) in the control group was 18.8 ± 1.9, and at the concentrations of 1 mg/L 17.2 ± 3.1, 10 mg/L 19.6 ± 4.8, and at 100 mg/L 14.3 ± 2.9, respectively (Fig. 3).

After statistical analysis by Dunnett's test and Tukey's test, no significant differences were observed between the tested groups and the control group at level of $p < 0.05$.

The exposure of egg masses to γ-Fe_2O_3 magnetic nanoparticles to evaluate embryonic development and hatching was performed. Four to five egg masses, i.e., approximately 50

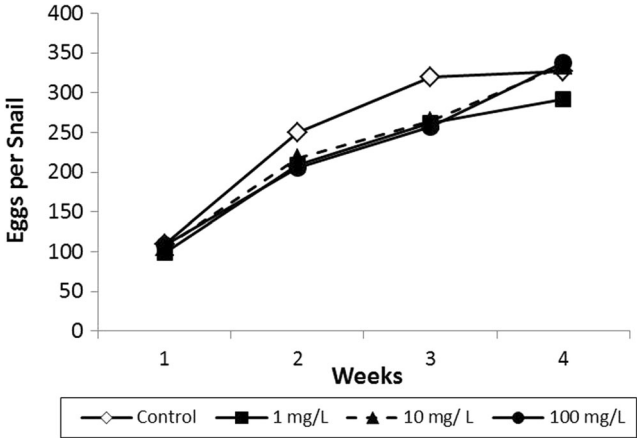

Fig. 2 Effects of γ-Fe_2O_3 magnetic nanoparticles on the fecundity of *B. glabrata* snails. Data are shown as cumulative means of eggs laid per snail. Differences ($p < 0.05$ ANOVA and Dunnett's multiple comparisons test) between tested groups and control group were not significant at the fourth week

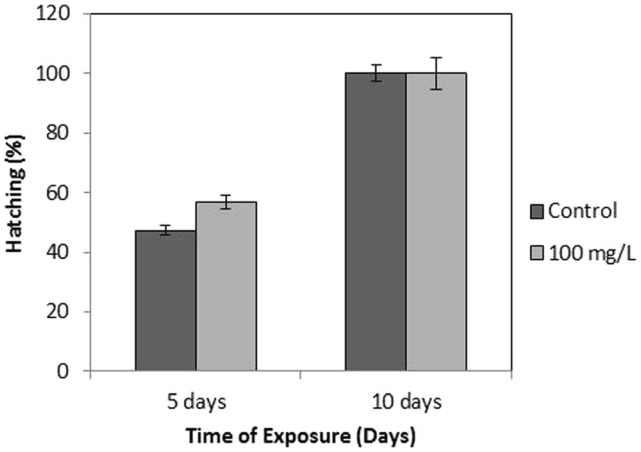

Fig. 4 Proportion of hatching until the 5th and 10th day after spawning of eggs exposed to water and to 100 mg/L of γ-Fe_2O_3 magnetic nanoparticles. Values are means ± S.E. of percentages of hatching [(no. of successfully hatched snails/no. of eggs) × 100] per egg mass. No difference was found ($p < 0.05$ ANOVA and Dunnett's multiple comparisons test) between the exposed group and unexposed control group (0 mg/L)

eggs per concentration, were exposed for 10 days after spawning. Egg masses were examined under a stereomicroscope, and developmental toxicity of the tested material was investigated by the observation of dead embryos (embryo lethality), the incidence of malformed snails (teratogenicity), and the proportion of hatching (hatching delay) until day 10 after spawning.

We found no statistically detectable difference between unexposed controls and the group exposed to 100 mg/L of γ-Fe_2O_3 magnetic nanoparticles regarding the proportion of hatchings (Fig. 4). There was no embryo lethality observed or incidence of malformed embryos (teratogenicity) at the tested exposure concentrations.

On the other hand, Zhu et al. (2012) observed considerable mortality and significant reduction in the hatching rate of zebrafish embryos after exposure to 50 and 100 mg/L of

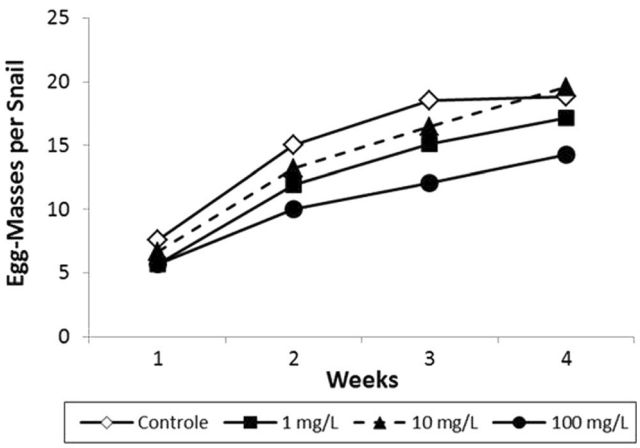

Fig. 3 Effects of γ-Fe_2O_3 magnetic nanoparticles on the fecundity of *B. glabrata* snails. Data are shown as cumulative means of egg masses laid per snail. Differences ($p < 0.05$ ANOVA and Dunnett's multiple comparisons test) between tested groups and control group were not significant at the fourth week

Fe_2O_3 nanoparticles. Malformations were observed in embryos and larvae exposed to these same concentrations, and these were related to embryo lethality and low hatchability rate. This comparison shows a higher susceptibility of zebrafish eggs to Fe_2O_3 magnetic nanoparticles.

In previous chronic studies with aquatic snails *Biomphalaria sp*., the hatching rate was observed as an important long-term endpoint after the exposure to chemicals (Oliveira-Filho et al. 2009a, 2009b, 2010). However, due to the physical characterization of metallic nanoparticles, snail eggs were not so easy for these xenobiotics to penetrate. In agreement with the present study, Musee et al. (2010) observed the absence of adverse effects on the survival, reproduction, and development of the aquatic snail *Physa acuta* after a 4-week exposure to M-TiO_2 and C-TiO_2, but significant effects of γ-alumina and α-alumina on the embryonic growth and hatchability of this species. The authors related these effects to the reduction of peroxidase activity in adult snails.

With vertebrates, results presented by Nations et al. (2011) indicate that TiO_2 and Fe_2O_3 nanomaterials had relatively few effects on the development of the embryos of the frog *Xenopus laevis* during its 96 h of life.

Oliveira-Filho et al. (2010) suggested that the snails' embryo lethality in that study was due to the easy penetration of chemical compounds inside the eggs. When a toxic chemical penetrates easily and is acutely toxic (eg. classic molluscicides) it kills the embryos. On the other hand, if it does not penetrate so easily (larger molecules) but presents some toxicity, it may cause a teratogenic effect (e.g., *Euphorbia milii* latex). This phenomenon can indicate the absence of effects of some metallic nanoparticles on snail embryos. However, the

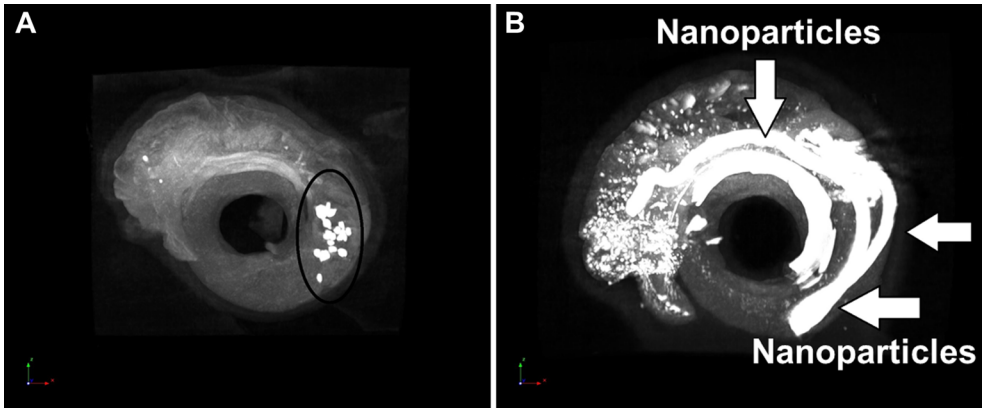

Fig. 5 Microtomography of the snail's body without shell after the end of the exposure time (30 days). An individual from the control group (**a**) and an individual from the 100 mg/L group (**b**). A *black circle* marks calcium phosphate or silicate stones in the stomach of the snail

effects on the hatching rate could have another explanation. Hathaway et al. (2010) demonstrated that the protein components of the mollusks' egg masses possess antimicrobial activity, thereby protecting the offspring inside the eggs from bacteria, fungi, and other potential pathogens. Furthermore, based on further studies, Benkendorff et al. (2001) stated that the need for antimicrobial protection is highest in the early stages of mollusks' embryonic development, and the loss of antibiotic activity during the growth of the embryo can have an important role in the hatching process and release of young snails. Thus, as suggested by Oliveira-Filho et al. (2010), it might be expected that the exposure to a chemical that presents a microbiocide effect could somehow cause a delay in egg hatching, not by a direct effect on the embryo, but by an indirect effect, eliminating the microorganisms that are consumers of the egg mass. Studies of the antimicrobial activity of metal oxide nanoparticles could show interesting data to confirm this position. Azam et al. (2012) showed the least bacterial activity of Fe_2O_3 nanoparticles, and the present study shows that Fe_2O_3 has no effect on embryos or hatching. In a thorough review, Hajipour et al. (2012) showed that TiO_2 did not present bacterial activity in a dark environment and is also dependent on light, and Al_2O_3 had an effective bacterial effect. In Musee et al. (2010), TiO_2 presented a weak embryonic effect, and γ- and α-alumina presented embryotoxic and hatching delaying effects. Several authors (Mukherjee et al. 2011; Geoprincy et al. 2012) consider alumina as a good antibiotic and a special nanomaterial for potential clinical applications.

Microtomography of the snails

For the achievement of these analyses, snails were removed from their shell to avoid interference from mineral elements present. Even so, some pieces of the shell were still stuck in the body of the mollusk and can also be seen in the photos. Figure 5a and Fig. 5b show the X-ray microtomography of one organism from the control group and another from the 100-mg/L group at the

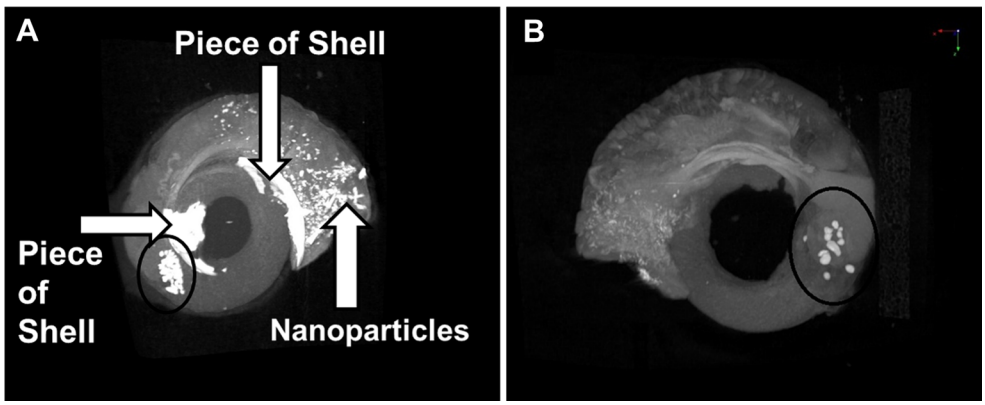

Fig. 6 Microtomography of the snails after the end of the exposure time and kept in clean water for 15 (**a**) and 30 (**b**) days. **a** After 15 days in clean water with pieces of shell fixed outside and nanoparticles near the mouth and the opening of the shell. **b** After 30 days with absence of nanoparticles (**b**). A *black circle* marks calcium phosphate or silicate stones in the stomach of the snail

end of exposure time (i.e., 30 days). In the snails presented in Fig. 5a and Fig. 6b, it is possible to see solid objects in the stomach; these were identified by Marxen et al. (2008) as calcium phosphate or silicate stones present in the snail's stomach. These materials were not identified as nanoparticles. In Fig. 5b, it is clearly observable that the intestine of the exposed individual (100 mg/L) is completely full of metallic nanoparticles.

After 30 days kept and exposed in 100 mg/L of γ-Fe_2O_3 magnetic nanoparticles, the remaining organisms were transferred to clean water and maintained for 15 and 30 days for a new observation by microtomography to evaluate the elimination of the nanoparticles. These observations show that 15 days in clean water was not enough for *B. glabrata* snails to excrete 100 % of the nanoparticles in their body (Fig. 6a), but it is possible to see a considerable reduction in the organisms at the end of 30 days' exposure (Fig. 5b).

With regard to the ingestion of iron oxide nanoparticles by aquatic species, the accumulation was evaluated with the aid of X-ray microtomography, and it was possible to observe that the snails ingested the material sedimented from the bottom and had a digestive tract completely full of the tested nanoparticles at the 30th day of exposure to the concentration of 100 mg/L (Fig. 5). However, there was no adverse effect arising from the presence of this material in the body of the organism. Maybe, if the exposures were longer, some effect could be observed, including on reproductive performance. At 15 days after the end of exposure in clean water, the amount of material was reduced (Fig. 6a), and completely eliminated after 30 days in clean water (Fig. 6b).

The residual presence of metallic nanoparticles in the body of aquatic organisms after a long-term clearance period has also been observed in several studies (Petersen et al. 2009; Tervonen et al. 2010; Zhu et al. 2010; Hu et al. 2012). The excretion of Fe_2O_3 nanoparticles in clean water and after food consumption was also observed by Hu et al. (2012) in assays with the microcrustacean *Ceriodaphnia dubia*. These authors emphasized that food consumption accelerated the process of clearance. With this prerogative, it should be noted that if an organism is unable to eat because its digestive tract is completely full, the process of elimination could become slower. In research with TiO_2 nanoparticles, Zhu et al. (2010) stated that the high accumulation and slow depuration of the nanomaterial were consistent with a significant reduction in the food ingestion and filtration rates of the microcrustacean *Daphnia magna*. These authors reported chronic toxicity behavior as a consequence of lower food intake and malnutrition.

Conclusions

Data from the present study show that after 30 days of exposure to γ-Fe_2O_3 nanoparticles coated with DMSA stabilizer at the concentration of 100 mg/L, the reproduction and embryonic development of aquatic snails *B. glabrata* were not affected. Results of the X-ray microtomography show the consecutive elimination of the nanomaterial after 15 and 30 days in clean water.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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