

Aluminum and Iron nanoparticles Bioaccumulation in the Land Snail (*Helix aspersa*)

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Abstract: In current work we have investigated the toxic effects of alumina oxide (average grain size of 93 nm) and iron oxide nanoparticles (average grain size of 99 nm) on a terrestrial bioindicator model, *Helix aspersa* snail using the phenomenon of bioaccumulation of these particles in digestive gland and kidney tissues using atomic absorption spectrophotometry (AAS) method. Adult land snail were exposed to 50, 100, 200 and 400 µg/g of both NPs for eight weeks. The results have shown that Al₂O₃ NPs accumulation increased from 1.3 to 11.13 µg/g dry weight in digestive gland with increasing exposure concentration from 0 µg/g (control group) to 200 µg/g of Al₂O₃ NPs, the accumulation in the kidneys tissue increased from 1.7 µg/g dry weight in control snails to 10.66 µg/g dry weight in treated with the 400 µg/g concentration. Concerning iron NPs accumulation in both organs increase was consistent with the exposure concentration. Indeed, the accumulated quantities of iron in the digestive gland are slightly higher than those recorded in the kidney. Furthermore, by comparing the bioaccumulation rates, we noticed that the highest amounts of accumulated NPs were observed in aluminum oxide-treated snails compared to those treated with iron oxide. Therefore, the results of this study have confirmed a high bioaccumulation capacity of both nanoparticles, Al₂O₃ and Fe₂O₃ in the digestive gland and kidney tissues of the land snail *Helix aspersa*.

Keywords: Nanoparticles, Al₂O₃, Fe₂O₃, Bioaccumulation, *Helix aspersa*.

INTRODUCTION

Nanosciences and nanotechnology provides a fundamental understanding of phenomena at the nanoscale level in order to create devices and systems that have novel properties and functions.

Indeed, the nanotechnology has been hailed as one of the key of the new industrial revolution affecting all spheres of society [1]. Nanoparticles are at the leading edge of the rapidly developing field of nanotechnology. NPs are used in a wide range of fields such as medical, industrial, aeronautical, electronics, cosmetics, textile, agricultural, food packaging and environmental applications [2, 3].

The emission of metallic oxides NPs in the environment from contaminated sources occurs mainly during implementation (before marketing or incorporation into matrices) and possibly during aging of the products in which they were dispersed. NPs can interact with different environmental components, undergo transformations and exhibit different behavior from their original form, and interaction with macromolecules via various mechanisms [4]. However, little is known about their environmental fate, reactivity, bioavailability and effects. It is for this

reason that the scientists have been worried for some time about the increased use of NPs which are likely to be released into the environment and they can accumulate in soils and threaten our food supply [5]. It is, however, unclear if these particles are harmful to living organism?

A high rate of nanotoxicological studies on the interaction of NPs with biological systems, both in cell line and different organs, have boosted a growing awareness of the need to predict the possible hazard of NPs with a necessity of interdisciplinary research [6]. In this regard, the studies of Ray *et al.*, [7] and Trouiller *et al.*, [8] have focused on the toxicity of NPs to the environment. Increasingly, investigations and assessment on NPs toxicity showed that the fate, bioavailability and bioaccumulation of these particles in different ecosystems was influenced by several factors including particle composition, size, distribution, solubility and agglomeration, shape and crystal

structure, surface area, mass and concentrations, charge chemistry and the presence of impurities [9-11]. Nanoparticles and cellular components, such as nuclei, mitochondria, and lysosomes are almost the same size. Certainly, NPs may be able to bypass cellular barriers, thereby leading to unexpected adverse tissue reactions or cellular dysfunction [12]. Nevertheless, the toxicological effects of NPs were manifested through mechanisms related to inflammation and generation of reactive oxygen species that can alter the surrounding tissues [13, 14], if the antioxidant systems of the body were not in sufficient quantity to inactivate the reactive oxygen species [15, 16].

The use of bioindication was primarily a consequence of the need to assess the impact of very dynamic nanotechnology progress on living organism, where information on the volume and air contamination type, water or soil was no longer sufficient. Many plants and animals were able to give indications on the natural features of a site and qualitative and quantitative information on the changes caused by anthropic activities [17]. The juveniles and adults snail of *Helix aspersa* were used in many toxicological and Eco toxicological studies. Indeed, because of their place within the terrestrial ecosystem, they represented a significant biomass within the soil invertebrate community [18], snails were capable of integrating multiple sources of contamination (soil, atmosphere and plants) by various ways: digestive, respiratory and / or cutaneous and they occupy a privileged situation at the soil-plant-atmosphere interface [19]. The resistance and accumulation capacities of metals have been demonstrated in these species [20].

Recently, studies on the accumulation of metal oxide nanomaterials in mollusks have been carried out. Nevertheless, Montes *et al.*, [21] observed the accumulation of Ce and Zn in soft tissues of a marine suspension-feeder, *Mytilus galloprovincialis* exposed to nano-CeO₂ and nano-ZnO. The accumulation of nano-CuO was demonstrated in digestive gland of *Mytilus galloprovincialis* in the study of Gomes *et al.*, [22] and the same observations are mentioned in the freshwater snail *Potamopyrgus antipodarum* [23] and in the land snail *Achatina fulica* [24].

As a common model organism, land snail (*Helix aspersa*) is recently used in ecological toxicity research for NPs [25]. The aim of this study was to investigate the accumulation of aluminum and iron oxides nanoparticles (Al₂O₃ and Fe₂O₃) in land snail under chronic exposure.

MATERIALS AND METHODS

Collection and selection of samples

Experimental subjects were the gastropod land snail *Helix aspersa*, were obtained from a snail farm at Bouchegouf *Guelma* (Northeast Algeria) in 2014. Adult snails collected were sorted by size and weight in the

laboratory with an average weight of 12 ± 0.35 g, placed and kept in microcosms before being selected in lots for different treatments and experiments.

Were raised in the following optimal environmental conditions: photoperiod 18h light/24h temperature ($20 \pm 2^\circ\text{C}$), humidity 80 to 95% wheat flour in food. The animals were divided into transparent polystyrene boxes (25 x 15 x 15 cm) with perforated lid; each box contains a wet sponge to retain humidity. During the exposure period food is supplied in petri dishes regularly every day [26].

Chemical Equipment

Aluminum oxide and iron oxide bulk powder used in our experiments were obtained from the laboratory of magnetism and spectroscopy of solids (LM2S) in the University of Annaba, Algeria.

Aluminum oxide nanoparticles powder

Aluminum oxide is an amphoteric oxide of aluminum with the chemical formula Al₂O₃, also commonly referred to as alumina.

Iron oxide nanoparticles powder

Iron oxide, also called ferric oxide, is the chemical compound with the formula Fe₂O₃, whose mineral form is hematite. It is a stable paramagnetic oxide of iron.

Characterization of the average grain size of Al₂O₃ and Fe₂O₃

X-ray diffraction analysis was performed on powder using the Scherrer method. We used a diffractometer type APD-15 Philips 2134 scan with Cu anode radiation at wavelength $\lambda = 1.5406 \text{ \AA}$ produced by a Ceramic X-ray tube with selection of K α radiation. From the Bragg's law $\lambda = 2d \sin\theta$, we deduce the value of the lattice distance for each of the spectral lines of the samples and compare them with those given by ASTM (American System Testing Metal) numbers 11-549 and 25 -447 respectively of β and α -PbO₂ (Figure 1 & 2).

The crystalline formation was determined from the diffraction pattern and the crystallite size was calculated using the Scherrer formula.

$$d = K\lambda / (\beta \cos\theta)$$

Where d is the mean crystallite size (in A°), K is the grain shape dependent constant 0.94, λ is the wavelength of the incident beam (in nm), θ is the Bragg reflection angle (in degrees), and β is the line broadening at half the maximum intensity (in radians).

Calculations of the average grain size by the Scherrer method showed a 93 nm size of aluminum oxide, whereas the iron oxide has a size of 99 nm (Figure-1, 2).

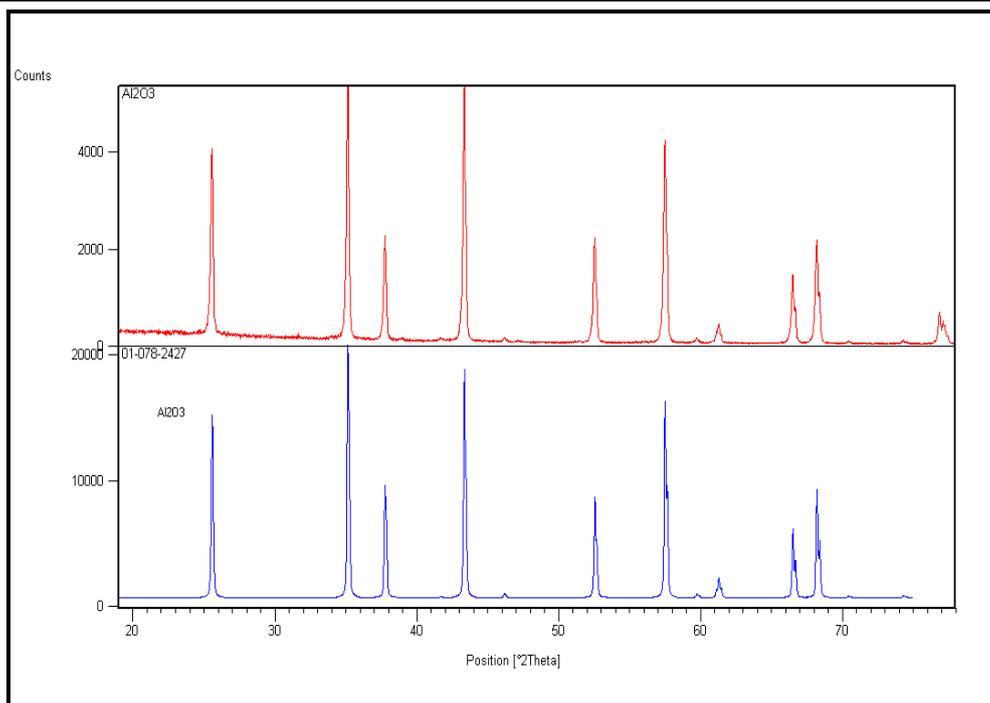


Fig-1: X-ray diffraction spectrum of Aluminum oxide.

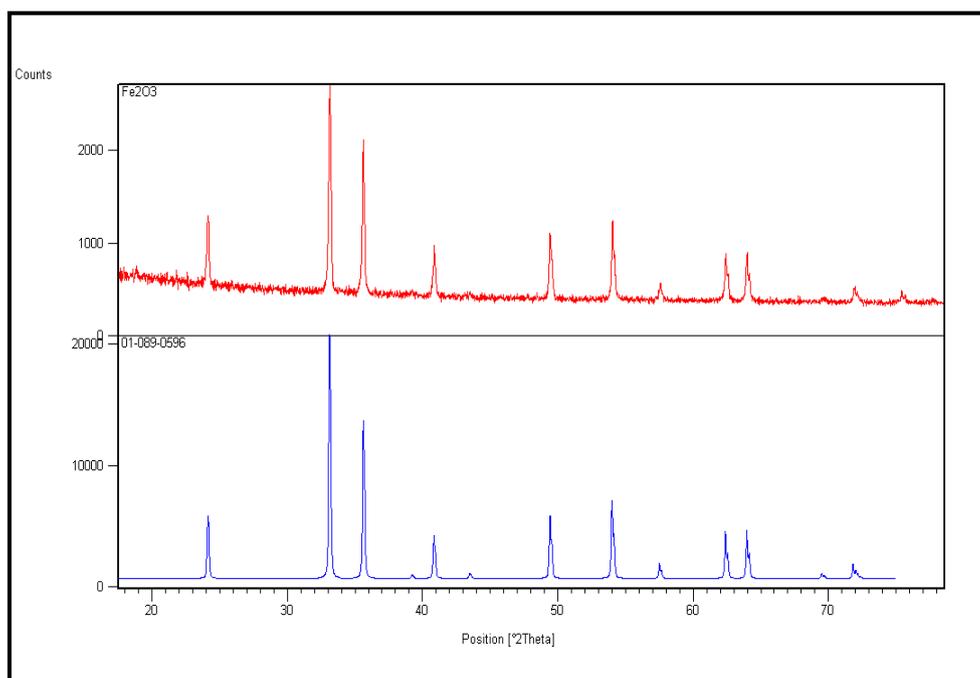


Fig-2: X-ray diffraction spectrum of iron oxide.

Treatment protocol

After 15 days for acclimatization to laboratory conditions, processing snails was made by adding various concentrations (50, 100, 200 and 400 mg/g diet such as wheat flour) of aluminum oxide nanoparticles (Al_2O_3) and iron oxide nanoparticles (Fe_2O_3). Snails were distributed into 5 groups of 12 snails in each, and they were treated daily for eight weeks.

Dissection and preparation of organs for measurement of the bioaccumulation

After eight weeks of treatment, the snails were sacrificed after freezing (-80°C), without prior fasting which could alter the expression levels of molecules sought. After dissection and removal of the two organs (digestive gland and kidney), from seven randomly chosen snails of each experimental group. We evaluated the bioaccumulation phenomenon using atomic absorption spectrophotometry (AAS) method. The digestive gland and kidney of the land snail were used to determine the possible bioaccumulation of Al_2O_3 and Fe_2O_3 NPs tissues.

Mineralization and extraction of digestive gland and kidney

Before the mineralization, the organ fragments were thawed, immersed in a 10 μM EDTA solution for 5 min. Subsequently the fragments were placed individually in screw tubes and then dried in an oven (50°C) for 48 to 72 hours [27]. The mineralization was a complete destruction of organic matter under the combined effect of temperature and concentrated nitric acid. We added 4 ml of concentrated nitric acid (50%) to the previously dried tissues. The whole is put at 60°C for (about 72 hours) until the solution becomes clear and the red nitrous vapor disappears. Then, each sample is made up to a volume of 19 ml with distilled water and stored at 4°C until analysis [27]. The minerals are then recovered in graduated tubes.

Statistical data analysis

The data obtained were subjected to analysis by ANOVA one way (treatment/ concentrations). The analysis over treatments was also performed by using CropStat 7.2. Software [28]. Significant difference between two means was measured using Fisher's test (LSD_{5%}). The linear regressions were performed with Microsoft excel 2010 (Microsoft, Redmond, WA, USA).

RESULTS

The analysis of the variances revealed a highly significant concentration effect for the bioaccumulation of alumina and iron nanoparticles in the digestive gland and the kidney of *Helix aspersa* snails (Table-1).

Table-1: Mean squares ANOVA analysis of bioaccumulation in digestive gland and kidney of snails treated with aluminum oxide and iron oxide.

Source of variation	DF	Bioaccumulation of Al ₂ O ₃ in digestive gland ($\mu\text{g/g}$ dry weight)	Bioaccumulation of Al ₂ O ₃ in Kidney ($\mu\text{g/g}$ dry weight)
Concentrations	4	62.95 ***	52.61***
Error	15	0.11	0.12
C.V %		4.43	4.55
Source of variation	DF	Bioaccumulation of Fe ₂ O ₃ in digestive gland ($\mu\text{g/g}$ dry weight)	Bioaccumulation of Fe ₂ O ₃ in Kidney ($\mu\text{g/g}$ dry weight)
Concentrations	4	32.08***	28.93***
Error	15	0.12	0.22
C.V %		6.05	7.96

DF=degrees of freedom, C.V: coefficient of variation, Level of significance: $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$, $p < 0.0001 = ****$.

The Fisher's test (LSD_{5%}) revealed that the bioaccumulation rate in digestive gland of *Helix aspersa* exposed to increasing concentrations of aluminum oxide, the emergence of five homogeneous groups with an overall average of 7.61 $\mu\text{g/g}$ dry weight. However, in snails treated with the tow highest concentrations 200 and 400 $\mu\text{g/g}$, the bioaccumulation rate was 11.13 $\mu\text{g/g}$ dry weight and 10.04 $\mu\text{g/g}$ dry weight respectively (Table 2).

Three homogeneous groups were observed after using the Fisher's test (LSD 5%) on the same parameter measured in the kidney. The first group (a) represents the snails exposed to concentrations of 50 and 400 $\mu\text{g/g}$, the second group (b) includes individuals exposed to concentrations of 100 and 200 $\mu\text{g/g}$, however the third group (c) represents controls (Table-2).

The Fisher's test (LSD 5%) showed the emergence of four homogeneous groups concerning the bioaccumulation of iron oxide in the digestive gland. The lowest concentration is recorded in the control

group with a value of 1.14 $\mu\text{g/g}$ of dry weight, whereas the highest bioaccumulation is observed in snails treated with the concentration of 200 $\mu\text{g/g}$ with a content of 8.12 $\mu\text{g/g}$ dry weight (Table-2).

In kidney, the Fisher's test showed four homogeneous groups for the bioaccumulation of iron nanoparticles. The first group (a) is represented by the snails treated with the concentration of 50 $\mu\text{g/g}$, where the bioaccumulation rate is highest with a value of 8.70 $\mu\text{g/g}$ dry weight, the second group (b) is characterized by snails exposed to the highest concentration of 400 $\mu\text{g/g}$, the third group (c) include snails treated with 100 and 200 $\mu\text{g/g}$, while the last group (d) represents controls with a bioaccumulation rate of 1.54 $\mu\text{g/g}$ dry weight (Table-2).

Regarding the bioaccumulation rate in digestive gland of *Helix aspersa* exposed to increasing concentrations of aluminum oxide, we distinguished five homogeneous groups with an overall average of 7.61 $\mu\text{g/g}$ dry weight (Table-2).

Table-2: Comparison of bioaccumulation means between digestive gland and kidney of snails treated with different concentration of aluminum and iron oxide.

Parameters	Concentrations ($\mu\text{g/g}$)	Aluminum oxide		Iron oxide	
		Digestive gland	kidney	Digestive gland	kidney
Bioaccumulation	0	1.315 (e)	1.776 (c)	1.143 (d)	1.544 (d)
	50	6.215 (d)	10.612 (a)	5.222 (c)	8.707 (a)
	100	9.354 (c)	7.683 (b)	7.544 (b)	5.994 (c)
	200	11.132 (a)	8.213 (b)	8.126 (a)	5.994 (c)
	400	10.046 (b)	10.669 (a)	7.074 (b)	7.337 (b)
Mean		7.612	7.790	5.822	5.915
LSD _{5%}		0.509	0.535	0.530	0.710

The variation of the bioaccumulation rates in the digestive gland and kidney of snails treated with iron oxide and aluminum oxide are illustrated in figure (3). We noted that the bioaccumulation of NPs is higher in snails treated with aluminum oxide than those treated

with iron oxide, and this difference is in the order of 40%. In addition, it should be noted that the amounts of accumulated NPs are slightly elevated in the digestive gland compared to those recorded in the kidney tissues.

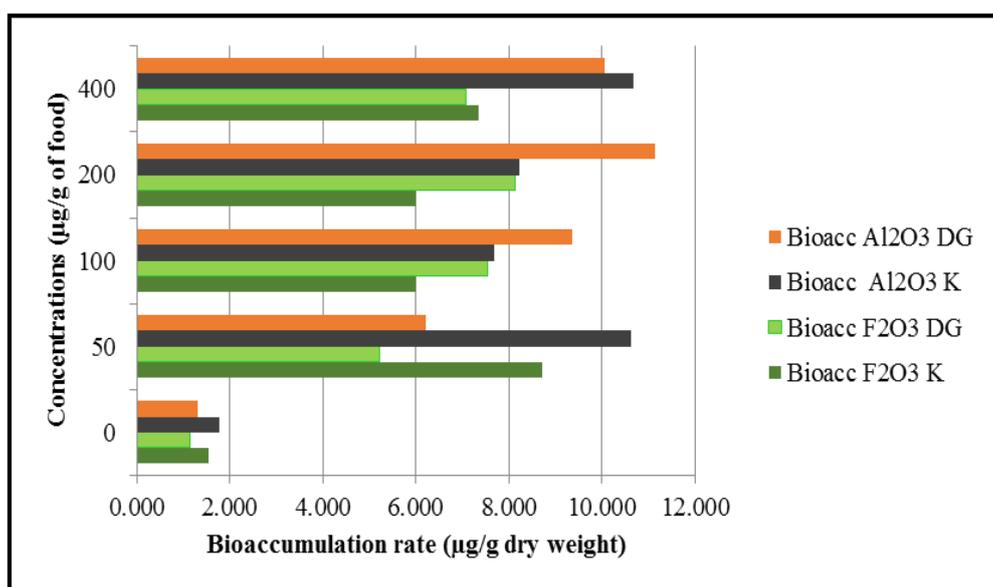


Fig-3: Variations of bioaccumulation rates depending on Fe₂O₃ and Al₂O₃ concentrations in both digestive gland and kidney of *Helix aspersa*.

In the digestive gland, accumulated alumina NPs tend to increase in a dose-dependent and highly significant manner in treated compared to controls ($r = 0.95^{***}$). As soon as they were treated with 50 $\mu\text{g/g}$ of

alumina NPs, bioaccumulation increased to about 11 $\mu\text{g/g}$ dry weight in snail's treated with 200 $\mu\text{g/g}$, whereas the Al₂O₃ controls is only 1.31 $\mu\text{g/g}$ dry weight (Figure-4).

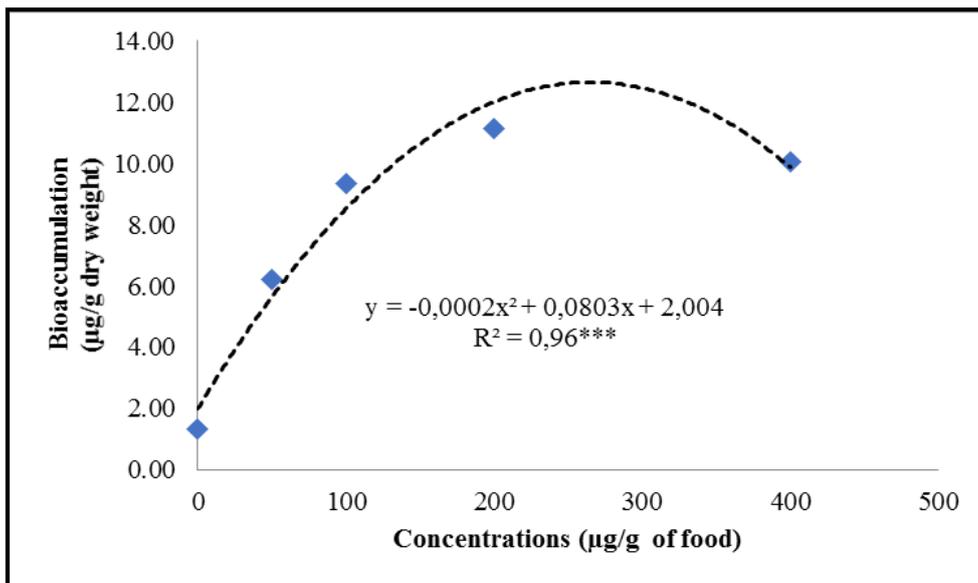


Fig-4: Amounts of Al₂O₃ NPs accumulated in the digestive gland of *Helix aspersa*.

As shown in figure (5), the linear regression analysis revealed a positive and significant correlation ($r = 0.60^*$) between the bioaccumulation capacity of Al₂O₃ particles and their concentration in the kidney tissue. The content of accumulated Al₂O₃ particles increases and reaches values that are all the higher as

the concentration of the NPs of alumina is important. Thus, the amount of accumulated particles increased from 1.77 µg/g dry weight in control group to 10.67 µg/g dry weight in the treated by the highest concentration (400 µg/g).

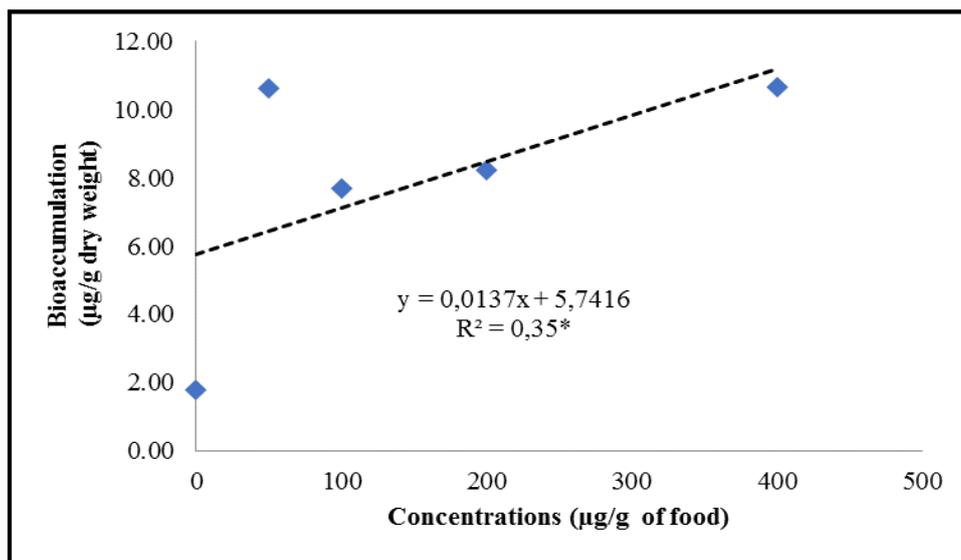


Fig-5: Amounts of Al₂O₃ NPs accumulated in the kidney of *Helix aspersa*.

Our results indicate that the bioaccumulation of iron NPs in the digestive gland is positively and highly significant related to the increasing

concentrations of Fe₂O₃. This is particularly highlighted for the treated with concentration of 200 µg/g, with ($r = 0.96^{**}$) (Figure-6).

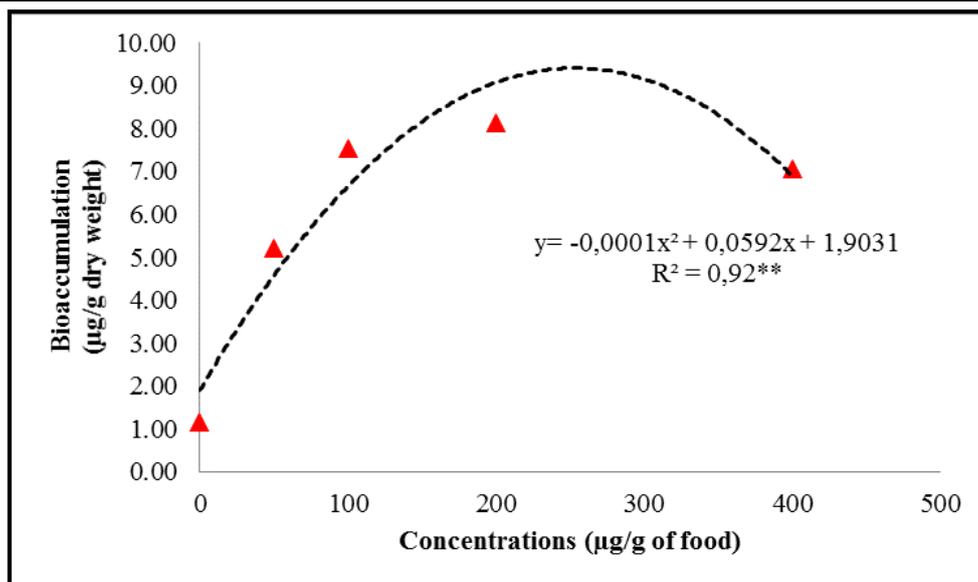


Fig-6: Amounts of Fe₂O₃ NPs accumulated in the digestive gland of *Helix aspersa*.

In the kidney, the concentrations of accumulated iron NPs after eight weeks of exposure are shown in Figure (7). We found a dose-dependent increase in the bioaccumulation of Fe₂O₃ particles. In fact, the regression analysis reveals the presence of a

significant concentration effect of NPs ($r = 0.55^*$). The accumulated iron concentrations increased from 1.54 µg/g dry weight in controls to 8.70 µg/g dry weight in snails treated with a concentration of 50 µg/g.

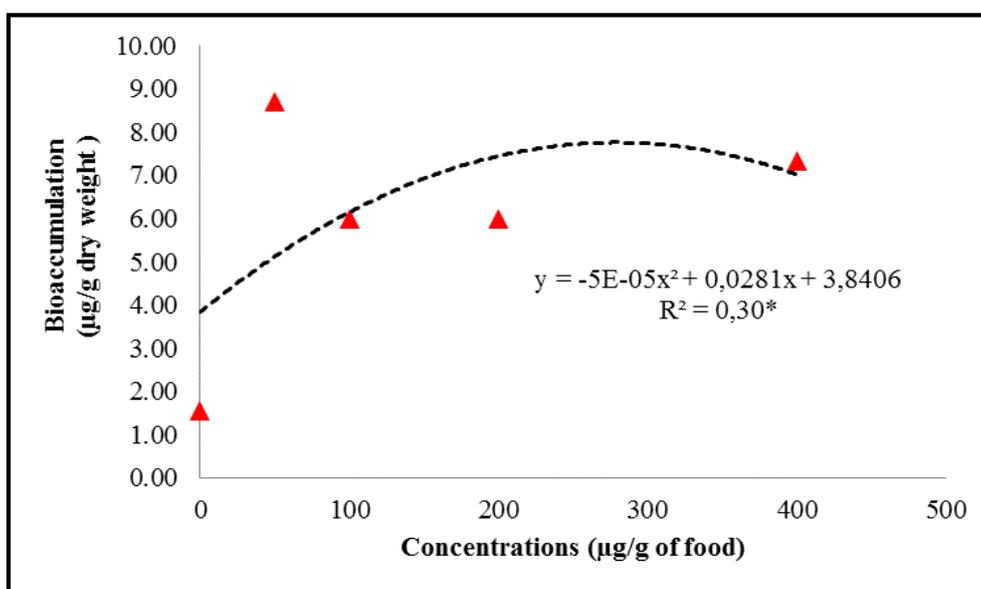


Fig-7: Amounts of Fe₂O₃ NPs accumulated in the kidney of *Helix aspersa*.

DISCUSSION

Considering the huge range of applications and systems using NPs, it seems reasonable to expect their dissemination in the environment, be bioavailable and taken up by several living organisms [29, 30] and consequently find their way into the food chain [31]. Our results demonstrated a significant accumulation of aluminum NPs, particularly in the digestive gland tissue of the land snail exposed for eight weeks to various concentrations of aluminum NPs. This result was consistent with that of Park *et al.* [32] who reported that

the highest accumulation of Al NPs have been recorded in the liver and kidneys of mice after 13 weeks of treatment. In addition, albino rats injected with Al NPs at a sublethal dose (1300 µg/g) for 28 days induced an important accumulation in the liver followed by the spleen, intestine, and kidney [33]. Moreover, morphological changes in cultured macrophages (RAW264), and the formation of many Al₂O₃ NPs aggregates in vesicles were observed at NPs exposure concentrations of 200 and 400 µg/ml [34]. The algae *Pleurotus eryngii* and the mushroom *Trametes*

versicolor showed a high capacity for accumulation of Al₂O₃ [35]. The bioaccumulation phenomenon was observed in the terrestrial snail *Cipangopaludina chinensis* after a 17 day exposure to titanium NPs [36]. Similar results were obtained in soft tissues of freshwater snail, *Potamopyrgus antipodarum* exposed to different-shaped copper oxide nanoparticles [23].

Overall, our results show that the digestive gland is the main tissue for aluminum oxide accumulation. Indeed, the studies of Gomes *et al.*, [22] and Barmo *et al.*, [37] showed a high bioaccumulation capacity in the digestive gland of *Mytilus galloprovincialis* exposed to copper oxide and titanium dioxide. The later observations were also confirmed by Canesi *et al.*, [38].

On the contrary, Farkas *et al.*, [39] did not observe any titanium dioxide accumulation in the gills, in the digestive gland and also in the rest of the soft tissue of mussels *Mytilus edulis*. The same observations was reported by Falfushynska *et al.*, [16] for the *Unio tumidus* mold exposed to low concentrations of ZnO nanoparticles, and also reported by the study of Tian *et al.*, [40] in bivalves *Scapharca subcrenata* treated with TiO₂ NPs. In fact, Di-Virgilio *et al.*, [41] and Radziun *et al.*, [42] mentioned that the cytotoxic effects of Al₂O₃ NPs are nonexistent or less than other NPs.

A high accumulation rate of iron in the digestive gland and kidney have been recorded after eight weeks exposure of snail *Helix aspersa* to various concentrations of iron oxide NPs. The current results corroborate with those of Zhang *et al.*, [43] that they observed an important accumulation of Fe₂O₃ NPs (size, 80-90 nm) compared to Fe₃O₄ NPs (size, 140-160 nm) in zebrafish *Danio rerio* exposed to 4.0 and 10.0 mg/l concentrations for 28 days. Furthermore, the highest concentration of nano-Fe₂O₃ (magnetic) was accumulated in the gut of *Ceriodaphnia dubia* [44]. In recent study, Chen *et al.*, [24] highlighted the importance of dietary uptake of Ag NPs during bioaccumulation in the land snails *Achatina fulica*. Wistar rats were reported to accumulate aggregate of iron nanoparticles in the pancreas, kidneys and liver [45]. Moreover, in this regard, exposures to silver NPs, Ali *et al.*, [46] revealed a high bioaccumulation of these particles in both digestive gland and kidney tissues of the land snail *Eobania vermiculata*. The same phenomenon has been reported in earthworms *Eisenia andrei* and *Eisenia fetida* [47, 48]. Unlike, no effect was observed in reproduction (fecundity and fertility) of aquatic snail *Biomphalaria glabrata* after four weeks exposure to meso-2, 3-dimercaptosuccinic acid (DMSA) coated γ -Fe₂O₃, and accumulation of iron NPs was not present after 30 days in clean water [49].

Using quantitative analysis by AAS method, we found that the highest amounts of accumulated NPs are recorded in aluminum oxide-treated snails compared

to those treated with iron oxide. In fact, we determined a variation of the bioaccumulation rate depending on the different concentrations used and the organ tested their digestive gland or kidney. Nevertheless, the variation of bioaccumulation in snails treated with nanoparticles of alumina and iron can be explained at the expense of several other factors which should be considered. Among these factors we note the critical chemical and physical properties and the concentration of NPs, bioavailability, interactions with macromolecules, tissues, biodistribution and their accumulations in different organs of biological systems, on one hand. On other hand, the physiological state and the tolerance and adaptation abilities of the body can positively or negatively influence the response to exposure to NPs generally and particularly on the bioaccumulation phenomenon. Recently, Lei *et al.*, [11] concluded that the toxicity of iron-based NPs is a function of their properties, tolerance of test organisms, and environmental conditions. According to Morsy *et al.*, [33], the bioaccumulation of NPs is closely related to duration, concentration, and evidently the target organ, whereas Jakubiak *et al.*, [35] showed that the accumulation phenomenon of NPs depends solely on the duration of exposure. However, the bioaccumulation of nanoparticles can be influenced by the exposure pathway as suggested by the work of Stebounova *et al.*, [50], Cho *et al.*, [51] whose results revealed that the properties of NPs are modified and significantly altered after their penetration and interactions with biological systems. However, the extent of cell surface aggregates of Al₂O₃ NPs was greater than the SiO₂ NPs. In contrary, the intercellular dissolution rate of SiO₂ NPs may be greater than that of Al₂O₃ NPs [34]. Furthermore, the nano-surface chemistry and dose govern the bioaccumulation and toxicity of metal nanoparticles [9], and can affect the lipid, glucose and amino acid metabolism pathways, by disturbance of renal, hepatic and cardiac performance [52, 53]. The cumulative toxic effects of NPs in various organisms may be influenced by storage or excretion of the NPs in a benign form [54, 55]. In contrary, Di-Virgilio *et al.*, [41] and Radziun *et al.*, [42] considered that categorized Al₂O₃ NPs as a safe material. All these studies confirm that the available information on toxicity and NPs accumulation by living organisms is still confusing and difficult to compare [56].

CONCLUSION

In our work we have investigated the nanotoxicity of alumina oxide and iron oxide nanoparticles on a terrestrial bioindicator model snail *Helix aspersa* through the determination of the concentrations of these NPs accumulated in the digestive gland and kidney tissues. After eight weeks of exposure, we recorded a significant accumulation of aluminum oxide NPs in snails treated with the two highest concentrations of 200 and 400 μ g/g. Indeed, the bioaccumulation rate in the digestive gland is slightly higher than those recorded in the kidney tissue.

However, the clear variation in the amounts of iron nanoparticles accumulated in the two organs tested according to the different concentrations used has been demonstrated.

Finally, we can conclude that the bioaccumulation capacity of the *Helix* snail varies according to the concentrations of the two NPs tested. Therefore, in nanotoxicology studies the bioaccumulation phenomenon in living organisms may be influenced by many factors which should be considered. We suggest that the land snail *Helix aspersa* could be used as effective terrestrial accumulation bioindicators for risk environmental assessment and biomonitoring program of both aluminum oxide and iron oxide nanoparticles.

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