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Monitoring the ecotoxicity of γ-Al_2O_3 and Ni/γ-Al_2O_3 nanomaterials by means of a battery of bioassays

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ABSTRACT

The increasing application of nanoparticles (NPs) to a variety of new technologies has become a matter of concern due to the potential toxicity of these materials. Many questions about the fate of NPs in the environment and the subsequent impact on ecosystems need to be answered. The aim of this work was to evaluate the ecotoxicity of two alumina-based nanoceramics, γ-Al2O3 (NC) and Ni/ γ-Al2O3 (NiNC) by means of three different standardized tests: Biochemical Oxygen Demand (BOD5), bioassay with luminescent bacteria (Vibrio fischeri; Microtox), and bioassay on amphibian larvae (Rhinella arenarum) (AMPHITOX). BOD5 values of a very biodegradable mixture (glucose/glutamic acid) decreased with the addition of NiNC(43.8%) and NC (31.6%) with respect to control samples (52.9%). Microtox test results indicated that NiNC presents higher toxicity than NC, with EC50s values of 16.1% and 29.9% respectively; a reduced toxicity was observed, however, in presence of organic matter, thus obtaining EC50s of 37.8% and 19.4%. The results of AMPHITOX test showed a significant increase in the toxicity of both substances over time, the NiNC toxicity being greater than that of NC. The values of 96 h-LC50 and 504 h-LC50 determined for NiNC were 1.58 and 0.83 mg/L, respectively, and 14.5 and 10.5 mg/L for NC samples. Amphibian larvae exhibited collapsed cavities, edema, axial flexures, and behavioral alterations as hyperkinesia and reduced movements. These results evidence the vulnerability of wildlife to xenobiotics and the need to develop specific standardized ecotoxicity tests in order to help environmental sustainability and natural species conservation.

1. Introduction

In the last years, many novel nanotechnology applications have been developed and, much information about the properties of nanomaterials has been provided. Therefore, nanoparticles (NPs) are not a novelty per se; they have always existed in our environment. Nanoparticles in soil including clays, organic matter, iron oxides, and other minerals play an important role in biogeochemical processes. In urban atmosphere, diesel- and gasoline-fueled vehicles contribute to pollution by the emission of a wide range of particles, where more than 36% are NPs (APHA, 2012). The background concentration in atmosphere is low but an increase can be expected in the near future due to potential release of manufactured NPs. Moreover, several authors reported the toxicity of these materials (Buzea et al., 2007; Kahru and Dubourguier, 2010; Oberdörster et al., 2000; Sahu and Casciano, 2009). Nevertheless, the fate, behavior, and transport of NPs in different water matrixes are still poorly understood (Buzea et al., 2007; Keller et al., 2010; Oberdörster et al., 2000). The high mobility and high specific area of NPs might favor the transport of chemical pollutants in rivers and lakes.

Alumina NPs have many industrial uses because of their excellent dielectric and abrasive properties; nanoporous gamma-alumina is utilized as adsorbent material, in separation membranes, and also as metal support in catalytic reactors (DeFriend et al., 2003). In contrast, these

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highly adsorptive materials are chemically active and potentially hazardous to the environment, where they can be released during the synthesis and their use. These and other NPs (e.g.: cerium oxide; nickel oxide) have been commercially distributed worldwide for many purposes (e.g.: NaBonds, Hong Kong; Kemix, Australia; Markettech Int., EEUU; Lintec, Germany etc.). In this economic and technological context, only a few studies about their potential toxicity have been published. Most of the works regarding the impact of Al_2O_3 NPs have focused on the toxicity by using in vitro cellular models on human or rat cells (Böhme et al., 2014; Karunakaran et al., 2015). Ecotoxicity studies have demonstrated Al_2O_3 NPs toxicity on autotrophic and heterotrophic organisms. Recent studies suggested that the toxicity mechanism of Al_2O_3 NPs is through the induction of oxidative stress in algae (Pakrashi et al., 2013a), microinvertebrates (Pakrashi et al., 2013b) and fishes (Kovrižnych et al., 2013).

Metal oxide NPs are of particular concern because they are non-biodegradable materials and can rapidly be distributed throughout the environment. The toxicity of metal oxide NPs has been reported as depending on the chemical composition, size, surface charge, and shape of the particles (Simon-Deckers et al., 2009). As a result of these studies, NPs stability and characteristics appear to be the key for designing and interpreting nanoparticle ecotoxicity experiments in several environmental conditions.

The interaction of metal oxide NPs with bacteria has already been demonstrated as well as its periplasmic accumulation and toxicity in some strains (Simon-Deckers et al., 2009). *In vitro* experiments showed cytotoxic effects of Ag and Al_2O_3 NPs on *Bacillus cereus* and *Pseudomonas stutzeri* cells. In soil experiments, NPs induced changes in the microbial gene expression profile (Fajardo et al., 2013). Bacterial isolates from freshwater exposed to 1 g/mL Al_2O_3 NP for 2 h showed a 17% decreased in cell viability (Pakrashi et al., 2011). Also some studies have shown the toxic effect of various NPs on *Vibrio fischeri* (Heinlaan et al., 2008, Jośko et al., 2016, Strigul et al., 2009). This is a luminescent bacteria used for in vitro test system Microtox®, which is applied for toxicity identification of pure or mixed chemicals and environmental samples (Bond and Martin, 2005).

Amphibians play a key role in food webs as they live near or in water reservoirs that can be affected directly or indirectly by many xenobiotics. They are extremely sensitive due to their permeable skin that readily absorbs chemicals from the environment; the same applies to their eggs (Boyer and Grue, 1995). Because of this high sensitivity amphibians can be very good indicators of environmental health (Welsh and Ollivier, 1998). Moreover, given the importance of amphibians in both aquatic and terrestrial ecosystems, the population decline can have large-scale consequences through alterations in food webs (Regester et al., 2008). Amphibian larvae tend to be more sensitive to contaminants than adults (Bridges, 1997). They graze from sediment surface incorporating particulate matter, thus making them good bioindicators of NP-contamination as NPs tend to form aggregates. In this context it is important to conduct studies on amphibians during larval development for assessing the toxicity of substances in order to obtain useful information for future prevention projects (Boyer and Grue, 1995). The study of sublethal effects is important due to indirect effects on population viability as, for example, the increasing vulnerability to predation and fitness reduction, which affect the projection of amphibian populations (Egea-Serrano et al., 2012). This information can be even more significant than the information provided by studies of lethal effects. Yslas et al. (2012) and Ibarra et al. (2015) assessed the lethal and sublethal effects of polyaniline NPs (PANI-NP) in embryos and larvae of *Rhinella arenarum*. They reported lethal effects on larvae exposed to concentrations from 1395 mg PANI-NP/L and sublethal effects such as abnormal axis and edema from 500 mg/L. Salvaterra et al. (2013) reported that *Pelophylax perezi* larvae exhibited no significant mortality after exposure to 20 mg/L titanium silicate nanoparticles ($TiSiO_4$ NP).

Understanding the relationship between nanoparticles and the environment has become an important area in ecotoxicology research. Despite the emerging reports on the potential toxicity of NPs, there is still a lack of scientific results, particularly of proof procedures, analytics and characterization, which provide information for normative regulation. The aim of this work was to evaluate the ecotoxicity of two different nanomaterials (NMs), γ-Al_2O_3 and Ni / γ-Al_2O_3, by means of bioassays like Biochemical Oxygen Demand (DBO5), Microtox and AMPHITOX, applied to bacteria and amphibians. Sublethal effects of these NMs on *Rhinella arenarum* larvae were also qualitatively evaluated. Although some toxicity studies of alumina NMs on bacteria, e.g. *Vibrio fischeri*, can be found in the literature (Strigul et al., 2009), results of Ni / γ-Al_2O_3 NMs, and activated sludge (BOD5) and AMPHITOX bioassays have not been reported up to now.

2. Materials and methods

2.1. Nanoceramic dispersion and characterization

Samples of γ- Al_2O_3 were prepared via sol-gel following the Yoldas technique modified by Perez Catán and Guraya (2015). Boehmite colloidal suspension was prepared, by hydrolysis at 80 °C of aluminum *sec*-butoxide/*sec*-butanol solution into an excess of Milli-Q water (100 water/alkoxide molar) and subsequent peptising with nitric acid, the ratio acid/boehmite being 0.07 mol/mol. The reaction was carried on for 30 min at temperature above 75 °C while briskly stirring. In case of Ni / γ-Al_2O_3 NMs aliquots of 9.3% solution of nickel nitrate (Fluka $NiNO_3{\cdot}6H_2O$, 99.7%) were incorporated to the suspension, inducing gelation in a very short time. Gels were dried at 90 °C for 12 h and calcined afterwards 4 h at 600 °C.

In order to characterize these nanoceramics, unsupported γ- Al_2O_3 (NC) and Ni/γ-Al_2O_3 (NiNC) were analyzed by nitrogen adsorption-desorption isotherms at liquid nitrogen temperature (77 K) after degassing at 450 °C for 2 h, in a Digisorb 2600 from Micromeritics Int. Corp. The algorithm used by the equipment is based on Faass's implementation of the BJH method with straight, non-interconnected cylindrical pores closed off at one end. Powder X-ray Diffraction (XRD) measurements ($10^\circ < 2\Theta < 90^\circ$) were conducted in a Philips PW1700 diffractometer with Cu-Kα radiation and a graphite monochromator. Observations in Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) were performed in a Tecnai F20 (FEG) and FEI Nova Nano SEM 230 and Philips 515 SEM with energy dispersive X-ray analyser (EDAX) Genesis 2000, respectively.

Test suspensions were prepared by dispersion of NCs in MiliQ water to an approximate concentration of 0.1 wt%. The suspensions were used after sonication with a probe sonicator (TESLAB® Digital Sonifier). The sonicated suspensions were place to decanted for some period of time (0, 24, 48 and > > 72 h) and the supernatants were separated. Then, the suspensions were centrifuged at 6000 rpm for 20 min followed by filtration through MF-Millipore (Millex-GS 0.22) membrane. The element concentrations were measured in suspensions and supernatants by Electrothermal Atomic Absorption Spectrometry (ETAAS) and nanoparticle sizes were determined, simultaneously with Z-potential, by light scattering techniques. The concentrations of dissolved Al and Ni (ions) from NMs dissolved fraction, and the characterization of crystalline patterns with particle size of NP, were examined according to time trends in order to evaluate aging effects. The dissolved fractions were calculated for both elements Ni and Al, as the element concentration in the supernatant solution respect to the total concentration in the NMs suspension.

2.2. Toxicity bioassays

2.2.1. Bacterial assays –BOD5 Test

The standard BOD5 test was used to assess growth inhibition, using a standard solution of glucose/glutamic acid (GlcGlt) as substrate, which was stirred with NPs for 60 min. Concentrations tested were

21.14 mg/L NC and 32.12 mg/L NiNC. BOD5 tests were conducted by following the Standard Methods for the Examination of Water and Wastewater (APHA, 2012). Dissolved oxygen was measured electrochemically, with a probe (YSI, model 508B) at the beginning and after 5 days as final time of the experiment (BOD5). The BOD5 determination was followed by inoculum addition of environmental communities of microorganisms, especially heterotrophic bacteria from activated sludge plants. Triplicate samples were incubated in 300 mL BOD5 bottles (mineral medium) during 5 days in darkness and at 20 ± 1 °C.

2.2.2. Bacterial assays –Microtox test

The toxicity tests on the marine luminescent bacteria *Vibrio fischeri* were performed according to ISO 11348-3 (ISO, 2007) and the methodology developed for Microtox 500 equipment. The toxic effect of an aqueous sample is determined as the concentration of the sample causing a 50% reduction on the light emitted by the bacteria, after a predetermined exposure time. In this work, measurements were made at 30, 60 and 80 min. Dilutions of the NP solution tested were between 2.8% and 90%. In this procedure, the freeze dried-bacteria was reconstituted with water, to provide a stock suspension of test organisms, which was kept at 5 °C and used to perform the test. A correction factor was applied due to the loss of luminescence of the control sample (reduction in light emitted without exposure to the toxicant). A 10% of the Microtox Osmotic Adjusting Solution was added to all samples to provide osmotic protection to test organisms. The 60 min-Inhibitory Concentration or EC50 (%) values were estimated by the equipment software.

2.2.3. Amphibian bioassays – AMPHITOX test

To examine the potential effects of the NCs on the larval development of *R. arenarum*, three mating pairs of adults weighing approximately 200–250 g per animal, were acquired in a non-impacted site, Lobos (Buenos Aires province, Argentina: 35° 11´ S; 59° 05´ W). Toad care, breeding, embryo acquisition and analysis were conducted according to the methods described in the AMPHITOX protocols (Herkovits and Pérez-Coll, 2003). Briefly, the ovulation of females was induced by means of an intraperitoneal injection of a suspension of one homogenized toad pituitary gland in 1 mL of AMPHITOX solution (AS) per female, plus 2500 IU human chorionic gonadotropin (hCG). The composition of AS was: NaCl 36 mg/L, KCl 0.5 mg/L, $CaCl_2$ 1 mg/L, and $NaHCO_3$ 2 mg/L, prepared in distilled water. Oocytes were fertilized in vitro using a testicular macerate homogenate suspended in AS resulting in a spermatozoid suspension of 10%. Sperm viability was confirmed by observing the spermatozoid morphology and movements under optic microscope. The egg quality and fertility were inspected and considered acceptable if the fertility rate was greater than 75% and embryo survival at the neurula stage was greater than 70%. Embryos were kept in AS and maintained at 20 ± 2 °C until reaching the complete operculum stage, S.25 (Del Conte and Sirlin, 1951). The AS was replaced entirely every three days and monitored weekly to ensure that the pH remained at acceptable levels (7 ± 0.5).

Ten larvae were randomly placed, in triplicate, in 10 cm-diameter glass Petri- dishes containing 40 mL of AS with γ-Al_2O_3 (NC) and NiO.γ-Al_2O_3 (NiNC). Nanoparticle (NP) concentrations tested ranged between 0.005 and 50 mg/L. Larvae were continuously exposed from the beginning of complete operculum stage (S.25) for acute (96 h), short-term chronic (168 h) and chronic (336, 504 h) periods. Organisms were maintained at 20 ± 2 °C, and a 12:12 h light: dark photoperiod. Tadpoles were fed with 3 granules (6 ± 0.5 mg) of balanced fish food TetraColor® per Petri-dish. Test solutions were entirely replaced every 48 h. Control groups were simultaneously maintained in AS without additions. Lethal and sublethal effects were evaluated every 24 h and the alterations compared with the normal development and behavior of controls. Morphological effects such as developmental delay, irregular surface, wavy tail and edemas, were evaluated. Neurotoxicity endpoints included spasmodic contractions and alterations in swimming. Smooth movements of the Petri-dishes, followed by stimulation with a light source were performed. In case of no response, soft mechanic stimulation with a glass rod was made and finally heartbeat was checked. Also feeding behavior was qualitatively assessed. Abnormalities and neurotoxic effects were observed under a binocular stereoscopic microscope (Zeiss Stemi DV4) and identified according to Bantle et al. (1998). For ultrastructural observations, larvae were fixed in 4% formol, dehydrated in a gradient of acetone, prepared for SEM (Philips 515 scanning electron microscope with energy dispersive X-ray analyser (EDAX) Genesis 2000, operated at 5 kW).

2.3. Data analysis

2.3.1. Bacterial assays –BOD5 test

Descriptive statistics were used to evaluate the experiments which were performed with three replicates and compared through Friedman test at a significance level of $p < 0.05$.

2.3.2. Bacterial assays –Microtox test

Microtox EC50 values for light inhibition were obtained as instrument output. The data were then analyzed with the ToxCalc™ software, high- end statistical data reduction software that compares results from exposed and control populations for reduced light emission. There are two major types of data analysis that can be performed with the ToxCalc™ software: point estimation and hypothesis testing procedures. Point estimation was used to determine the toxicant concentration that would cause an observable adverse effect in a given percentage of the organisms (such as 50% Inhibitory Concentration, EC50).

2.3.3. Amphibian bioassays – AMPHITOX test

Lethal concentrations (LC50) were statistically estimated by the USEPA Probit Program (USEPA, 1988). The LC values were considered substantially different when the higher/lower ratio exceeded the critical value established by American Public Health Association (2005). We conducted generalized linear mixed models (GLMMs) assuming a binomial distribution of the error to evaluate the effect of concentration and exposure time on lethality. Di Rienzo, Guzmán and Casanoves (DGC) test (Di Rienzo et al., 2002) was used to compare treatments at a significance level of $p < 0.05$. This analysis was conducted using InfoStat statistical software (Di Rienzo et al., 2015). The value of No Observed Effect Concentration (NOEC) was determined as the highest concentration without statistically significant differences, compared to the control group.

3. Results

3.1. Nanoceramic characterization

BET surface area, pore volume, mean pore diameter and porosity parameters of NMs are shown in Table 1. Regarding the porous structure all parameters showed a decrease by NiO addition with respect to those of the pure γ- Al_2O_3.

Fig. 1 shows the XRD spectra and the structures corresponding to NiO, spinel-like nickel aluminate, and gamma-alumina. The same spectra were obtained for the aging samples.

Total Al and Ni concentrations of NMs suspensions were measured in order to verify the capacity of dissolution of the NPs vs. the age of the

Table 1
Porous structure derived from nitrogen adsorption/ desorption experiments.

Sample	BET surface area (m^2/g)	Pore volume (cm^3/g)	Mean pore diameter (Å)	Porosity (%)
NC	217 ± 3	0.346	25 – 75	56
NiNC	200 ± 3	0.203	38	22

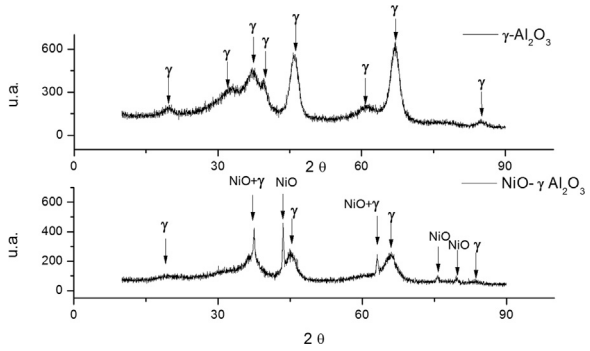

Fig. 1. XRD spectra of γ-Al_2O_3 (NC) and NiO γ-Al_2O_3 (NiNC).

Table 2
Dissolution rate, Z-potential and Particles size in relation to ageing of the suspension.

Time (h)	NiNC			NC		
	Dissolution rate (%)	Z-pot (mV)	Particle size (diam., nm)	Dissolution rate (%)	Z-pot (mV)	Particle size (diam., nm)
0	0.58	38.9	290.9	0.26	47.3	278
24	0.49	39	1325	0.54	37.9	368
48	0.41	23.5	1641	0.50	17.8	1311
> 72	< 0.4	41.1	249.2	< 1.4	43.7	297.5

suspension. Solubility rate, particle size and Z-potential (*z*-pot) of samples are shown in Table 2. Suspended NPs become stable after around 72 h; Al and Ni dissolved from the materials always were less than 1.4% (Table 2) which means a Ni content below to 2 μg/g and Al content of 7 μg/g.

Z-potential is the potential required to break the ion layer that encloses the NPs and therefore gives information of the hydrodynamic stability. Z-pot values at pH 7.5 for NC and NiNC suspensions were 41.1 and 43.7 mV, respectively, and both were reduced by the addition of GlcGlt solution. Moreover, the particle size distribution of NCs had only one mode and became bimodal by the end of the bioassay.

The shape and size of NPs were evaluated by TEM (Fig. 2); the average particle size was below 50 nm. Nickel NPs in the stock solution were primarily hexahedral-type crystals and γ-Al_2O_3, spherical NPs, which formed the matrix structure. Polyhedral particles have larger surface areas than spherical ones; this may affect the reactivity of the NPs. Particles in both NC and NiNC suspensions after treatment with GlcGlt, showed agglomerates of 200 nm in size or bigger. The agglomerates of NCs with organic matter (OM) were clearly defined in Fig. 2D as bright spots and in Fig. 2E as dark ones. Fig. 2F shows the results of NiNC-GlcGlt samples in transmission mode; in this case other agglomerates were observed probably as consequence of microorganism association to agglomerations.

3.2. Bacterial assays

Table 3 shows the results of biodegradation (BOD5) and Microtox test at 60 min of NMs associated with GlcGlt and the suspensions of post-biodegradation assay. Biodegradation values were 43.8% and 31.6% for NiNC-GlcGlt and NC-GlcGlt, respectively, which were very different from results obtained with GlcGlt (52.9%) (Friedman test, $p = 0.05$; critical value: 4.8), thus indicating that biodegradation rates were reduced in the case of OM associated to NMs.

Only the Microtox assays performed at 60 and 80 min gave positive results, without significant differences between them (Table 3). Both suspensions, NiNC and NC, were toxic to bacteria with EC50 of 29.9% (V/V) and 16.1% (V/V) respectively. The nanomaterial associated with Glc-Glt showed EC50 of 37.8% for NiNC and 19.4% for NC, which were more than 20% significantly higher than the values determined for NCs alone. Moreover, the Microtox test on suspensions of post-biodegradation assay were not toxic with EC50 values > 84% (Table 3).

3.3. AMPHITOX test

Fig. 3 shows the LC50s of both matrixes for *Rhinella arenarum* larvae at different exposure times. By extending the larval exposure to the NC an increasing toxicity was observed. In addition, LC50 for larvae in all NiNC samples was one order of magnitude lower than for the NC; thus representing a much higher toxicity. Acute lethal effects were not detected until 0.5 mg/L in NC suspensions and it was possible to calculate the LC50s only from 96 h onwards. The LC50s at the end of chronic exposure (504 h) were 10.52 and 0.83 mg/L for NC and NiNC, respectively, while the empirical 504 h-NOEC (No Observed Effect Concentration) values for lethality were 5.02 and 0.05 mg/L.

Sublethal effects caused by both NP materials to *R. arenarum* larvae were mainly behavioral alterations expressed as hyperkinesia and reduced swimming movements. In addition, collapsed cavities, edema and axial flexures were observed. These effects were evident from 48 h onwards and were concentration-dependent. Furthermore, essential functions such as feeding were deeply affected even at the lowest concentrations (0.005 mg/L). Despite the small value of the lowest concentration tested (0.005 mg/L), NOEC for sublethal effects could not be determined, thus demonstrating the high toxicity of these nanomaterials.

The results of elemental SEM-EDS characterization showed higher contents of Al and Ni in larval heads with respect to tails. Both Al and Ni were determined in the oral disc by means of SEM-EDS mapping as shown in Fig. 4. The oral disc is an adhesive apparatus located anteriorly on the ventral surface of the larval head that surrounds keratinized jaw sheaths and labial teeth.

4. Discussion

Bioassays have been used to evaluate the toxicity levels of contaminants by measuring the response of exposed organisms relative to controls. Bacteria are the most important microorganisms able to defend the environment against chemical contaminants. There is evidence that bacterial community structure undergoes changes after long-term exposure to NMs (Chen et al., 2012; Zheng et al., 2012). The studies indicated that decreasing abundance of denitrifying bacteria and declining activities of nitrate and nitrite reductases were due to the exposure to Al_2O_3 NPs.

Microtox test is conducted on a desktop analyser that measures the light emission of the microorganisms in contact with the samples, under automatically controlled temperature and test conditions. *Vibrio fischeri* is sensitive to a large number of toxic compounds and pollutants. These luminescent bacteria produce visible light as a result of their normal metabolic processes. Luciferase catalyzes the reaction of light energy production from chemical energy, linked to the respiratory metabolism of the microorganism. Toxic samples produce the inhibition of the metabolism of the bacteria which results in a reduction of bioluminescence. This change in light emission is directly proportional to the toxicity of the sample. We considered that Microtox test alone would not be conclusive because of the presence of very small particles, which causes turbidity of suspensions and, consequently, false-positive results. BOD5 test is an electrochemical-based technique and it is not influenced by the particle size, thus giving reliable results regarding toxicity.

Comparison of toxic effect on bacteria of both alumina phases, alpha- and gamma- alumina, were perfomed by Pakrashi et al. (2011, 2014); their results showed the higher toxicity of gamma phase. In addition, Jiang et al. (2009) have found that the mortality rate due to Al_2O_3 NPs was 57% to *B. subtilis*, 36% to *E. coli*, and 70% to *P. fluorescens*. Our results of BOD5 and Microtox test are consistent with these toxic responses of freshwater bacteria to gamma-alumina NPs. As

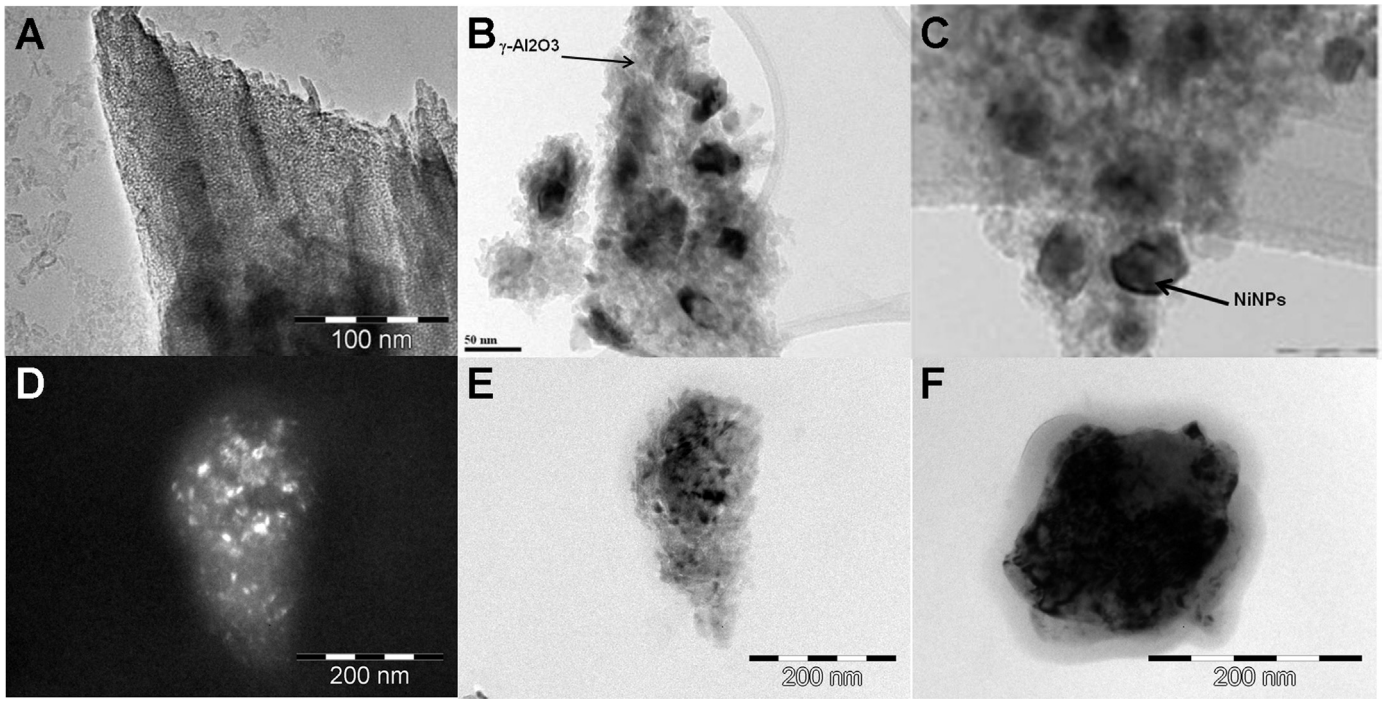

Fig. 2. TEM images of: A) γ- Al_2O_3 (NC); B and C) NiNC samples prepared by dispersing the powder in deionized water by ultrasonication stirring. NiO particles are inside γ- Al_2O_3 matrix, with particle sizes below 50 nm and 10 nm for NC and NiNC respectively; D, E and F) NiNC-GlcGlt post biodegradation samples with NiNC defined as bright points (D), as dark spots (E) and image of NiNC agglomerates (F) in transmission mode.

Table 3
Mean values (n = 3) of EC50-Microtox, biodegradation, Z-potential and particle size results of suspensions. EC50 values in bold indicate NC-GlcGlt and NiNC-GlcGlt performed with microorganisms associated with BOD5 tests.

Samples	Concentration (ppm)	EC50 (%v/v)	Biodegradation (%) (uncertainty)	Z –pot (mV)	Particle size (nm)
GlcGlt	–	–	52.9 (5.7)		
NC		29.9		43.7	297.5
NiNC		16.1		41.1	249.2
NC-GlcGlt	32.20	37.8 **100**	31.6 (3.7)	16.9	Bimodal 104.9 (33%); 282.7 (67%)
NiNC-GlcGlt	21.14	19.4 **84**	43.8 (1.0)	−0.2	350.8

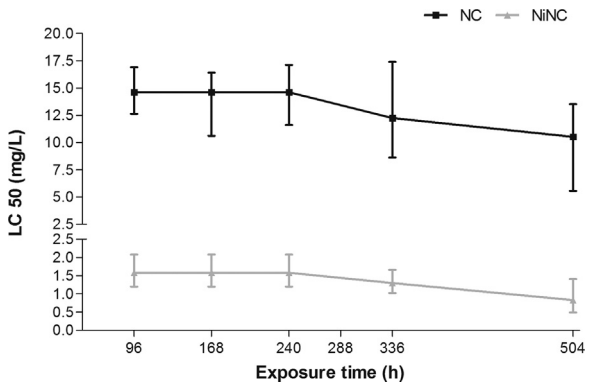

Fig. 3. Lethal Concentration (LC50) profiles of NC and NiNC materials for *Rhinella arenarum* larvae at different exposure times.

bacterial responses to NPs might be different from those obtained from their bulk counterparts, NP toxicity mechanisms need to be studied thoroughly.

The toxicity results of bacterial assays to NPs alumina, NC and NiNC, were also confirmed for *R. arenarum* larvae. Nickel is a dietary requirement to some animals, although it is toxic at high concentrations. Its excess in coastal environment can have significant and detrimental effects for example on the reproduction of copepods (Eisler, 1998; Mohammed et al., 2010).

Particle size and Z-potential of samples (Table 2) showed agglomeration of the nanomaterial with OM, as observed in TEM images (Fig. 2). After 24 h, the particle size of NiNC suspension was about 1300 nm, whereas for NC sample only 370 nm was obtained. These values of particle size indicated that NiNC agglomerated much faster than NC particles, i.e. a higher agglomeration rate of Ni-containing NCs. Although Microtox test was conducted within two hours of the suspension preparation, an agglomeration effect could be expected. Particulate matter in the sample can interfere with bioluminescence by absorbing light and yield misleading results in Microtox tests; the fast agglomeration and further flocculation of the NiNC could give place to an overestimation of the actual toxicity of this material, among other possible effects. Aggregation and flocculation effects in alumina NPs due to interaction with bacterial cells have been already reported (Jiang et al., 2009; Pakrashi et al., 2011). Biodegradability of GlcGlt solution (52.9%) was reduced when adding NCs (31.6%) and NiNC (43.8%). These results would indicate a higher toxicity of the NC as compared to NiNC, due to a toxic effect of the inoculum or a reduction of the bioavailability, in relation to the NMs added. On the contrary, Microtox assay EC50 from NC and NiNC were 29.9% and 16.1% v/v, respectively (Table 3), thus indicating a toxic effect which was higher for NiNC. In addition, the samples after BOD5 tests showed in Microtox assay an important increase in 1 h-EC50 values; 100% for NC-GlcGlt sample and 84% for NiNC-GlcGlt sample, the latter being the most toxic. Microtox test is sensitive to light interferences caused by the presence of particles and particle agglomeration while BOD5 results are influenced by both toxicity and bioavailability of NCs. The higher biodegradability value

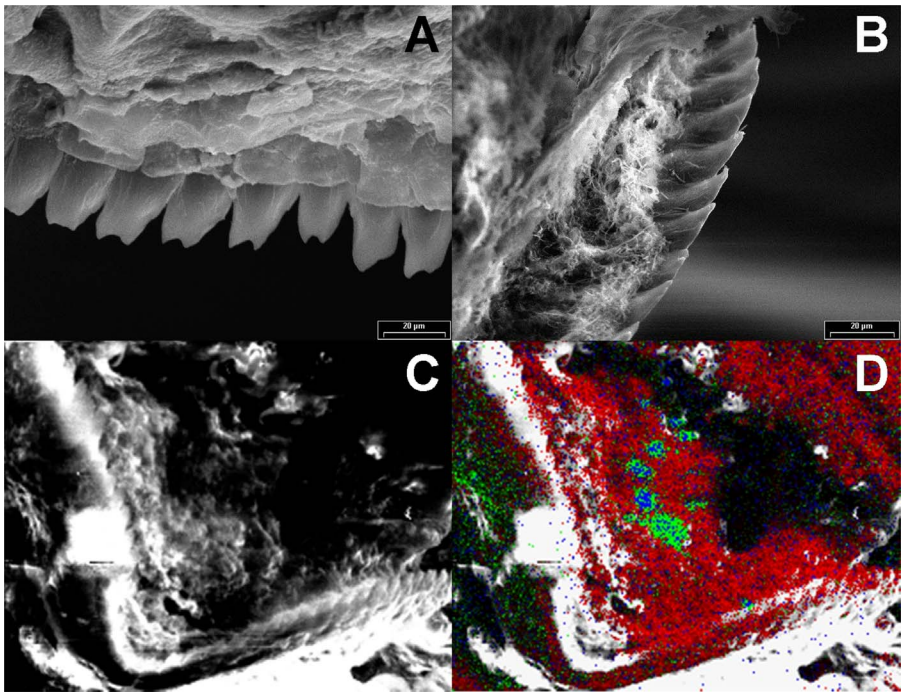

Fig. 4. SEM-EDS micrographs of the keratinized jaw sheath and labial teeth in the oral area of *Rhinella arenarum* larvae: A) control; B-D) exposed to NiNC. EDS shows Al in green and Ni in blue in NiNC exposed larvae (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

obtained in the case of NiNC could be mainly due to the lower bioavailability of these NMs than NC; higher toxicity of NiNC, as Microtox results indicated. Toxicity overestimation due to the presence of agglomerates in Microtox test, already discussed, is not expected according to the particle size values determined for both NC-GlcGlt - and NiNC- GlcGlt samples.

The toxicological risk of the gamma-alumina NPs has been attributed to their inherent chemical and structural characteristics (Brunner et al., 2006). The results of the solubility of suspensions and the NM structure assessed in this work did not show important differences between NC and NiNC (Table 3). On the other hand, it was found that biodegradation values were higher in samples containing Ni. The presence of Ni in the matrix of NMs could produce changes in chemical composition, which influence the toxicological response during the tests, as reported by Pakrashi et al. (2014). Also the much lower porosity in the NiNC (22%) than in NC (56%) could be responsible of the worse bio-response of the latter; bacteria can be immobilized, trapped in pores, which is more likely in particles with a higher porosity.

Taking into account the LC50 values of *R. arenarum* larvae, NC could be classified as harmful to aquatic organisms and NiNC, as toxic to aquatic organisms according to the grid applied for the potential ecotoxicological hazard evaluation by Sanderson et al. (2003) and Blaise et al. (2008). This classification is based on the mean LC50 value for the most sensitive aquatic organisms. Thus, values < 0.1 mg/L are extremely toxic; 0.1–1 mg/L = very toxic; 1–10 mg/L = toxic; 10–100 mg/L = harmful; > 100 mg/L = non-toxic.

Nickel as bulk component exhibited a slightly higher toxicity than NiNC to *R. arenarum* larvae as reported by Sztrum et al. (2011). In that study, the LC50s for *R. arenarum* larvae exposed to solutions of $NiCl_2$ at different exposure times were determined, thus obtaining 1.14 (96 h), 0.60 (168 h), and 0.48 (240 h) mg Ni^{2+}/L. In the present work, LC50–96 h was 1.58 mg NiNC/L, then remaining constant up to 240 h, these results indicate a different behavior of NPs besides the chemical nature of the material. In $NiCl_2$ solution, the longer the exposures, the lower the LC50 concentrations; on the contrary, NiNC effect proved independent of exposure time, thus indicating a rapid stabilization. This result is consistent with the solubility behavior of NiNC (Table 2), a rapid dissolution followed by a stable state with no further dissolution.

It was shown that these NMs formed aggregates that exceeded the nanoscale range (> 100 nm) as determined by the particle size and Z-potential results. This aggregation would modify the NP-toxicity lowering the bioavailability of these materials. Ispas et al. (2009) observed that spherical Ni-NPs of different diameter (30 nm, 60 nm, 100 nm) resulted in similar levels of toxicity to zebrafish embryos. However, exposure to dendritic clusters consisting of aggregates of 60 nm particles resulted in higher toxicity, thus suggesting that differences in shape and aggregation could be responsible for the increased toxicity of Ni NPs as synthesis and composition are similar. Therefore, aggregation capacity comes into sight as an important factor to be considered when assessing NMs toxicity. In case of Ni-NP toxicity, Ni is known as a promoter of free radical reactions, which adds a high surface reactivity to the NMs, besides its agglomeration capacity.

Presently, there is no information about levels of these NPs in the environment, only freshwater levels of bulk Ni in the range of $10^{-3} - 10^{-2}$ mg/L have been determined in undisturbed areas (Eisler, 1998; WHO, 1992). In this work, the NOEC values for lethality after 504 h exposure were determined as 5.02 mg/L for NC and 0.05 mg/L for NiNC; the 504 h-NOEC value for sublethal effects was lower than 0.005 mg/L. Sublethal toxicity was mainly evident by behavioral alterations of larvae as hyperkinesia followed by reduced swimming movements; also essential functions as feeding were deeply affected. These developmental disorders can make amphibians more vulnerable to predation and other environmental stressors such as infectious agents and invasive species (Egea-Serrano et al., 2012) that may represent an indirect effect on the species conservation. In addition, changes in physical and chemical parameters of the environment can influence the physical condition of the animals and/or their reproduction. Our results showed that environmental bulk nickel concentrations exceed the level of the sublethal 504 h-NOEC, therefore the viability of *R. arenarum* larvae is compromised by the presence of NPs, which would represent a threat for this amphibian species.

The results of this study demonstrate NMs can be harmful to aquatic life although toxicity differs significantly among the species used in the bioassays. The evidence presented here of the vulnerability of wildlife to NPs indicates the need to develop specific standardized ecotoxicity tests to reveal it.

5. Conclusions

Toxic effects of NMs would not be related to structural characteristics but to chemical properties. NiNCs also have a higher tendency to agglomerate as compared to gamma-alumina particles, which makes them more stable in water and therefore more toxic to aquatic organisms. Agglomeration and flocculation of NMs can give place to misleading results of the toxic effect of the material especially in methods which use photoelectric sensors and photocell as Microtox test.

Toxic effects of both gamma-alumina and Ni/gamma-alumina nanoporous materials have been demonstrated for bacterial communities and also for *R. arenarum* larvae. NPs containing Ni were more toxic to these aquatic organisms.

Further studies are needed in order to determine NP resistant and sensitive bacteria and the potential cellular damage of NC. In addition, the results of *R. arenarum* alert on the importance of considering NPs freshwater pollution, and also the need to develop specific standardized toxicity tests.

Conflict of interest

The authors declare that there are no conflicts of interest and affirm that this paper consists of original and unpublished work.

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