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Assessment of polyaniline nanoparticles toxicity and teratogenicity in aquatic environment using *Rhinella arenarum* model

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ABSTRACT

With the rapid growth of nanotechnology and the applications of nanoparticles, environmental exposure to these particles is increasing. However, their impact in human and environmental health is not well studied. Anurans, with life stage comprising embryos, tadpoles and adults, have an extremely permeable skin which makes them excellent indicators of environmental health. This study evaluated the acute toxicity effects of polyaniline nanoparticles (PANI-Np) in different dispersant on embryos and larvae of *Rhinella arenarum*. The results showed that LC_{50} of PANI-Np dispersed in polyvinylpyrrolidone (PVP) were 1500 mg/L, while LC_{50} by PANI-Np dispersed in PVP+PNIPAM (polyN-isopropylacrilamide) showed a highest toxicity (1170 mg/L). The embryo teratogenicity increased with increasing exposure concentration in both kinds of PANI-Np although in PANI-Np1, there is an increased teratogenic effect associated with the polymer stabilizer PVP.

1. Introduction

Polyaniline (PANI) is one of the most promising electrically conducting polymers because of its chemical stability and relatively high conductivity (MacDiarmid and Epstein, 1994, 1995). Recently, special interest has developed in the area of nanostructured conducting polymers (nanoparticles, nanotubes, nanowires and nanofibers) since they combine the advantages of organic conductors with low-dimensional systems, and therefore create interesting physicochemical properties and potentially useful applications (Gangopadhyay, 2004; Chiou and Epstein, 2005; Chiou et al., 2007; Lange et al., 2008). The ability to build and control engineered polymeric materials at the nanometer (nm) scale is important for current and future development of materials for a wide range of applications, from drug delivery to electronic applications. Due to various physico-chemical properties of nanomaterials that differ from those of bulk materials, new nanoscale materials may present toxic effects on living organisms. Surface area and chemical properties influence material interactions with biological systems. This increased biological activity can be either positive and desirable (e.g., antioxidant activity, carrier capacity for therapeutics, penetration of cellular barriers for drug delivery), negative and undesirable (e.g., toxicity, induction of oxidative stress or of cellular dysfunction), or a mix of both. Also, these nanomaterials may have increased toxicological effects due to their reduced size and increased surface area.

Though PANI is a conducting polymer with wide potential application in biotechnology and medicine, data on its biocompatibility are scarce (Bidez et al., 2006; Wang et al., 2008; Liu et al., 2010). This is possibly due to the fact that PANI has often been regarded with caution because the monomer (aniline) and reaction intermediates (aniline dimers and oligomers) are aromatic amines that can be physiologically active or even harmful.

The most widely recognized threat is the carcinogenic effect of benzidine, the aniline dimer (Humpoliceka et al., 2012). Moreover, PANI alone, being insoluble in aqueous media, can hardly be cytotoxic, but the possible impurities after polimeratization like low-molecular-weight reaction by-products may cause toxicity issues.

Polyaniline is only soluble in few solvents (N-methylpyrrolidone, sulfuric or formic acid). To overcome this problem and therefore expand the performance of the polymer in biological applications, the preparation of stable dispersion of polymer nanoparticles is possible which can be coated with different

Abbreviations: PANI-Nps, polyaniline nanoparticles; NOEC, no observed effects concentration; LC_{50}, median lethal concentration; LC_{99}, ninety-nine lethal concentration; PANI, polyaniline; RS, ringer's solution; PVP, polyvinylpyrrolidone; PNIPAM, polyN-isopropylacrilamide; PBS, phosphate buffered saline; TC_{50}, median teratogenic concentration; TC_{99}, ninety-nine teratogenic concentrations; NMP, pyrrolidone; TI, Teratogenic Index; TiSiO4-Np, titanium silicate nanoparticles

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polymers. Stabilization with biocompatible polymers such as PVP (poly(vinylpyrrolidone), PNIPAM (N-isopropylacrilamide) or HCP (hydroxypropylcellulose) allows use of the nanoparticle dispersions in biological applications. Additionally, an extended coil form of the polymeric stabilizer is necessary to avoid aggregation and precipitation of the polyaniline nanoparticles (PANI-Np). During this process either to achieve the polymerization of aniline onto solid substrates for technological applications or the synthesis of colloidal particles in aqueous media, water is contaminated with PANI-Np. Accordingly, there is a strong possibility that these nanoparticles will ultimately enter into aquatic ecosystems through wastewater discharges, and wash off during the industrial production.

One of the more promising applications of PANI-Np is in the field of nanomedicine, even more in the next generation of cancer treatments. In our laboratory, the effectiveness of these nanoparticles was demonstrated in the implementation of a new therapy that combines light and conductive nanomaterials, called photothermal therapy (Ibarra et al., 2013). In addition, there is a potential application of this nanoparticles to dissolve protein aggregates of neurodegenerative diseases (e.g. Alzheimer) (Bongiovanni et al., 2014) and in the field of cell imaging (Choi et al., 2014). The synthesis of this type of polymer nanoconjugated are increased and hence, could be potential candidates to be applied for medical applications (Zhou et al., 2013; Stejskal et al., 2014).

The increasing number of cytotoxicology studies of polyaniline nanostructures reflect the importance of the toxicology data on this kind of materials. However, only in vitro biocompatibility tests were used to assess these types of nanomaterials (Humpoliceka et al., 2012; Khan et al., 2012; Villalba et al., 2012; Li. et al., 2014). While cytotoxicity studies are essential for biomedical applications, since both the fabrication of the nanoparticles and its medical application are likely to involve release of nanoparticles, there is an urgent need for the information about their potential health and environmental effects of nanoparticles. In actuality many *in vitro* and *in vivo* biological models for studying the interactions of nanomaterials in biological systems have been reported (Cheng et al., 2009; Fako and Furgeson, 2009; Manabe et al., 2011). *In vitro* studies, which are based on cell culture, have the drawback that they provide an incomplete assessment of the interactions with the whole organism (Rashidi et al., 2014, Laha et al., 2014). *In vivo* systems address the overall effect on the physiology and anatomy of the organism, and thus constitute a more immediately relevant platform for translational clinical studies (Roy et al., 2014; Mohammad et al., 2014; Naksuriya et al., 2014). *In vivo* models evaluating the environmental impact of nanomaterials have been used, focusing mainly on aquatic organisms representing main food-web levels (bacteria, algae, crustaceans, ciliates and fish) (Lapresta-Fernández et al., 2012; Faria et al., 2014; Baumann et al., 2014). An extensively used laboratory model for toxicological and ecotoxicological studies is *Rhinella arenarum* (Herkovits et al., 2002), a species widely distributed in South America (Frost, 2009). Among amphibians, anurans have a biphasic life cycle, with aquatic embryos and tadpoles and generally terrestrial adults. Anurans have an extremely permeable skin, making them excellent indicators of the health of the environment. It is widely accepted that amphibian embryos, because of their high susceptibility to physicochemical agents, have been successfully used for hazard assessment of a large number of single physicochemical agents, as well as of complex mixtures (Herkovits and Perez-Coll, 1993, 1999; Ankley et al., 2002). By means of AMPHITOX, a standardized test customized for acute and early life stages toxicity assessment, the hazard of either a single agent or complex mixtures can be reported according to the more convenient end point in each case (Herkovits and Perez-Coll, 2001, 2003; Herkovits et al., 2002). The aim of this study was to evaluate the toxicity of PANI nanoparticles stabilized with different polymeric stabilizers such as PVP (poly(vinylpyrrolidone) and PNIPAM (N-isopropylacrilamide) *in vivo* toxicological models that employed embryonic and larval stages of *R. arenarum*.

2. Materials and methods

2.1. Polyaniline nanoparticle dispersions and characterization

PANI-Np were synthesized by dispersion polymerization in the presence of polymeric stabilizers. The method of synthesis was performed according to Stejskal and Sapurina (2005). To achieve greater stability in aqueous media and prevent precipitation of the nanoparticles, different polymers such as polyvinylpyrrolidone (PVP), Poly N-isopropylacrilamide (PNIPAM) were used in different percentages w/v at the moment of synthesis: PANI-Np dispersed in PVP 2% only (PANI-Np1) and PANI-Np dispersed in a combination of PVP 1%+1% PNIPAM (PANI-Np2). These polymers were adsorbed onto the nanoparticles to create the polymer stabilization effect (Joanny et al., 1979) and were chosen due to their biocompatibility (Hayama et al., 2004; Zhang et al., 2013; Kavanagh et al. 2005; Xu et al., 2004). The nanoparticles are present as aqueous dispersion, stabilized by hydrophilic polymers. Therefore, we studied the toxicity of these dispersions. To determinate PANI-Np concentration, a calibration curve was obtained with stock solution. To this end, 4 aliquots of 500 μl of the stock PANI-Np solution were dehydrated, to reduce pressure in a vacuum oven in a pre-weighed recipient. The mass of the obtained solid residue was calculated by difference and this was used to estimate the average concentration (mg/L) of PANI-Np in the stock solution. A calibration curve was constructed as follows. Different volumes of PANI-Np stock solution were diluted into 5 ml of buffer (sodium acetate/acidic acid, 0.1 M). The absorption of the resulting dilutions was measured at 636 nm and the absorbances were plotted as a function of the PANI-Np concentration. The calibration curve showed a lineal relationship with a slope of $\sim 2.99\ cm^3\ mg^{-1}\ cm^{-1}$. The same procedure was performed for the other PANI-Np dispersion and the calibration curve also showed a lineal relationship with a slope of $\sim 1.31\ L/mg^{-1}$. Samples of PANI-Np1 and PANI-Np2 stock solution were suspended in distilled water, sonicated for 1 min and vortexed. Aliquots were immediately pipetted and deposited onto a graphite-coated SEM grid, and finally dried for analysis by a scanning electron microscope (SEM, Carl Zeiss Evo10MA). For transmission electron microscopy (JEOL JEM-1010) studies, samples of both PANI-Np were placed onto a Formvar-covered copper grid and evaporated slowly and additionally, dynamic light scattering and zeta potential (Malvern Instruments, Zetasizer Nano S-90) measurement were performed using PANI-Np stock solution and PANI-Np dispersed onto PBS and Ringer solutions and water respectively.

2.2. The amphibian species

R. arenarum, previously known as *Bufo arenarum,* is widely distributed in Argentina, Uruguay, Bolivia and the south of Brazil (Frost et al., 2006). Many species included in the genus *Bufo* have been accommodated in other genera because of a series of large scale taxonomic changes in amphibian systematic recently proposed by several authors (Frost, 2007). The adult anurans used here were maintained in aquaria with tap-water at 20 ± 2 °C, alternating 12 h light/dark cycles and were fed with a homogenate of bovine liver three times a week.

2.3. Obtaining R. arenarum embryos and tadpoles

R. arenarum embryos and tadpoles were obtained from *in vitro* fertilized eggs by using the method described by Casco et al. (1992). In order to obtain *R. arenarum* embryos, adult females and males weighing approximately 200–250 g were collected in Río Cuarto (Córdoba Province, Argentina).

Ovulation of the females was induced by intraperitoneal injection of a suspension containing one female homologous hypophysis (Riede et al., 1998; Ferrari et al., 2005) and 300 IU of Human Chorionic Gonadotrophin (Endocorion5000, ELEA) (Mann and Bidwell, 2000) in 8 mL of 10% Ringer's solution (RS). The composition of Ringer's solution at pH 7.5 is NaCl 0.66 g, KCl 0.015 g, $CaCl_2$ 0.015 g, $NaHCO_3$ 0.03 g and distilled water Csp. 100 cc. This ovulation-combined procedure was performed in order to optimize female's ovulation. Oocyte strings were then collected from the ovisac and hydrated in RS. Oocytes were fertilized in vitro with a sperm suspension made by mincing testes in 10% RS. After fertilization, the embryos obtained were maintained in RS at 20 ± 2 °C until they reached the adequate development stage to perform the teratogenic test (S.2–S.4 embryonic stages) or toxicity test (S.25 larval stage with complete opercula. Developing embryos were staged according to the procedure described by Del Conte and Sirlin (1951, 1952).

2.4. Acute toxicity analysis

Bioassays were carried out with *R. arenarum* larvae following the AMPHITOX test conditions (Herkovits et al., 2002). Both PANI-Np stock solutions were diluted in phosphate buffered saline (PBS $1\times$) and Ringer's solution to achieve final concentrations that were used with the larvae. Ten larvae with closed opercula, which is the last stage of embryonic development (S.25), were placed in triplicate in 10 cm diameter glass Petri dishes containing 15 mL of PANI-Np dispersions. The concentrations tested for PANI-Np1 were 182, 530, 830, 980, 1240, 1395 and 1550 mg/L; while for PANI-Np2 were, 500, 750, 1000, 1250 and 1500 mg/L. In all cases, PANI-Np concentrations were determined by comparison with a calibration curve obtained with PANI-Np stock solution in water. Four different control solutions were used: (a) Ringer solution, which is recommended for AMPHITOX test conditions, (b) PBS, which was used to assess the effect of the media used to prepare PANI-Np dispersions, (c) 2% PVP in PBS, which was used to assess the effect of the stabilizer used to prepare one kind of PANI-Np1 and (d) 1% PVP plus 1% PNIPAM in PBS to assess the effect of polymers used to prepare PANI-Np2. For this acute toxicity analysis, larvae at S.25 of development were placed in the different PANI-Np concentrations or control solutions for 96 h, and the mortality of the tadpoles was checked every 24 h. Those larvae exhibiting no reaction towards gentle prodding were considered dead. The number of dead tadpoles was registered. The solutions were replaced once a day and dead larvae were removed to avoid altering the tested solutions. Experiments with larvae (S.25) were performed independently four times.

2.5. Teratogenic assay

For the early life stages test, embryos at 2–4 blastomeric stages (S.2–S.4) were used. Jelly coats were dissolved by treatment with 2% thioglycolic acid solution at pH 7, during 2 min, followed by egg washing with RS. 10 Embryos were placed in triplicate in 6 cm diameter glass Petri dishes containing 10 mL of PANI-Np1 dispersions (153, 182, 530 and 1240 mg/L) or PANI-Np2 dispersions (125, 250, 500 and 1000 mg/L) for 96 h. The control solutions used were the same as described in the previous experiment (Ringer solution, PBS, 2% PVP and 1% PVP+1% PNIPAM). Embryos were maintained at 20 ± 2 °C and the solutions were renewed once a day. Dead organisms were removed from test solutions to avoid quality alterations of solutions.

Survival and sublethal effects were evaluated every 24 h. Abnormalities were identified according to the "Atlas of Abnormalities" (Bantle et al., 1998). The primary endpoints included mortality, malformations, and growth inhibition. To assess the abnormalities the embryos were observed using a digital optical microscope (Motic DM39). Dead embryos were removed and survival in these embryonic stages was evaluated every day. Experiments with embryos (S.2–S.4) were performed independently four times.

2.6. Excretion

Three batches of ten larvae (S.25) were placed into 10 cm diameter glass Petri dishes containing 15 mL of RS with fish food (control) or 15 mL of PANI-Np at a concentration of 530 mg/L during 24 h. Then, all the larvae were gently washed with RS. Finally, the stool of the tadpoles was observed under the microscope. The stool samples were recollected and dissolved in N-Methyl-2-pyrrolidone (NMP), due to the major solubility of PANI in this solvent and finally to determine the UV–visible spectra of both stool control and treated with both PANI-Np dispersions.

On the other hand, to determine the fate of PANI nanoparticles in the larvae gut, three batches of 10 larvae at stage 25 were treated by quadruplicate, with a concentration of PANI-Np1 or PANI-Np2 of 500 mg/L during 96 h. The larvae were placed in 10 cm diameter glass Petri dishes containing 15 mL of tested solutions. Larvae were removed from each set at 96 h, each group of larvae was washed with 50 mL RS, dried with filter paper, and homogenized in 100 μL of N-methyl-2-pyrrolidone (NMP), where PANI is soluble. Homogenized samples were diluted to 1 mL with NMP, and contents of both nanoparticles (PANI-Np1 and PANI-Np2) were quantified using UV–visible spectroscopy. UV–visible spectra measured from 800 to 200 nm were obtained on a Shimadzu UV–vis 1601PC spectrometer using a quartz cell of 1 cm path length.

2.7. Statistical analysis

All the data were analyzed using one way analysis of variance (ANOVA) and Duncan post hoc test, in all cases $p < 0.05$ denoted significance. Based on mortality data obtained through acute toxicity analysis, we calculated the concentration to kill the 50% of exposed larvae (LC_{50}), the concentration to kill the 99% of exposed larvae (LC_{99}) and no observed lethal effects concentration (NOEC). Based on malformation data obtained over a range of dose levels, we calculated the concentration to induce malformations in 50% of exposed embryos (TC_{50}), the concentration to induce malformations in 99% of exposed embryos (TC_{99}) and no observed effects concentration (NOEC). These values were estimated by means of EPA Probit Analysis Program (US EPA, 1998; Verwey, 1947; Paisio et al., 2009) according to the American Society for Testing and Materials (ASTM) (ASTM E729-96, 2007). The results were reported as values for median lethal concentration (LC_{50}), ninety-nine lethal concentration (LC_{99}), median teratogenic concentration (TC_{50}), ninety-nine teratogenic concentrations (TC_{99}), and no observed effects concentration (NOEC), after 96 h of exposure. The Teratogenic Index (TI), which is useful for estimating the teratogenic risk associated with the tested compounds, was the LC_{50}/TC_{50} ratio (Dawson and Bantle, 1987).

3. Results and discussion

3.1. Characterization

The size distribution and the morphological characterization for PANI-Np1 were estimated by TEM, SEM, (Fig. 1A and B), zeta potential and DLS (Table S1). SEM images showed spherical structure and homogeneous size distribution with an average size about 200 nm and the same result was observed trough DLS (224 nm) with a polydispersity index (PdI) of 0.145 maintained for several measurements along months. This result suggests the good stability of the polymer conjugated nanoparticles regarding aggregation/agglomeration parameters and an acceptable primary size too. The distribution of size and the morphology for PANI-Np2 were analyzed by TEM, SEM, (Fig. 1C and D), zeta potential and DLS (Table S1). The SEM images showed a homogeneous size distribution with an average size $\bar{X} = 93.870\ \text{nm} \pm SD = 25.253$ calculated through ImageJ software analysis. The sizes of the nanoparticles by TEM were as similar as had been estimated by SEM. Moreover, the appearance of such nanoparticles did not present a regular spherical shape rather than PANI-Np1. However, the hydrodynamic diameter obtained by DLS showed an average size $\bar{X} = 300\ \text{nm}$ with a PdI of 0.082. The greatest difference in size could be attributed to the lack of the hydration layer of nanoparticles resulting of the assembly procedure for analysis by SEM or TEM, which interfere with the interaction of the polymer stabilizer and the solvent molecules (water). Taking into account the importance of the hydrodynamic diameter, many studies suggest that it is an important parameter for understanding and optimizing the nanoparticle performance in biological assays as well as understanding the *in vitro* migration of the particles. The zeta potential in the aqueous medium (water) of different nanoparticles had values by PANI-Np1 and PANI-Np2 of −7.58 mV and −6.07 mV s, respectively. Neutral surface charges were found for both PANI-Np and this characteristic could be attributed to the presence of PVP, which is a neutral polymer (Shannigrahi and Bagchi, 2005).

On the other hand, nanoparticle dispersions were stable in aqua mediums (water, PBS and RS) for several months and when started to agglomerate and sediment, sonication procedure restored the dispersibility of the nanoparticles.

3.2. Acute toxicity (lethal effects on S.25 larval stage)

The objective of this research was to determine the environmental hazard associated with PANI-Np in terms of acute toxicity in *R. arenarum*. Due to the increasing use of nanomaterials, the evaluation of potential adverse effects on humans and the environment is a matter of particular importance.

To our knowledge, *R. arenarum* have not been used to characterize the toxic effects of nanoparticles in the aquatic medium. This organism is widely distributed in our region (Río Cuarto, Cordoba Province, Argentina). Therefore, it is easily available for the tests without affecting biodiversity. On the other hand, the

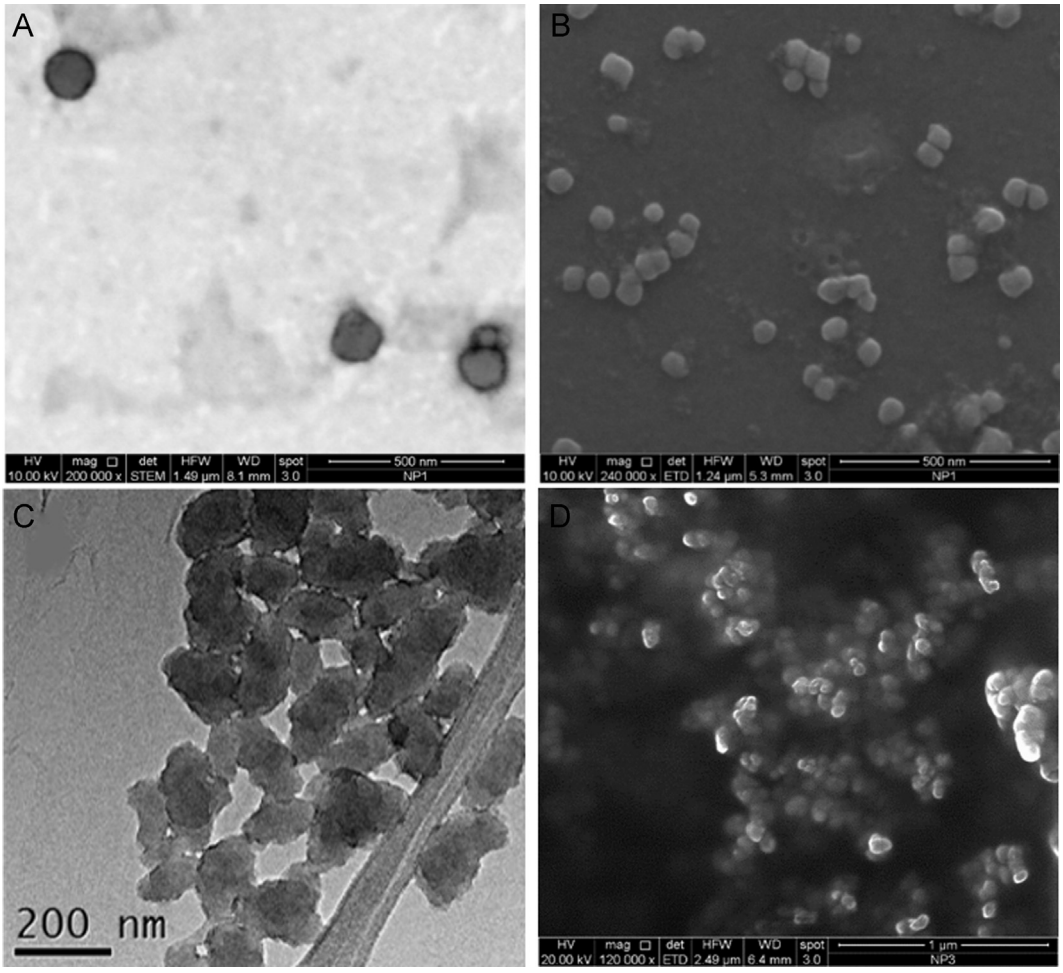

Fig. 1. (A) TEM photograph of PANI-Np1, (B) SEM photograph of PANI-Np1, (C) TEM photograph of PANI-Np2 and (D) SEM photograph of PANI-Np2.

presence of the species makes the evaluation of possible harmful effects of nanoparticles relevant for the regional environment.

The susceptibility of *R. arenarum* larvae to PANI-Np during the 25th stage of development was evaluated by exposing the larvae to different PANI-Np concentrations for 96 h and then assessing percentage of survival (Fig. 2). This test is a standardized test employing amphibian larvae that can be used to evaluate acute toxicity. The larval survival was not affected by PANI-Np1 at concentrations of 182–1240 mg/L (Fig. 2A). The results showed that there was no statistically significant difference in the percentage of viability relative to control. At concentration of 1395 mg/L was observed a statistically significant decrease in the survival reaching a value of 70% ± 0.12. Consistent with this, at higher concentration of PANI-Np1 (1550 mg/L), the survival rate decreased to 16.66% ± 1.2. Furthermore, PVP, the polymer used as dispersant in the synthesis of PANI-Np1, did not decrease the viability at all concentration tested. On the other hand, no statistically significant lethality was registered between control groups; however, a statistically significant lethality was registered between the control groups and the larvae treated with PANI-Np1. In summary, these results indicated that there is a tolerance to PANI-Np1 exposure in larval stage S.25 at concentration < 1240 mg PANI-Np/L. The lethal concentrations were also studied by Probit analysis (Table S2).

Moreover, when toxicity of PANI-Np2 was assessed; significant differences were recorded compared to PANI-Np1. A starting lethal effect for these nanoparticles was determinate at a lower concentration than PANI-Np1. This lethal effect was first observed at 750 mg/L (Fig. 2B). The toxicity from both kinds of nanoparticle dispersions was dose dependent and almost twice higher for PANI-Np1 (lethal effect starting at concentrations ≥ 1395 mg/L) compared with PANI-Np2 (lethal effect starting at concentrations ≥ 750 mg/L). Additionally, no lethal effect of the combination of polymers used as dispersants in the synthesis PANI-Np2 was found assuming its innocuousness. According Probit both LC_{50} and non-toxic concentrations (NOEC) were obtained at values lower for PANI-Np2 compared to PANI-Np1 (Table S2). Hence, we could demonstrate a dependent toxic effect on the size of PANI-Np.

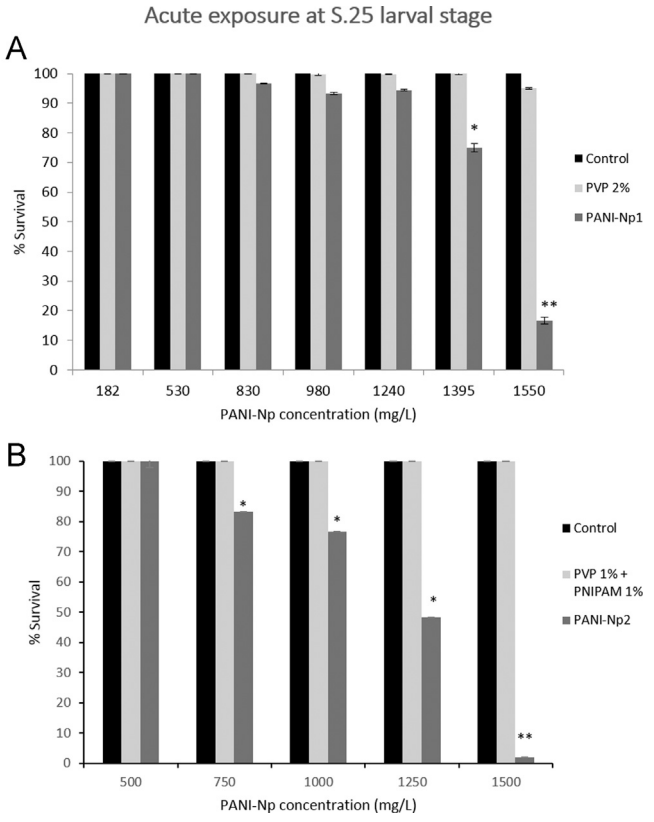

Fig. 2. Percentage survival of *Rhinella arenarum* larvae following a 96 h exposure period with different concentrations of (A) PANI-Np1 and (B) PANI-Np2 compared to controls (PBS and Ringer solutions) (mean ± SEM) $*p \leq 0.05$ with ANOVA test.

Considering LC_{50} of both PANI-Np dispersions, PANI-Np1 and PANI-Np2 could be classified as non-toxic to aquatic organisms according to the grid applied by Sanderson et al. (2003) and Blaise et al. (2008) for the potential ecotoxicological hazard evaluation. Classification is based on median LC_{50} value of the most sensitive organism used; < 0.1 mg/L = extremely toxic to aquatic organisms; 0.1–1 mg/L = very toxic to aquatic organisms; 1–10 mg/L = toxic to aquatic organisms; 10–100 mg/L = harmful to aquatic organisms; > 100 mg/L = non-toxic to aquatic organisms. Salvaterra et al. (2013) evaluated lethal effect of a short-term exposure to titanium silicate nanoparticles (TiSiO4-Np), which did not cause a significant mortality in *Pelophylaxperezi* tadpoles at a concentration of 20 mg/L. Hence, these nanomaterials (TiSiO4-Np) were considered harmful to aquatic organism according to the grid previously mentioned (Kahru and Dubourguier, 2010). Various ecotoxicological tests have been previously developed as good biomarkers for aquatic organisms to evaluate the lethality and sublethal effects caused by nanomaterials (King Heiden et al., 2007; Wiench et al., 2009; Nelson et al., 2010; Nations et al., 2011).

It is well-known that nanosize range may lead to increased toxicity due to increased specific surface area (Nel et al., 2006). Studies with some fine and ultra-fine (< 100 nm) particles have exhibited more toxicity than larger particles of the same chemical composition (Borm, 2002). Our results are in agreement with a previous study using another PANI nanomaterial with different shape, due to fact that both PANI-Np1 and PANI-Np2 were up to 6.56 and 3.94 fold more toxic, respectively than PANI-Nanofibers in larvae of *R. arenarum* based on data from NOEC of these nanomaterials.

3.3. Teratogenic assay (early life stage test-developmental effects)

The teratogenic assay of PANI-Np1 and PANI-Np2 to *R. arenarum* embryos was carried out by observing the embryotoxic effects at continuous exposure from early blastula (S.2–S.4) onwards during a 96 h period. The susceptibility of embryos continuously exposed from early blastulae was evaluated by exposing them to different concentrations of PANI-Np1 and PANI-Np2. Sublethal effects were expressed as morphological abnormalities, which involve teratogenesis and reduced body size. During the test, the embryo teratogenic rate increased with increasing exposure concentration in both kinds of dispersion of PANI-Np (Fig. 3A and B).

Concerning PANI-Np1, no statistically significant differences were observed about sublethal effects between the different concentrations of PANI-Np1 and its polymer dispersant (PVP 2% in PBS in different proportion according PANI-Np tested solutions) (Fig. 3A). These results suggest a sublethal effect from both tested solutions and therefore this effect can be attributed to the dispersant of PANI-Np1. For this nanoparticle dispersion, a lethal effect was observed in embryonic stages. The lethal effect of PANI-Np1 on the exposed embryos increased gradually as the concentrations increased (Fig. 4). Early larvae in continuous treatment were more resistant than blastula during the acute period. In this case, the LC_{50} indicates that PANI-Np1 concentration necessary to cause lethal effect is higher for amphibian larvae than embryos. The percentages of mortality (lethal effects) as well as the percentages of malformations (sublethal effects) on early stage embryos were also investigated by Probit analysis at the end of the test. For PANI-Np1, sublethal concentrations were NOEC 26.9 mg/L, TC_{50} of 299.5 mg/L, TC_{99} 3327.6 mg/L. The TI_{50} of 5 was derived. On

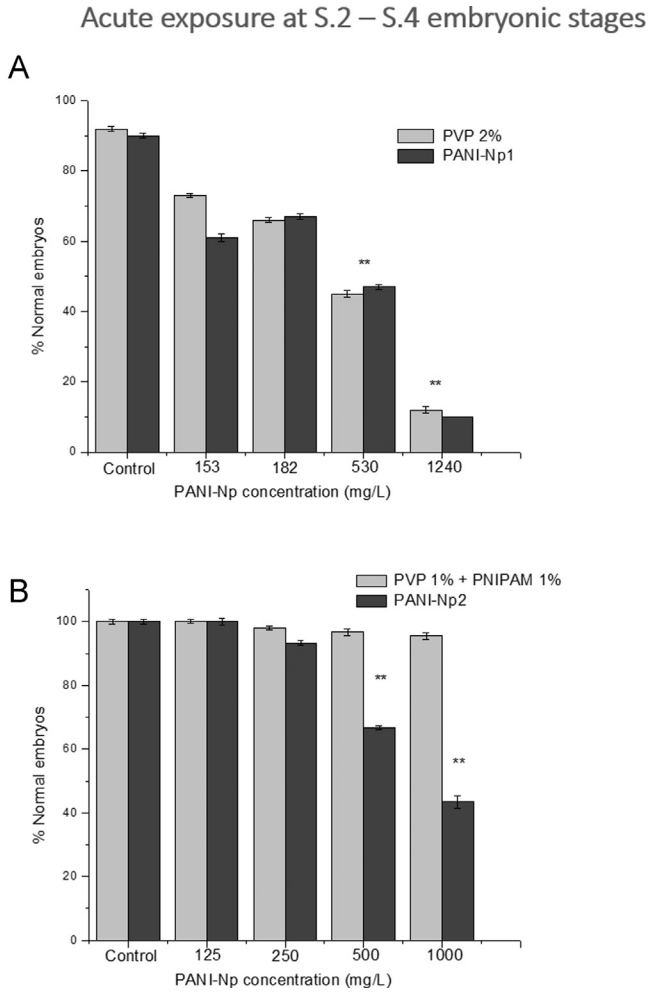

Fig. 3. Percentage of morphologically normal embryos following a 96 h exposure period with different concentrations of (A) PANI-Np1 and (B) PANI-Np2 compared to controls (PBS and Ringer solutions) (mean ± SEM) $*p \leq 0.05$ with ANOVA test.

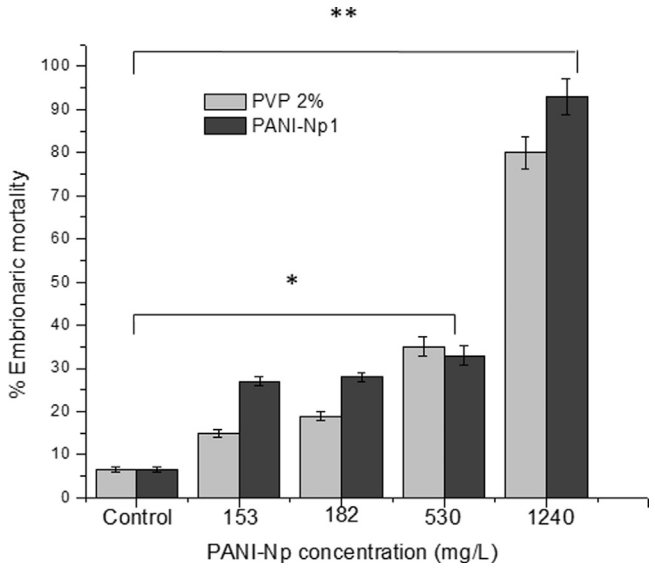

Fig. 4. Percentage of mortality of embryos following a 96 h exposure period with different concentrations of PANI-Np1 or dispersant (PVP) compared to control (mean ± SEM) $*p \leq 0.05$ with ANOVA test.

the other hand, for PVP sublethal concentrations were NOEC 44.7 mg/L, TC_{50} 426.6 mg/L, TC_{99} 4073.6 mg/L.

On the other hand, regarding PANI-Np2, the sublethal effect of these nanoparticles were less severe that PANI-Np1; the combination of two dispersants did not produce sublethal effects in any concentration tested and neither lethal effect. This result suggests that the two polymers stabilizers, (a lower percentage of PVP 1% plus the addition of a new polymer PNIPAM 1%) decrease and eliminate the additional teratogenic effect of polymer stabilizer of PANI-Np1. This is the first report that describe the effect toxic of PVP in *R. arenarum*.

However, malformations product from the PANI-Np2 exposure were observed starting at concentration of 500 mg/L (Fig. 3B). According to the Probit analysis, the sublethal concentrations for PANI-Np2 were NOEC 142.8 mg/L, TC_{50} of 868.3 mg/L, TC_{99} 5279.1 mg/L. The TI_{50} calculated was 1.35. All the estimate sublethal concentrations from PANI-Np2 were higher than concentrations calculated for PANI-Np1 (Table S3). For instance, at a concentration of 500 mg/L, about 30% of embryos exposed to PANI-Np2 had malformations, while sublethal effects of PANI-Np1 were higher, reaching 50% of the embryos. The Teratogenic Index (TI) is a measure of hazard of a determined xenobiotic on the early development of a species; if it is higher than 1.5 signifies a greater potential to obtain all embryos malformed in absence of significant mortality (Bantle and Sabourin, 1991). The Teratogenic Index (LC50/TC50) for PANI-Np2 was 1.35 while for PANI-Np1 was 5.01, which indicates a great potential to exert malformation in all embryos in the absence of significant embryo mortality for this last PANI nanoparticle dispersion. According to the sublethal results, PANI-Np1 had more teratogenic effects and this effect could be attributed to the toxicity added by PVP 2% used as stabilizer of these nanoparticles. It should be noted that while the TI decreased with PANI-Np2 to a minor significant value (TI = 1.35), the teratogenic effect remains and it is due to exposure to nanoparticles.

To evaluate the abnormal development of embryos in the different groups (control ringer; control PBS; dispersant PVP; dispersant PVP + PNIPAM; PANI-Np1 and PANI-Np2), embryos at different stages: early blastula (S.2–S.4), gastrula (S.10–S.12), rotation (S.15) were morphologically examined daily, and abnormalities were recorded (Fig. 5).

In this study, the most common development abnormalities found in embryos treated with dispersant of PANI-Np1 were abnormal axis (Fig. 5C) and edema (Fig. 5D) at rotation (S.15). However, in embryos treated with PANI-Np1, predominance of detent growth in late gastrula stage (S.12) (Fig. 5E), underdevelopment at rotation (S.15) (Fig. 5F) and, to a lesser extent, edema (Fig. 5G) were observed. In most cases malformations observed with this treatment led to the death of embryos. Contrarily, in the control embryos the presence of malformations was not observed (Fig. 5A and B). The predominant type of malformation recorded in embryos treated with PANI-Np2 was the underdevelopment at rotation (S.15) (Fig. 5H) and to a lesser extent, edema (Fig. 5I). These finding were in accordance with malformations observed in embryos treated with PANI-Np1. Although, the detent growth in late gastrula stage (S.12) was not observed with the PANI-Np2, malformation that led to the death of the embryos. A stage-dependent susceptibility were featured in this study, as reported by Yslas et al. (2012) for PANI-nanofibers. Early stage embryos are more sensitive than more mature embryos. Similar findings were recorded by Hutler Wolkowicz et al. (2013).

3.4. Excretion

The elimination of labeled PANI-Nps was evaluated in toad larvae by analysis of its stool when the larvae were feed with fish food or 500 mg/L dispersion of PANI-Np. The UV–visible spectra of

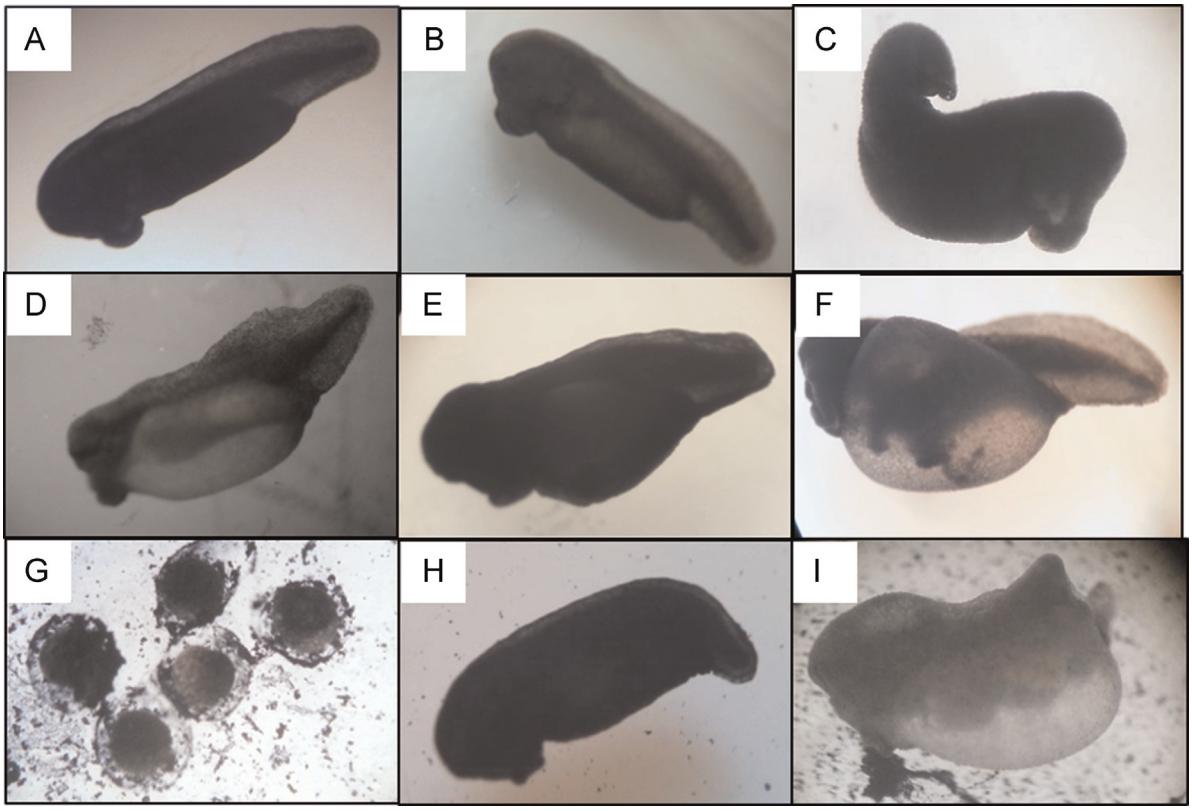

Fig. 5. Photographic recording of malformations detected in embryos (A) Control embryo in Ringer and (B) PBS solutions (magnification 4 ×), (C and D) PVP-treated embryo (4 ×); (E, F and G) PANI-Np1 treated embryo (4 ×) and (H and I) PANI-Np2 treated embryo.

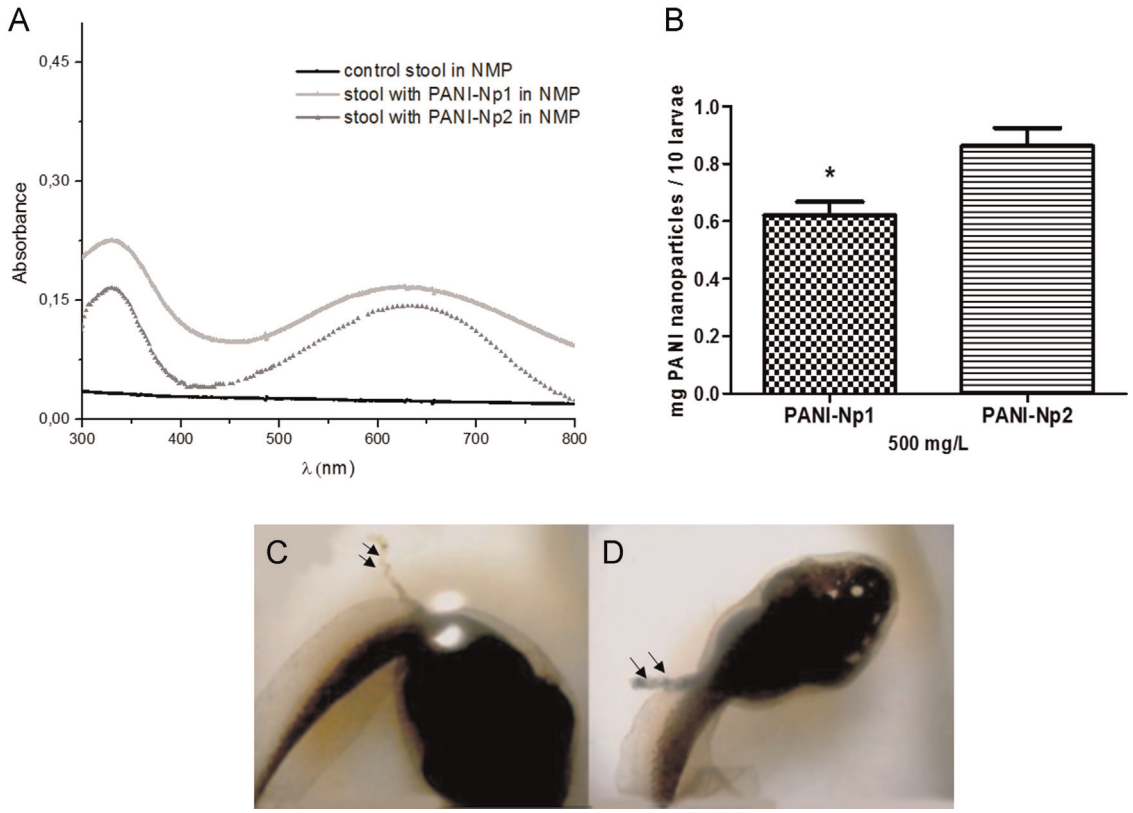

Fig. 6. (A) The UV–visible spectra of stool samples dissolved in NMP, (B) gut contents of PANI-Np in premetamorphosis (S.25) larvae of *Rhinella arenarum* exposed to 500 mg/L of both PANI-Np after 96 h. The data represent the mean ± SEM. Asterisk indicate significant differences between PANI-Np1 and PANI-Np2 exposure. $*p < 0.05$, (C) optical micrographs of *Rhinella arenarum* larvae grown with food fish and (D) with PANI-Np1 as the only carbon source. Arrows show control stool and stool with PANI-Np, respectively.

stool samples dissolved in NMP correspond to PANI. PANI nanoparticles could clearly be detected in all observed stool of exposed animals, while the control samples show no absorption (Fig. 6A). The results agree with the visual observation of color changes of the stool by microscopy (Fig. 6C and D).

The determinations of PANI-Np1 and PANI-Np2 accumulation in larvae with a concentration of 500 mg/L indicate that, when S.25 larvae were incubated with PANI-Np1 the amount of retained was $\bar{X} = 0.621$ mg = 10 larvae ± SEM = 0.046 while for PANI-Np2 the amount of retained was $\bar{X} = 0.862$ mg= 10 larvae ± SEM = 0.062 . Based on the results it is possible to suggest that gut contents in larvae increased when the size of nanoparticles is small (Fig. 6B). The mayor toxicity of this PANI-Np2 perhaps it could be attributed to the minor size of the nanoparticles and therefore tend to accumulate more in organisms (Lanone and Boczkowski, 2006; Kreyling, and et al., 2006; Chen et al., 2006). Besides the fact that it is clear, that exposure of larvae to the combination of dispersants of PANI-Np2 caused no lethal effect on any of the concentrations tested.

4. Conclusions

Larval stage survival of *R. arenarum* was not affected by concentrations of 182–1240 mg PANI-Np1/L, while PANI-Np2 dispersed in PVP plus PNIPAM showed toxic effect at concentration lower (nearly half of concentration). Through AMPHITOX, the lethal concentrations of both PANI-Nps dispersions on larvae of *R. arenarum* were obtained at concentrations as high, so PANI-Np can considered as nontoxic nanoparticles to this aquatic organisms but with a significant teratogenic potential founded with one nanoparticle dispersion. The dispersant PVP at 2% resulted possess teratogenic effects, which disappeared when the combination of PVP at 1% plus PNIPAM 1% was used as dispersant agent in the synthesis of PANI-Np. Kato and Nagao (2009) have reported that PVP solution exhibited a potentially hazardous effect on embryos and clearly suppresses the development of the embryo. Moreover, it was possible to determine a size-dependent toxicity between the two type of PANI nanoparticles. This *in vivo* model (AMPHITOX) might serve as the basis for determining the toxicity of other nanomaterials so this popular alternative organisms can be extensively used as models in nanotoxicology.

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Appendix A. Suplementary Information

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