Chemosphere 87 (2012) 1374–1380

Contents lists available at SciVerse ScienceDirect

Chemosphere

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

Polyaniline nanofibers: Acute toxicity and teratogenic effect on *Rhinella arenarum* embryos

Edith I. Yslas [a,b,*], Luis E. Ibarra [a], Damián O. Peralta [b], César A. Barbero [b], Viviana A. Rivarola [a], Mabel L. Bertuzzi [a]

[a] Departamento de Biología Molecular, Universidad Nacional de Río Cuarto, Agencia Postal Nro 3, X580BYA Río Cuarto, Argentina
[b] Departamento de Química, Universidad Nacional de Río Cuarto, Agencia Postal Nro 3, X580BYA Río Cuarto, Argentina

ARTICLE INFO

Article history:
Received 19 September 2011
Received in revised form 6 February 2012
Accepted 8 February 2012
Available online 3 March 2012

Keywords:
Nanomaterials
Nanofibers
Ecotoxicology
Toxicity test
AMPHITOX

ABSTRACT

The fate and effect of nanomaterials in the environment is of paramount importance towards the technological application of the materials. This work shows the ecotoxicological potential of polyaniline (PANI) nanofibers in the larvae *Rhinella arenarum* by means of AMPHITOX test. Acute toxicity of PANI nanofibers towards embryos of the common South American toad *R. arenarum* (Anura: bufonidae) was evaluated in the premetamorphosis (stage 25) larvae. The exposure of *R. arenarum* larvae to at dose of 150, 250 and 400 mg L^{-1} resulted in 100% viability within 96 h exposure. The embryos at 2–4 blastomers stage (early life stage teratogenic test) revealed that embryos were not killed and no teratogenic effects were observed when embryos were incubated with PANI nanofibers (150 and 250 mg L^{-1}), while only a growth retardation of embryos was induced at levels of 250 mg PANI nanofibers L^{-1}. On the other hand, at 400 mg L^{-1} concentration, a reduction in the body length of larvae and tail malformation was observed. This results suggest that a concentration-dependent toxicity is operative, typified by phenotypes that had abnormal body axes. The presence of PANI nanofibers in gut contents and its excretion by larval stages of *R. arenarum* was confirmed by UV–visible spectroscopy.

1. Introduction

Research in the field of nanomaterials (defined as substances or structures which exhibit at least one dimension of smaller than 100 nm) has lead to the discovery that at this scale, dramatically different properties may be present with respect to bulk materials (Daniel and Astruc, 2004; Curtis et al., 2006). Therefore, it is possible for materials innocuous in macroscopic form, to be harmful in nanometric size. Accordingly, in the last few years, the number of research studies on the toxicity of different types of nanomaterials has increased dramatically (Nel et al., 2006; Fischer and Chan, 2007). Since research on new applications of nanomaterials is increasing it seems necessary to evaluate their environmental and health impacts before actual industrial production begins.

Nanotubes or nanofibers of conducting polymers have attracted interest because of their novel properties and wide potential application in nanoscale engineering. Among the family of conducting polymers, polyaniline (PANI) has been one of the most widely studied due to its simple synthesis, easily varied physical, chemical and electronic properties, and good environmental stability (Morales et al., 2006; Bhadra et al., 2009). PANI has shown biocompatibility in several biological applications (Tahir et al., 2005; Kim et al., 2009). Recently, PANI nanofibers have been developed to be used in technological applications (Tseng et al., 2005). Polyaniline nanofibers have been used in sensors (Huang et al., 2004; Virji et al., 2006; Sadek et al., 2007), electronic devices (Tseng et al., 2007; Yen-Fu et al., 2011), charge storage (Hyder et al., 2011) and catalysis (Gallon et al., 2007). Both the synthesis and the deposition of polyaniline nanofibers, to produce the devices, are made in aqueous media. Therefore, water contaminated with polyaniline nanofibers will be a waste from those processes which will end up in aquatic bodies. Unlike bulk polyaniline, polyaniline nanofibers are dispersible in water and conventional filters of water treatment plants are likely to be ineffective to retain them, due to its small size. Therefore, polyaniline nanofibers are potential hazards to aquatic organisms and its toxicity should be studied.

Abbreviations: PANI, polyaniline; TC_{50}, median teratogenic concentration; TC_{99}, ninety-nine teratogenic concentration; NOEC, no observed effects concentration; PVP, polyvinyl pyrrolidone; RS, Ringer's solution; PBS, phosphate buffer solution; FTRI, fourier transform infrared; DLS, dynamic light scattering; TEM, transmission electron microscopy; NMP, N-methyl-2-pyrrolidone.

* Corresponding author at: Departamento de Biología Molecular, Universidad Nacional de Río Cuarto, Agencia Postal Nro 3, X580BYA Río Cuarto, Argentina. Tel.: +54 0358 4676437; fax: +54 0358 4676232.
E-mail addresses: eyslas@exa.unrc.edu.ar (E.I. Yslas), vrivarola@exa.unrc.edu.ar (V.A. Rivarola).

0045-6535/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2012.02.033

However, in the best of our knowledge the possible health effects of PANI nanofibers have not studied. While that in that sense, polypyrrole nanoparticles have been studied recently and shown to be limited cytotoxic (Kim et al., 2011).

Amphibians are being increasingly used for toxicity screening purposes due to their high sensitivity to physicochemical stress (Blaustein et al., 1994) and useful indicators of freshwater contamination (Kamalesh et al., 2000; Bidez et al., 2006). Amphibians are particularly sensitive to numerous environmental contaminants during their embryonic stages due to their position in the food chain, the permeability of their skin to toxic substances, and the vulnerability that metamorphosis causes in larval development (Vitt et al., 1990). Moreover, some studies suggest that amphibians can be more sensitive, than other vertebrate species when exposed to aquatic contaminants (Hall and Henry, 1992). Much of the amphibian toxicological literature describes studies using representatives of the genera *Rana, Rhinella and Xenopus* (Mann and Bidwell, 1999). Embryos of *Rhinella arenarum*, a South American toad, due to their high sensitivity to environmental pollutants (Herkovits and Perez-Coll, 1990, 1993; Hall and Henry, 1992), could be very useful for ecotoxicological studies and are widely used in our country to perform various bioassays (Herkovits and Pérez-Coll, 1999, 2001). Moreover, young tadpoles of *R. arenarum* constitute a very useful tool for toxicity studies, because these organisms available throughout the year, genetically homogeneous and easy to handle in the laboratory (Ferrari et al., 2005).

Embryo development and early life stages, provides information that might be useful in estimating the chronic toxicity of a test material to aquatic organisms. Thanks to its three endpoints (embryo mortality, malformation, and growth inhibition), the amphibian test can detect the xenobiotics that affect embryonic development a weak link in the life circle of an organism (Prati et al., 2000). AMPHITOX is a set of customized toxicity test for acute, short term chronic, chronic and early life stages of amphibian embryos of *R. arenarum* (Herkovits and Pérez-Coll, 2001; Stockert and Herkovits, 2003) which allow to select the most appropriate exposure period and end points according to the toxicity of the sample and the purpose of the study (Pérez-Coll and Herkovits, 2004).

The main purpose of this study was to evaluate the susceptibility of embryos at 2–4 blastomeric stage (s2–s4) and larvae in premetamorphosis (stage 25) (AMPHITOX bioassay) to different concentrations of PANI nanofibers dispersed using polyvinyl pyrrolidone (PVP). PVP is used as polymeric stabilizer to maintain the nanofibers dispersed in neutral media (Riede et al., 1998). Malformations, mortality and growth inhibition exerted by PVP and PANI nanofibers were evaluated, to assess the toxic and genotoxic potential of PANI nanofiber. Furthermore, the fate of the nanofibers in the gut of the toad *R. arenarum* and its excretion in larval stages was also investigated.

The hazard identification base set is an evolving concept developed to characterize the inherent hazards related to nanomaterial exposures in ecological environments using aquatic species. Nanotoxicology is an interdisciplinary field in which scientists in material sciences and the biological and toxicological sciences need to cooperate in order to understand adverse nano-bio interactions. In essence, a fundamental understanding of nanomaterial toxicology is highly desirable both from the material's stand point as well as from the biological system's point of view (Oberdörster et al., 2005, 2007). A comprehensive material characterization is a critical requirement for each nanotoxicological study and will lead to a better understanding on how different nanomaterials properties affect their biological response (Warheit, 2008). Nanotechnology is growing at an exponential rate and will undoubtedly have both beneficial and toxicological impact and consequences on health and environment.

2. Materials and methods

2.1. Animals

Adult females and males *R. arenarum* were maintained in aquariums with tap-water at a 22 ± 2 °C, alternating 12 h light/dark cycles and fed with homogenate of liver three times a week. The species has been called previously: *Bufo arenarum*, the name that has been used in nearly most publications. 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).

2.2. Obtaining R. arenarum embryos

R. arenarum embryos were obtained from *in vitro* fertilized eggs by using the method described by Casco and co-workers (1992). In order to obtain *R. arenarum* (Hensel) 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 two female homologous hypophysis (Riede et al., 1998; Ferrari et al., 2005) with 300 IU of Human Chorionic Gonadotrophin (Endocorion 5000, ELEA) (Mann and Bidwell, 2000) in 8 mL of 10% Ringer's solution (RS). This combined procedure was performed by us 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 s2–s4 or toxicity test s25 (complete operculum) or by each experimental protocol. Developing embryos were staged according to the procedure described by Del Conte and Sirlin (1951, 1952).

2.3. Polyaniline nanofibers

Polyaniline nanofibers were synthesized by interfacial polymerization, using the method developed by Kaner and co-workers (Li et al., 2009). 24 mmoles of aniline were dissolved in chloroform (75 mL), while ammonium peroxydisulfate (6 mmoles) was dissolved in 75 mL of 0.8 M aqueous hydrochloric acid. The two solutions are then carefully transferred to a closed flask generating a liquid–liquid interface. The monomer diffuses into the aqueous phase, where it is oxidized and polymerizes. All the reactions are performed in the dark. After 24 h, the entire water phase is filled homogeneously with dark-green polyaniline fiberrs, while the organic layer (chloroform) appears red–orange due to the dissolution of byproducts. The aqueous phase is then collected and the remaining reactants and side products are removed by dialysis. To do that, the aqueous phase is dialyzed (using a cellulose membrane) by 4 d with deionized water and then 4 d with phosphate buffer solution (PBS) pH = 7.4. Then, PANI nanofibers were redispersed in acid media (pH < 2). In this media the fibers are protonated and the surface charge separates the individual nanofibers and stabilizes the colloid (Verwey, 1947). In neutral media, like in biological fluids, the nanofibers are deprotonated and agglomerate after some time. To avoid that, a water soluble polymer (poly(vinylpyrrolidone) (PVP)) is adsorbed onto the nanofibers to create the polymer stabilization effect (Joanny et al., 1979). The polymer was chosen due to its well known biocompatibility (Hayama et al., 2004). Polyaniline nanofibers require dispersion in fluid media to be deposited onto solid substrates for technological applications. Besides that, polyaniline nanofibers are produced in aqueous media. In both processes, water contaminated with polyaniline nanofibers is

produced. The nanofibers are present as aqueous dispersion, stabilized by hydrophilic polymers. Therefore, the toxicity of such kind of dispersions is studied.

The nanofibers were then characterized. The activity of the fibers to external pH changes was evaluated by UV–visible spectroscopy. The apparent size was measured using dynamic light scattering (DLS) and its morphology was evaluated using Transmission Electron Microcopy (TEM).

2.4. Acute toxicity analysis

Bioassays were conducted with *R. arenarum* embryos following the AMPHITOX test conditions (Herkovits and Pérez-Coll, 2001). PANI nanofibers dispersions were used prepared with PBS and dispersed with PVP (3%). Ten embryos with closed operculum, which is the last stage of embryonic development (s25), were placed in 6 cm diameter glass Petri dishes containing 10 mL of PANI nanofibers dispersion in concentrations of 150, 250, and 400 mg L^{-1} or control solutions (by triplicate in acute test). In all the cases, nanofibers amounts were determined by comparison with a calibration curve obtained with standard dispersions of PANI nanofibers. Three 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 nanofibers dispersions and (c) 3% PVP in PBS which was used to assess the effect of the stabilizer used to prepare PANI nanofibers dispersions, on the embryos. For this acute test, embryos at the 25th stage of development were placed in the different or control solutions for 96 h and the mortality of the tadpoles was checked every 24 h. Those exhibiting no reaction towards gentle prodding were considered dead. The number of dead tadpoles was registered at each stage. Dead larvae were removed and the solutions were replaced once per day. The experiments using larvae (s25) were repeated four times.

2.5. Teratogenic assay

For early life stage test, embryos at 2–4 blastomeric stage (s2–s4) were used. Jelly coats were dissolved by a treatment with 2% thioglycolic acid solution at pH 7, during 2 min, followed by eggs washing with RS. Batches of 10 embryos (by triplicate in teratogenic test) were placed in PANI nanofibers solutions (150, 250 and 400 mg L^{-1}) for 96 h. Three 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 nanofibers solutions and (c) 3% PVP in PBS which was used to assess the effect of the stabilizer used to prepare PANI nanofibers solutions, on the embryos. Embryos were maintained at 20 ± 2 °C and the solutions were renewed once a day.

Malformations and mortality were recorded each 24 h. Abnormalities were identified according to the "Atlas of Abnormalities". The primary endpoints include mortality, malformations, and growth inhibition. Based on malformation data obtained over a range of dose levels, the concentration to induce malformations in 50% of exposed embryos (TC_{50}), and the concentration to induce malformations in 99% of exposed embryos (TC99) and for no observed effects concentration (NOEC) were calculated. On the other hand, since growth inhibition is another indicator of toxicity during embryonic development, the growth was measured. At the end of the previous test, embryos were fixed in formaldehyde 3% and the head–tail length was measured. If the embryos are curved, the length is taken following the contour. To asses the abnormalities the embryos were observed using a digital optical microscope (Motic DM39). Dead embryos were removed and survival was evaluated every other day. The teratogenic tests were repeated four times.

2.6. Gut contents

To determine the fate of PANI nanofibers in the larvae gut, three batches of 10 larvae at stage 25, were treated by triplicate, with different concentrations of PANI nanofibers (150, 250 and 400 mg L^{-1}) during 96 h. The larvae were placed in 6 cm diameter glass petri dishes containing 10 mL of tested solutions. Experiments were repeated three times with embryos from different couples of parents. 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 PANI nanofibers contents 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. Excretion

Three batches of ten larvae (s25) were placed, in triplicate, into 6 cm diameter glass Petri dishes containing 10 mL of RS with or without PANI nanofibers (control). The larvae controls were fed during the experiment with fish food. Whereas the larvae treated were fed with 250 mg L^{-1} nanofibers during 24 h. After 24 h all the larvae, treated with or without PANI nanofibers, were gently washed with RS. Finally, the stool of the animals was observed under the microscope. The stool samples were recollected and dissolved in NMP to determine the UV–visible spectra of both stool control and treated with PANI nanofibers.

2.8. Statistical analysis

All the data were analyzed using two way analysis of variance ANOVA and Duncan post hoc test, in all cases $p < 0.05$ denoted significance. Malformations data for experiments were transformed by means of EPA Probit Analysis Program (US EPA, 1988; Paisio et al., 2009) according to the American Society for Testing and Materials (ASTM). The results were reported as values for median teratogenic concentration (TC_{50}), ninety-nine teratogenic concentrations (TC_{99}), and no observed effects concentration (NOEC), after 96 h of exposure.

3. Results

3.1. Nanofiber characterization and dispersion

The nanofibers showed FTIR and UV–visible spectra (not shown) in agreement with those previously reported for polyaniline (Huang and MacDiarmid, 1993; Werake et al., 2005). TEM images (Fig. 1) reveal that PANI nanofibers have ribbon like shapes with ca. 60 nm width and several micrometer of length. The dynamic light scattering measurements (Fig. 2) shows monomodal dispersion with an apparent (revolution sphere) size of ca. 600 nm. While this is not directly related to an actual dimension (length or diameter), it shows that stable dispersions of PANI nanofibers could be obtained.

3.2. Acute toxicity

The susceptibility of *R. arenarum* larvae to PANI nanofibers during the 25th stage of development was evaluated by exposing the embryos to different PANI nanofibers concentrations (150, 250 and

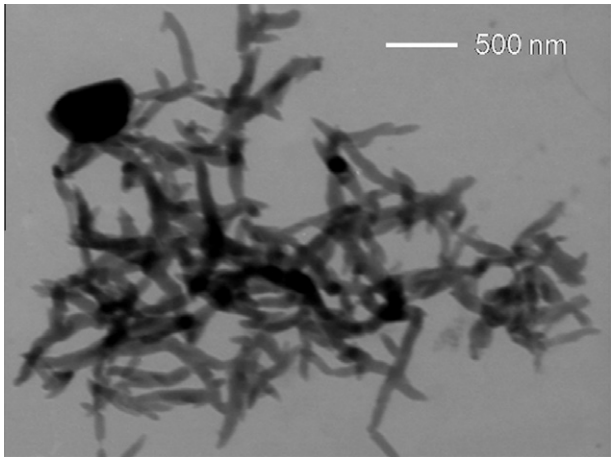

Fig. 1. TEM micrographs of PANI nanofibers.

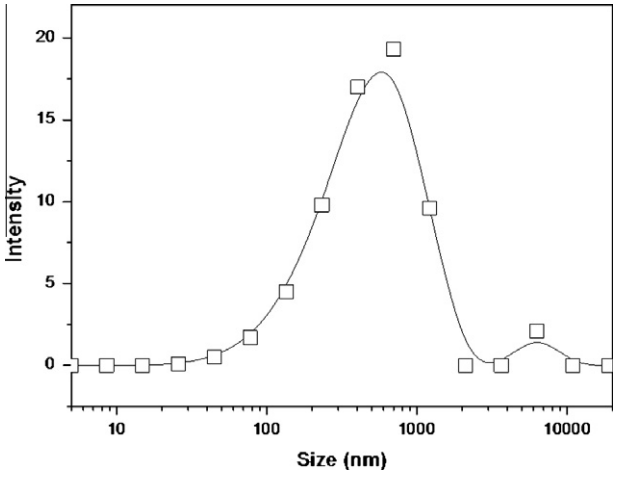

Fig. 2. Size distribution of dispersed PANI nanofibers obtained by dynamic light scattering (DLS).

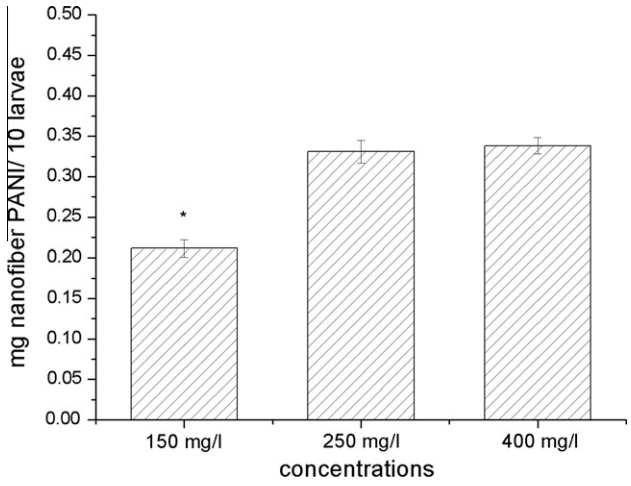

Fig. 3. Gut contents of PANI nanofibers in premetamorphosis (stage 25) larvae of *Rhinella arenarum* exposed to different nanofiber concentrations after 96 h. The data represent the mean ± SEM. Asterisk indicate significant differences between the 250 and 400 mg L^{-1} exposure. $^{*}p < 0.05$.

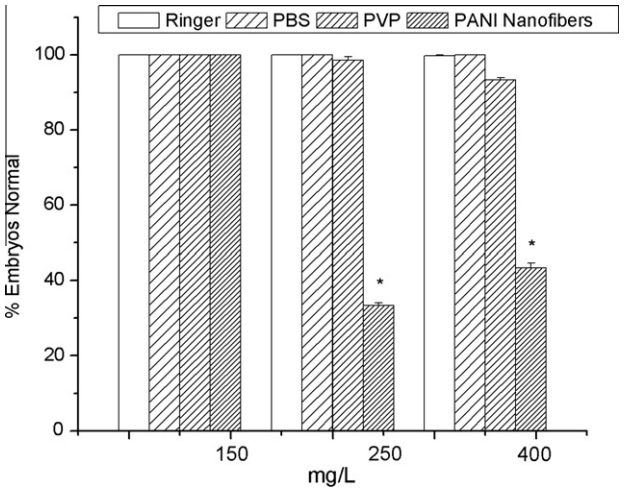

Fig. 4. Teratogenic effect produced by different concentration of PVP or PANI nanofibers on early life stage of *Rhinella arenarum* embryos. The data represent the mean ± SEM. Asterisk indicate significant differences between the control and PVP. $^{*}p < 0.05$.

400 mg L^{-1}) for 96 h and then assessing lethality. This test is a standardized test employing amphibian larvae that can be used to evaluate acute toxicity. The development and survival of the larvae were not significantly affected by PANI nanofibers concentrations ranging from 150 to 400 mg L^{-1}. The results show no lethality in different concentration of PANI nanofibers. The content of PANI nanofibers in the gut of larvae during the 25th stage of development was similar in value at concentrations of 250 mg L^{-1} and 400 mg L^{-1}, when the incubation time was 96 h (Fig. 3).

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

The teratogenic assay of PANI nanofibers to *R. arenarum* embryos was made by observing specific toxicological endpoints during the 96 h period. The developments of larvae were not significantly affected by PANI nanofibers or PVP in concentration below 150 mg L^{-1}. At concentrations of 250 mg L^{-1} of nanofibers seems to induce growth retardation of the larvae. The mean head–tail length of larvae in the control was $\bar{x} = 5.99$ mm± SEM 0.049 and in the treated sample with 250 mg PANI nanofibers L^{-1} was $\bar{x} = 5.59$ mm ± SEM 0.085. Therefore, there was a significant difference ($p = 0.00039$) between head–tail length of control and treated samples. At 400 mg L^{-1} the percentage of malformations (Fig. 4) was significantly higher than the ones for control ($p = 0.05$). PVP alone do not induce significant malformations in all studied concentrations (Fig. 5A). Fig. 5B and C showed the most common malformations occurred at concentration of 400 mg L^{-1} PANI such as incurvated body axis and underdeveloped gills, respectively.

The effect of PANI nanofibers on the exposed embryos increased gradually. At 150 mg L^{-1} no effect was observed, at 250 mg L^{-1} an induced inhibition of growth was found while at 400 mg L^{-1} high level of abnormalities were found. Accordingly the Probit analysis showed a TC_{50} value of 398.54 mg L^{-1}, teratogenic effect on all embryos (TC_{99}) at a concentration of 899.90 mg L^{-1} and a NOEC value of 176.50 mg L^{-1}. Also, at the 2–4 blastomeric stage (s2–s4), the susceptibility seems to be higher than at stage 25th, because no effect was found at the later growth stage.

3.4. Fate of PANI nanofibers in the gut

The UV–visible spectra (not shown) of PANI nanofibers extracted from larvae corresponds to that of PANI. Therefore, a calibration curve was used to evaluate the amount of PANI. The

Fig. 5. Photographic recording of malformations detected in embryos treated with 400 mg L^{-1} PANI nanofibers A – control without treatment B – embryo with incurvated body axis and C – embryo with underdeveloped gills.

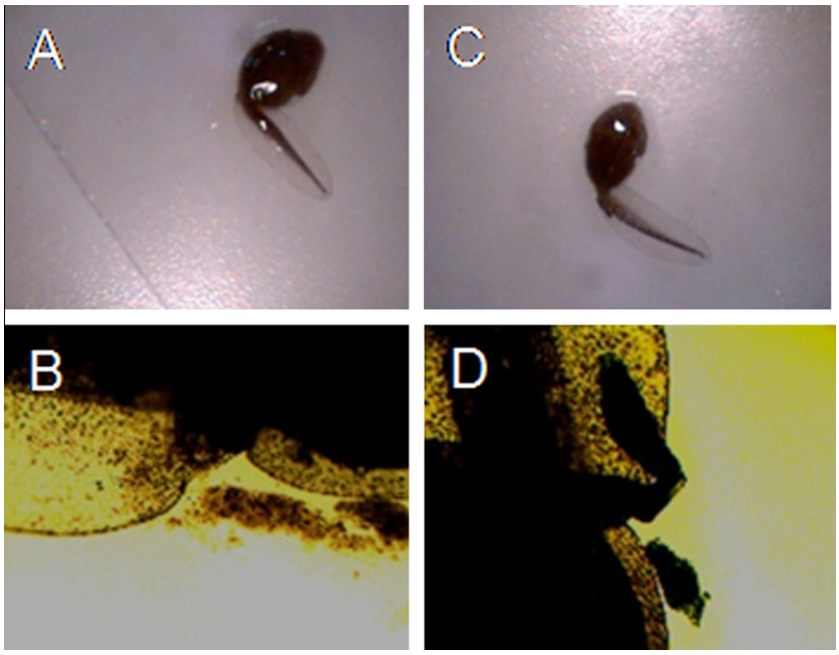

Fig. 6. Optical micrographs of *Rhinella arenarum* larvae grown: A and B with food fish; C and D with PANI nanofibers as the only carbon source.

determinations of PANI nanofibers accumulation in larvae with different concentrations indicate that, when were incubated with 150 mg L^{-1} the amount of retained PANI was $\bar{x} = 0.211$ mg/10 larvae ± SEM = 0.01 while that at 250 mg L^{-1} and 400 mg L^{-1} PANI nanofibers retention were $\bar{x} = 0.331$ mg/10 larvae ± SEM = 0.01 and $\bar{x} = 0.338$ mg/10 larvae ± SEM = 0.01, respectively. These last value of the gut contents concentration were significantly different ($p < 0.05$) that when the larvae were incubated with 150 mg L^{-1}. Based on this result it is possible to suggest that gut contents in larvae increased with PANI nanofibers concentration and tends to a saturation value when were incubated with a concentration of 250 mg PANI nanofibers L^{-1} (Fig. 3).

3.5. Excretion

On the other hand elimination of labeled PANI nanofibers was evaluated in toad larvae by analysis of its stool, when feed with fish food or with 250 mg L^{-1} dispersion of PANI nanofibers. The UV–visible spectra (see Supplementary Information) of stool samples dissolved in NMP correspond to that of PANI (Huang and MacDiarmid, 1993). PANI nanofibers could clearly be detected in all observed stool of exposed animals, while the control samples show no absorption. The results agree with the visual observation of color changes of the stool by microscopy (Fig. 6).

4. Discussion

Several organisms have been suggested as control populations in bioassays. Among them, amphibians such as *R. arenarum* which is a native South American species, showed to be suitable for toxicity evaluation of xenobiotics and environmental samples. Therefore, we evaluated lethal and teratogenic effects of PANI nanofibers on embryos and young tadpoles of this native species, under laboratory conditions.

The data showed no toxicologic effects of PANI nanofibers in *R. arenarum* larvae exposed at the 25th stage, even at the highest concentrations tested (400 mg PANI nanofibers L^{-1}). On the other hand, it is found that PANI nanofibers are slightly teratogenic to developing *R. arenarum* embryos at 400 mg PANI nanofibers L^{-1} and at 250 mg L^{-1} the nanofibers induced growth inhibition. A partir de esto se puede concluir que la Exposures embryo-larval stages of *R. arenarum* to PANI nanofibers revealed that the embryonic stage is more sensitive than larvae in premetamorphosis stage. In addition the incidence of malformations and the degree of adverse effects were concentration dependent. Probit analysis showed a TC_{50} value of 398.54 mg L^{-1}. Nanofibers killed all embryos at concentrations of 899.90 mg L^{-1} (TC_{99}) and the NOEC value is 176.50 mg L^{-1}. The teratogenic concentrations obtained by means of PROBIT analysis point out the proportionality of the response to the doses administrated.

Stage-dependent susceptibility between embryos and larval state features found in this study with *R. arenarum* were also reported in the case of other chemical (Herkovits et al., 1997). This susceptibility could be related to specific morphogenetic and cellular differentiation processes that occur at this early stage of development. A similar survey was reported by Nelson et al. (2010) that silica nanomaterial in the embryonic period was more sensitivity causing abnormalities and embryonic death. This manuscript suggests that the nanowires may interfere with the morphogenetic processes of gastrulation and/or neurulation. Poland et al. (2008) reported toxic effects of carbon nanotubes within the abdominal cavities of mice that were directly related to nanotube aspect ratio. It is possible that materials with large surface areas and elongated exhibit more toxicity that spherical materials (Grabinski et al., 2007). The data on toxicity of nanomaterials are contradictory and evidently require additional verification. the toxicity of nanomaterial is determined by the multitude of variablestheir physicochemical properties (particle size, roughness, shape, charge, composition and surface coating) or specific features of their manufacture and preparation of aqueous suspensions. Most likely, the toxic effects of nanomaterials can be associated not only with their size, but also of other parameters.

Our results suggest the toxicity of nanofibers to aquatic species is concentration dependent and early stage-dependent susceptibility. The use of acute test allows obtaining integrated information about mortality, malformations, and grown retardation of the tested organisms, which implies a vast knowledge of the toxicity of certain substances. In this sense, it is important to quantify toxicity at early embrionary stage because in most cases the concentrations of xenobiotics which can affect embryos are so different than those affect adult organisms. Before these novel materials can be safely applied in a clinical setting, their biocompatibility, biodistribution and biodegradation needs to be carefully assessed. Also, more work is needed to investigate the long-term fate and biocompatibility of such engineered nanomaterials or nano-systems. Present results suggest that embryotoxic potential of polyaniline nanofibers exists at concentrations above 176.50 mg L^{-1}. This result is relevant because nanofiber. has been used by significant applications in circuits and devices, displays, lighting, chemical, biological and environmental sensors, and scaffolds for tissue growth (Baughman, 1996; Novak et al., 1997; Frackowiak et al., 2006; Spinks et al., 2006; Mao et al., 2012). In all these application the nanofibers are made in aqueous media using different dispersants. This way, the nanofibers can reach the aquatic environment as conventional filters of water treatment plants are likely to be ineffective to retain them, are potential hazards to aquatic organisms. For this reason, due to the wide application of this nanomaterial technological devices these results provide new data to the literature that would help prevent aquatic toxicity prior to their massive industrial use.

5. Conclusion

The present work evaluates the ecotoxicity and teratogenic effect of PANI nanofibers in *R. arenarum* larvae and embryos in controlled laboratory conditions, according to different concentration after 96 h of exposure. In the current acute study, the highest dose of saturation of nanofiber retention in larvae (400 mg L^{-1}) did not show any adverse effect. On the other hand the treated embryos with PANI nanofibers in this concentration showed teratogenic effects and exhibited phenotypic defects as severe lateral flexure of the tail and underdeveloped gills. To our knowledge, this is the first study to describe the acute toxicity and teratogenic study of PANI nanofibers. To summarize the findings, the results of most of the studies demonstrated low hazard potential for *R. arenarum* following acute exposures to the PANI nanofibers.

Acknowledgments

Authors are grateful to Secretaría de Ciencia y Técnica (SECYT) of Universidad Nacional de Río Cuarto and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) for financial support. L. Ibarra and D. Peralta thanks SECYT and CONICET, respectively for research fellowships. V. Rivarola, C. Barbero and E.I. Yslas are members of CONICET.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2012.02.033.

References

American Society for Testing and Materials. Annual Book of ASTM Standard. Designation E729-80. Standard Practice for Conducting Acute Toxicity Test with Fishes, Macroinvertebrates, and Amphibians.

Baughman, R.H., 1996. Conducting polymer artificial muscles. Synth. Met. 78 (3), 339–353.

Bhadra, S., Khastgir, D., Singha, N.K., Lee, J.H., 2009. Progress in preparation, processing and applications of polyaniline. Progr. Polym. Sci. 34, 783–810.

Bidez, P.R., Li, S., MacDiarmid, A.G., Venancio, E.C., Wei, Y., Lelkes, P.I., 2006. Polyaniline, an electroactive polymer, supports adhesion and proliferation of cardiac myoblasts. J. Biomater. Sci. Polym. 17 (1–2), 199–213.

Blaustein, A.R., Wake, D.B., Sousa, W.P., 1994. Amphibian declines: judging stability, persistence and susceptibility of populations to local and global extinctions. Conserv. Biol. 8, 60–71.

Casco, V.H., Paz, D.A., Ruiz, G., Maldonado, C., Pisanó, A., Aoki, A., 1992. Differentiation of endocrine myocardiocytes in the developing heart of the toad (Bufo arenarum Hensel). Int. J. Dev. Biol. 36, 537–542.

Curtis, M., Greenberg, J., Kester, S., Phillips, G., Krieger, G., 2006. Nanotechnology and nanotoxicology: a primer for clinicians. Toxicol. Rev. 25, 245–260.

Daniel, M., Astruc, D., 2004. Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology. Chem. Rev. 104, 293–346.

Del Conte, E., Sirlin, J.L., 1952. Pattern series of the first embryonary stages in Bufo arenarum. Anat. Rec. 112, 125–130.

Del Conte, E., Sirlin, L., 1951. The first stages of Bufo arenarum development. Acta Zool. Lilloana 12, 495–499.

Ferrari, L., De la Torre, F., Demichelis, S., García, M., Salibián, A., 2005. Ecotoxicological assessment for receiving waters with the premetamorphic tadpoles acute assay. Chemosphere 59, 567–575.

Fischer, H.C., Chan, W.C.W., 2007. Nanotoxicity: the growing need for in vivo study. Curr. Opin. Biotechnol. 18, 565–571.

Frackowiak, E., Khomenko, V., Jurewicz, K., Lota, K., Béguin, F., 2006. Supercapacitors based on conducting polymers/nanotubes composites. J. Power Sources 153 (2), 413–418.

Frost, D.R., 2007. Amphibian species of the world: an online reference. Version 5.1. American Museum of Natural History, New York, USA. <http://research.amnh.org/herpetology/amphibia/index.php>.

Gallon, B.J., Kojima, R.W., Kaner, R.B., Diaconescu, P.L., 2007. Palladium nanoparticles supported on polyaniline nanofibers as a semi-heterogeneous catalyst in water. Angew. C Chem. Int. Ed. 46, 7251–7254.

Grabinski, C., Hussain, S., Lafdi, K., Braydich-Stolle, L., Schlager, J., 2007. Effect of particle dimension on biocompatibility of carbon nanomaterials. Carbon 45, 2828–2835.

Hall, R.J., Henry, P.F.P., 1992. Review: assessing effects of pesticides on amphibians and reptiles: status and needs. Herpetol. J. 2, 65–71.

Hayama, M., Yamamoto, K.-i., Kohori, F., Sakai, K., 2004. How polysulfone dialysis membranes containing polyvinylpyrrolidone achieve excellent biocompatibility? J. Membrane Sci. 234, 41–49.

Herkovits, J., Cardellini, P., Pavanati, C., Pérez-Coll, C.S., 1997. Susceptibility of early life stages of Xenopus laevis to cadmium. Environ. Toxicol. Chem. 16 (2), 312–316.

Herkovits, J., Perez-Coll, C.S., 1990. Amphibian embryos as bioassays to evaluate environmental pollutants and to reduce their toxic effects. In: Barceló, E., (Ed.), Proceedings of the Environmental Contamination 4th International Conference PANI Nanofibers, Barcelona, pp. 623–626.

Herkovits, J., Perez-Coll, C.S., 1993. Stage dependent susceptibility to cadmium on Bufo arenarum embryos. Bull. Environ. Contam. Toxicol. 50, 608–611.

Herkovits, J., Pérez-Coll, C.S., 1999. Bioensayos para test de toxicidad con embriones de anfibios (“AMPHITOX”). Ingeniería Sanitaria y Ambiental 42, 24–30.

Herkovits, J., Pérez-Coll, C.S., 2001. Ecotoxicological studies of environmental samples from Buenos Aires area using a standardized amphibian embryo toxicity test (AMPHITOX). Environ. Pollut. 116, 177–183.

Huang, J., Virji, S., Weiller, B.H., Kaner, R.B., 2004. Nanostructured polyaniline sensors. Chem. Eur. J. 10, 1315–1319.
Huang, W.S., MacDiarmid, A.G., 1993. Optical properties of polyaniline. Polymer 34 (9), 1833–1845.
Hyder, M.N., Lee, S.W., Cebeci, F.Ç., Schmidt, D.J., Shao-Horn, Y., Hammond, P.T., 2011. Layer-by-layer assembled polyaniline nanofiber/multiwall carbon nanotube thin film electrodes for high-power and high-energy storage applications. ACS Nano 5 (11), 8552–8561.
Joanny, J.F., Leibler, L., De Gennes, P.G., 1979. Effects of polymer solutions on colloid stability. J. Polym. Sci.: Pol. Phys. 17, 1073–1084.
Kamalesh, S., Tan, P., Wang, J., Lee, T., Kang, E.-T., Wang, C.-H., 2000. Biocompatibility of electroactive polymers in tissues. J. Biomed. Mater. Res. 52 (3), 467–478.
Kim, H., Hobbs, H.L., Wang, L., Rutten, M.J., Wamser, C.C., 2009. Biocompatible composites of polyaniline nanofibers and collagen. Synt. Metals 159, 1313–1318.
Kim, S., Oh, W.-K., Jeong, Y.S., Hong, J.-Y., Cho, B.-R., Hahn, J.-S., Jang, J., 2011. Cytotoxicity of, and innate immune response to, size-controlled polypyrrole nanoparticles in mammalian cells. Biomaterials 32, 2342–2350.
Li, D., Huang, J., Kaner, R.B., 2009. Polyaniline nanofibers: a unique polymer nanostructure for versatile applications. Acc. Chem. Res. 42 (1), 135–145.
Mann, R.M., Bidwell, J.R., 1999. The toxicity of glyphosate and several glyphosate formulations to four species of south-western Australian frogs. Arch. Environ. Contam. Toxicol. 36, 193–199.
Mann, R.M., Bidwell, J.R., 2000. Application of the FETAX protocol to assess the developmental Toxicity of nonylphenol ethoxylate to xenopus laevis and two Australian frogs. Aquat. Toxicol. 51 (1), 19–29.
Mao, L., Zhang, K., Chan, H.S.O., Wu, J., 2012. Surfactant-stabilized graphene/polyaniline nanofiber composites for high performance supercapacitor electrode. J. Mater. Chem. 22, 80–85.
Morales, G.M., Salavagione, H.J., Grumelli, D.E., Miras, M.C., Barbero, C.A., 2006. Soluble polyanilines obtained by nucleophilic addition of arenesulphinic acids. Polymer 47 (25), 8272–8280.
Nel, A., Xia, T., Madler, L., Li, N., 2006. Toxic potential of materials at the nanolevel. Science 311, 622–627.
Nelson, S.M., Mahmoud, T., Beaux II, M., Shapiro, P., McIlroy, D.N., Stenkamp, D.L., 2010. Toxic and teratogenic silica nanowires in developing vertebrate embryos. Nanomed.: Nanotechnol. Biol. Med. 6, 93–102.
Novak, P., Muller, K., Santhanam, K.S.V., Haas, O., 1997. Electrochemically active polymers for rechargeable batteries. Chem. Rev. 97 (1), 207–282.
Oberdörster, G., Oberdörster, E., Oberdörster, J., 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. Health Perspect. 113 (7), 823–839.
Oberdörster, G., Stone, V., Donaldson, K., 2007. Toxicology of nanoparticles: a historical perspective. Nanotoxicology 1 (1), 2–25.
Paisio, C.E., Agostini, E., González, P.S., Bertuzzi, M.L., 2009. Lethal and teratogenic effects of phenol on Bufo arenarum embryos. J. Hazar. Mater. 167, 64–68.
Pérez-Coll, C.S., Herkovits, J., 2004. Lethal and teratogenic effects of naringenin evaluated by means of an amphibians embryo toxicity test (AMPHITOX). Food Chem. Toxicol. 42, 299–306.
Poland, C.A., Duffin, R., Kinloch, I., Maynard, A., Wallace, W.A.H., Seaton, A., Stone, V., Brown, S., MacNee, W., Donaldson, K., 2008. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat. Nanotechnol. 3 (7), 423–428.
Prati, M., Biganzoli, E., Boracchi, P., Tesauro, M., Monetti, C., Bernardini, C., 2000. Ecotoxicological soil evaluation by FETAX. Chemosphere 41, 1621–1628.
Riede, A., Helmstedt, M., Riede, V., Stejskal, J., 1998. Polyaniline dispersions. 9. Dynamic light scattering study of particle formation using different stabilizers. Langmuir 14, 6767–6771.
Sadek, A.Z., Baker, C.O., Powell, D.A., Wlodarski, W., Kaner, R.B., Kalantarzadeh, K., 2007. Polyaniline nanofiber based surface acoustic wave gas sensors – effect of nanofiber diameter on H2 response. IEEE Sens. J. 7, 213–218.
Spinks, G.M., Mottaghitalab, V., Bahrami-Samani, M., Whitten, P.G., Wallace, G.G., 2006. Carbon-nanotube-reinforced polyaniline fibers for high-strength artificial muscles. Adv. Mater. 18 (5), 637–640.
Stockert, J., Herkovits, J., 2003. Photodynamic toxicity and its prevention by antioxidative agents in Bufo arenarum embryos. Toxicology 192, 211–218.
Tahir, Z.M., Alocilja, E.C., Grooms, D.L., 2005. Polyaniline synthesis and its biosensor application. Biosens. Bioelectron. 20 (8), 1690–1695.
Tseng, R.J., Baker, C.O., Shedd, B., Huang, J.X., Kaner, R.B., Ouyang, J.Y., Yang, Y., 2007. Charge transfer effect in the polyaniline-gold nanoparticle memory system. Appl. Phys. Lett. 90, 053101.
Tseng, R.J., Huang, J., Ouyang, J., Kaner, R.B., Yang, Y., 2005. Polyaniline nanofiber/gold nanoparticle nonvolatile memory. Nano Lett. 5 (6), 1077–1080.
US environmental protection agency (US EPA), 1988. Users guide for a computer program for PROBIT analysis of date from acute and short-term chronic toxicity test with aquatic organism. Biological Methods, Environmental monitoring and Support Lab.
Verwey, E.J.W., 1947. Theory of the stability of lyophobic colloids. J. Phys. Chem. 51 (3), 631–636.
Virji, S., Kaner, R.B., Weiller, B.H., 2006. Hydrogen sensors based on conductivity changes in polyaniline nanofibers. J. Phys. Chem. B 110, 22266–22270.
Vitt, L.J., Caldwell, J.P., Wilbur, H.M., Smith, C.D., 1990. Amphibians as harbingers of decay. Bioscience 40, 418.
Warheit, D.B., 2008. Howmeaningful are the results of nanotoxicity studies in the absence of adequate material characterization? Toxicol. Sci. 101, 183–185.
Werake, L.K., Story, J.G., Bertino, M.F., Pillalamarri, S.K., Blum, F.D., 2005. Photolithographic synthesis of polyaniline nanofibres. Nanotechnology 16 (12), 2833–2837.
Yen-Fu, L., Chien-Hsiang, C., Wen-Jia, X., Sheng-Hsiung, Y., Chain-Shu, H., Minn-Tsong, L., Wen-Bin, J., 2011. Nano approach investigation of the conduction mechanism in polyaniline nanofibers. ACS Nano 5 (2), 1541–1548.