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Monitoring of toxicity of As(V) solutions by AMPHITOX test without and with treatment with zerovalent iron nanoparticles

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ABSTRACT

Changes in toxicity of As(V) solutions from acute to chronic exposure have been evaluated by the AMPHITOX test. This test employs *Rhinella arenarum*, a widely distributed toad in Argentine areas. LOEC values were 6.37 and 1.88 mg L^{-1} for embryos and larvae, respectively, and serious sublethal effects have been observed. Toxicity of As(V) solutions has been also evaluated after treatment with zerovalent iron nanoparticles (nZVI). After 60 min of treatment with nZVI, As(V) removal was 77%, and neither lethal nor sublethal effects were observed. However, nZVI had to be eliminated before the bioassay because they caused adverse effects in both embryos and larvae. This work highlights the high sensitivity of *R. arenarum* to As(V), the relevance to assess toxicity on different periods of the lifecycle, and the need to expand exposure to As(V) to chronic times. The utility of the test for monitoring toxicity changes in As(V) solutions after nZVI treatment has been also shown.

1. Introduction

Regions with high natural arsenic (As) levels in groundwater are well-known in different countries of the world such as China, Hungary, India, Bangladesh and Vietnam. In Latin America, the problem affects at least 14 countries (Argentina, Bolivia, Brazil, Chile, Colombia, Cuba, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Peru and Uruguay), and the number of exposed people can be calculated to be around 14 million. The most critical areas are in Argentina, Chile and Mexico (Figueiredo et al., 2010). It is currently estimated that the population living in areas with As contaminated water in Argentina rises to about 4,000,000 people (Bardach et al., 2015).

Although minor sources of As in water come from anthropogenic activities, As pollution is mainly natural (Litter et al. 2010). Concerns on human health due to consumption of waters containing high As concentrations have prompted numerous research studies worldwide (Bardach et al., 2015; Hughes et al., 2011; Mandal and Suzuki, 2002). The phenomenon, known as Chronic Endemic Regional Hydroarsenicism (*hidroarsenicismo crónico regional endémico*, HACRE, in Spanish) in Argentina and in some other Latin American countries, affects largely human health. In many provinces of Argentina such as Santiago del Estero and Santa Fe, elevated levels of As in water and food as well as elevated excretion of As in urine of the population have been reported (Swiecky et al., 2006). Moreover, the International Agency for Research on Cancer (IARC) classifies As in Group 1, as there is sufficient evidence of a relationship between exposure to As and human cancer (IARC, 2018), and the World Health Organization recommends a limit of 0.01 mg As L^{-1} in drinking water (WHO, 2011). Although the consequences in humans have been very much studied (Vahter and Concha, 2001; Bardach et al. 2015), the possible impacts of As-contaminated water on the ecosystems and wildlife have been less investigated. Particularly, amphibians play a key role in the food webs, living near or in water reservoirs affected by the presence of As. The decline and extinction of amphibians is a major concern for biodiversity protection worldwide, alerted since the 1960's (Blaustein et al., 2003; Pérez Coll et al., 2017), because these organisms are extremely sensitive as they have permeable skin and eggs that readily absorb chemicals from the environment. Because of their high sensitivity, mainly during the

Abbreviations: nZVI, zerovalent nanoparticles; nZVIR, nZVI removed; TOP, Toxicity Profile; AMPHITOX, Amphibian Toxicity Test; ASol, AMPHITOX Solution; LC, Lethal Concentration; EC, Effective Concentration; LOEC, Low Observed Effect Concentration; TI, Teratogenic Index

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developmental period of their life cycle, they are increasingly used for toxicity screening purposes. Standardized tests employing amphibian embryos and larvae are successfully used to evaluate the toxicity of hazardous substances and environmental samples (Herkovits and Pérez-Coll, 2003; Hoke and Ankley, 2005; Pérez Coll et al., 2017). The assessment of toxicity in both embryos and larvae allows to search an eventual differential susceptibility between them. The finding of the most sensitive period of the life cycle of a species is critical for conservative and ecological purposes, and these studies are important to make available the toxicity profile of a chemical as completely as possible (Pérez Coll et al., 2017).

Embryos and larvae of *Rhinella arenarum*, the common South American toad (Fam. Bufonidae), are used in AMPHITOX as a valuable biological material to perform toxicity tests (Herkovits et al., 2002; Pérez Coll et al., 2017). This toad exhibits an extensive neotropical distribution, including countries such as Argentina, Uruguay, Paraguay, Brazil and Bolivia. Contrary to other bioassays that only assess the acute toxicity of chemicals or the toxicity on a unique stage of the life cycle, AMPHITOX evaluates toxicity by using different endpoints (exposure times and developmental stages), offering a more complete information about the toxicity of a chemical species. Examination of Toxicity Profile (TOP) curves, based on the plot of the Lethal Concentration 10 (LC_{10}, considered as the Low Observed Effect Concentration value, LOEC, from a statistical approach), LC_{50} and LC_{90} (or LC_{100}), from acute to chronic exposure, allows the visualization of concentration- and time-exposure thresholds, as well as the range of concentrations that exert adverse effects in each case. These TOP curves provide, within a systemic toxicity approach, a more complete and appropriate set of data than the classical LC_{50}-48 h (Herkovits et al., 1997; Pérez-Coll and Herkovits, 2004).

It is well known that there is a differential susceptibility of living organisms to both As chemical species, As(III) and As(V), with the first form shown to be more toxic than the latest one (Gardner et al., 2017; Ventura-Lima et al., 2011). While As(III) dominates in reducing environments such as groundwater, As(V) is dominant in oxidizing environments such as surface waters (Chen et al., 2009), where amphibians live and breed. However, the toxicity of As(III) to several amphibians have been mainly reported, with LC_{50} values ranging from 0.04 mg L^{-1} for the eastern narrow-mouthed toad embryos (*Gastrophryne carolinensis*) (Birge et al., 1979), 0.25 mg L^{-1} for the green pond frog larvae (*Rana hexadactyla*) (Khangarot et al., 1985) to 4.45 mg L^{-1} for the marbled salamander (*Ambystoma opacum*) (Birge, 1978). For embryos of *Xenopus laevis*, the South African clawed frog, a 96-h LC_{50} of 470.5 mg L^{-1} for As(V) was estimated (Gornati et al., 2002), indicating a low sensitivity of *X. laevis* to As(V). Mortality values for As (V), for other freshwater organisms (microinvertebrates) have been reported, such as 48-h LC_{50} of 2.44 mg L^{-1} for *Daphnia carinata* (He et al., 2009) and 3.9 mg L^{-1} for *Daphnia pulex* (Shaw et al., 2007).

In recent years, several studies on As removal from water were performed based on the use of zerovalent iron nanoparticles (nZVI) as innovative treatment. In comparison with other methods, nZVI can simultaneously remove As(V) and As(III) without previous oxidative treatment and does not require the use of additional chemicals, attaining very good removal efficiencies in very short times (Levy, 2013; Litter et al., 2010; Morgada et al., 2009; Mosaferi et al., 2014; Rahmani et al., 2010; Rahmani et al., 2011; Ramos et al., 2009).

Previously, we have demonstrated the usefulness of the AMPHITOX test for monitoring the toxicity changes after the application of highly efficient technologies for the removal of difficult contaminants. In that case, TiO_2-heterogeneous photocatalysis was used to drastically decrease Cr(VI) concentrations from aqueous systems, and AMPHITOX represented an optimal test to detect the abatement of the heavy metal during the application of this innovative methodology (Hojman et al., 2015).

The aims of the present work have been: i) to evaluate the toxic effects of As(V) on *R. arenarum* embryos and larvae from acute to chronic exposure, and ii) to assess the usefulness of AMPHITOX for monitoring changes in the toxicity of As(V) solutions submitted to nZVI treatment.

2. Materials and methods

2.1. Reagents

The zerovalent iron nanoparticles (nZVI) were provided by NANO IRON S.R.O. (Czech Republic) as NANOFER 25 in aqueous suspension (C_{Fe} = 242 mg L^{-1}). Sodium arsenate dibasic 7-hydrate ($Na_2HAsO_4.7H_2O$, Baker) and all other chemicals were of the highest purity. In all experiments, Milli-Q water was used (resistivity = 18 MΩ cm).

2.2. As removal with nZVI

To 200 mL of a 10 mg L^{-1} As(V) aqueous solution (pH 7) in a thermostatted (25 °C) glass cylindrical cell open to air, drops of nZVI suspension were added to reach 745 mg L^{-1} nZVI (1:100 As:Fe molar ratio), and the system was stirred with a paddle stirrer, open to the air. Samples were taken at 5, 15, 30, 45 and 60 min, nZVI were removed by centrifugation using an Eppendorf AG 5810 centrifuge, and the supernatants were used in toxicity bioassays. The removal experiment was performed by duplicate and the experimental error was never higher than 2% of the initial value, as calculated by standard deviation among the duplicate experiments; error bars for the averaged experiments are shown in the corresponding figure. These samples will be called nZVIR (nZVI removed) 5–60 min. As(V) concentration was determined in each sample by spectrophotometry, using the arsenomolybdate complex, measuring at 868 nm (Lenoble et al., 2003).

2.3. Obtaining Rhinella arenarum embryos and larvae

Rhinella arenarum adults weighing approximately 200–250 g were acquired in Lobos, Buenos Aires province, Argentina (35°11′ S; 59°05′ W). Toad care, breeding, embryo acquisition and analysis were conducted according AMPHITOX protocols (Herkovits and Pérez-Coll, 2003; Pérez Coll et al., 2017). Oocytes were fertilized in vitro using fresh sperm suspended in AMPHITOX solution, ASol (36.0 mg L^{-1} NaCl, 0.5 mg L^{-1} KCl, 1.0 mg L^{-1} $CaCl_2$ and 2.0 mg L^{-1} $NaHCO_3$). For bioassays with larvae, embryos were kept in ASol and maintained at 20 ± 2 °C, until organisms reached the complete operculum stage, S.25 (Del Conte and Sirlin et al., 1951).

2.4. Toxicity bioassays

Three independent experiments were performed in which 2 groups of ten embryos (S.2) and early larvae (S.25) were randomly placed by triplicate in covered 10 cm-diameter glass Petri dishes containing 40 mL of solution under different conditions. To evaluate the lethal and sublethal effects of As(V) on *R. arenarum* embryos and larvae and to construct the survival and TOP curves, the following conditions were set: 1) absolute control: ASol, 2) 2–10 mg L^{-1} As(V) solution in ASol, assaying the following five As(V) concentrations: 2, 4, 6, 8 and 10 mg L^{-1}. To assess the usefulness of AMPHITOX for monitoring changes in the toxicity of As(V) solutions submitted to treatment with nZVI, the following conditions were used: 1) absolute control: ASol, 2) 10 mg L^{-1} As (V) solution in ASol, 3) 10 mg L^{-1} As(V) solution treated with 745 mg L^{-1} (13.34 mM) nZVI for the indicated reaction time and then centrifuged (nZVIR 5–60 min), 4) supernatant of a pure (no As) 745 mg L^{-1} nZVI aqueous suspension centrifuged after 60 min (nZVIR), and 5) pure (no As) 745 mg L^{-1} nZVI aqueous suspension without centrifugation, 60 min after its preparation (nZVI). In conditions 3), 4) and 5), AMPHITOX salts were added to the solutions before exposure to achieve the concentration of the absolute control. Prior to testing and renewal of

solutions, pH, conductivity and dissolved oxygen were measured to ensure acceptable levels. Embryos and larvae were continuously exposed to the solutions or suspensions for acute (96 h), short-term chronic (168 h) and chronic (336 h) periods. For embryos, the exposure began after fertilization, lasted 14 days (336 h) and covered embryonic and larval development. For larvae, the exposure began after the end of the embryonic development and lasted also 14 days (336 h). Bioassays were semistatic, and test solutions were entirely replaced every 48 h. Temperature was 20 ± 2 °C and a 12:12 h light:dark photoperiod was maintained throughout the experiments. Larvae were fed with Tetra Color Fin Sinking Granules for Goldfish ad libitum every other day, coincident with the changes of the solutions. The care and use of animals were conducted in accordance with the guidelines of the international standards on animal welfare (Canadian Council on Animal Care in Science, 1993). Lethal and sublethal effects under different conditions were evaluated every 24 h, and dead individuals were removed. Abnormalities were observed under a binocular stereoscopic microscope (Zeiss Stemi DV4), photographed with a Sony DSC-S90 digital camera, and identified according to the "Atlas of Abnormalities" (Bantle et al., 1991). To confirm the death of individuals, smooth movements of the Petri-dishes, followed by stimulation with a light source were done. In case of no response, soft mechanic stimulation with a glass rod was made and, finally, heartbeat was checked under the Zeiss microscope. Lethality data were analyzed statistically by the U.S. Environmental Protection Agency Probit Program (US EPA, 1988). TOPs, as isotoxicity curves, were plotted based on the LC_{10}, LC_{50}, and LC_{90} values at different times. Effective Concentration (EC) data were also analyzed statistically by the US EPA Probit Program. The Teratogenic Index (TI) was calculated as LC_{50}/EC_{50} at the end of the embryo development (168 h), EC_{50} being the Effective Concentration to cause sublethal effects in the 50% embryos. TI > 1.5 implies a high risk for embryos to be malformed in the absence of significant embryo lethality (ASTM, 1993). A 10% of difference with the absolute control was set as the significance level.

3. Results

3.1. Arsenic removal with nZVI

The initial As(V) concentration in the removal experiments, i.e., 10 mg L^{-1}, was selected after a preliminary bioassay that evaluated the toxicity to *R. arenarum* in the range 2–10 mg L^{-1}. This concentration allowed to follow clear changes during the toxicity tests. Then, 10 mg L^{-1} As solutions at pH 7 were put in contact with nZVI (As:Fe(0) molar ratio (MR)) = 1:100), and the evolution of the initial As(V) concentration with time was registered after centrifugation to eliminate the remaining nanoparticles or any solid iron reaction product (Fig. 1). It can be observed that the concentration fell from 10 to 2.3 mg L^{-1} after 60 min of contact, i.e., 77% As removal was achieved. Negligible changes in pH were observed during the reaction time.

3.2. Lethal effects of As(V) on Rhinella arenarum embryos and larvae

The lethal effects of As(V) on *R. arenarum* embryos and larvae are shown in Figs. 2A, B, 3 and 4 .

3.2.1. Embryos

There were no significant lethal effects on the amphibian embryos exposed to As (2–10 mg L^{-1}) during the first 120 h. However, survival began to decline mainly at the highest concentration as exposure continued, coincident with the development of embryos into larvae (Fig. 2A).

Fig. 3 shows the TOP curves of As(V) for *R. arenarum* embryos constructed from lethality data of exposed embryos. It was only possible to draw these curves from chronic exposure (216 h) because the earlier lethality data were too low and thus not appropriate for Probit analysis.

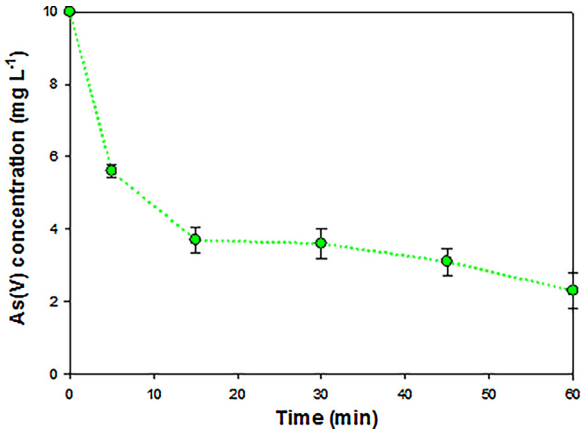

Fig. 1. Time profile of As(V) concentration during the treatment with nZVI (NANOFER 25 ®). Conditions: As(V) concentration = 10 mg L^{-1}, As:Fe(0) MR = 1:100, open to air, pH_0 7. The dotted curve is only for a better visualization of points and does not correspond to any fitting model.

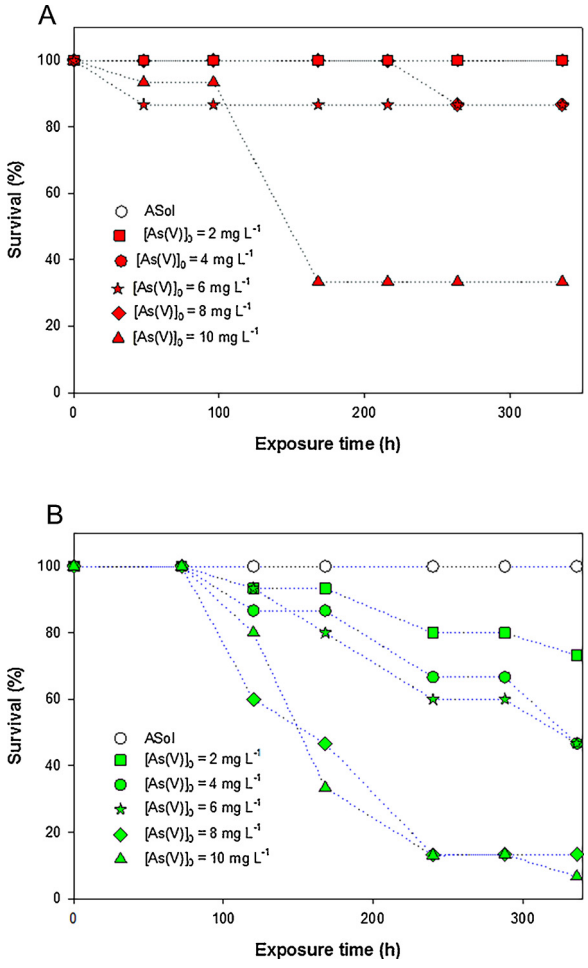

Fig. 2. (A) Survival curves for *Rhinella arenarum* embryos continuously exposed from S.2 to As(V) describing the concentration–response relationship from the beginning of the exposure up to 336 h. (B) Survival curves for *Rhinella arenarum* larvae continuously exposed from S.25 to As(V) describing the concentration–response relationship from the beginning of the exposure up to 336 h.

From the plots, it can be seen that the LC_{10} (LOEC), LC_{50} and LC_{90} values at 216 h were 6.37 mg As L^{-1}, 9.43 mg As L^{-1} and 13.95 mg As L^{-1}, respectively, and stayed almost constant up to 336 h.

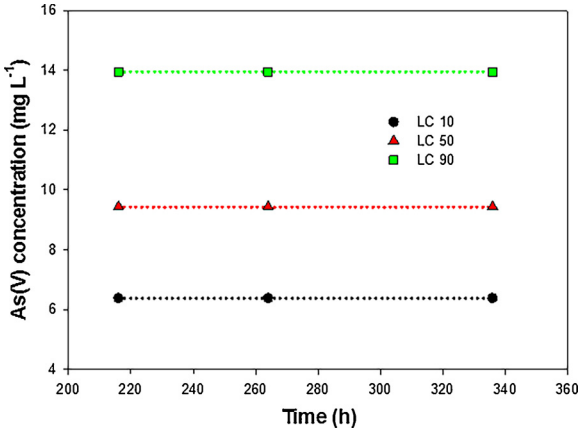

Fig. 3. As(V) Toxicity Profile (TOP) curves based on LC 10, 50 and 90 for *Rhinella arenarum* embryos (S.2) continuously exposed for 336 h.

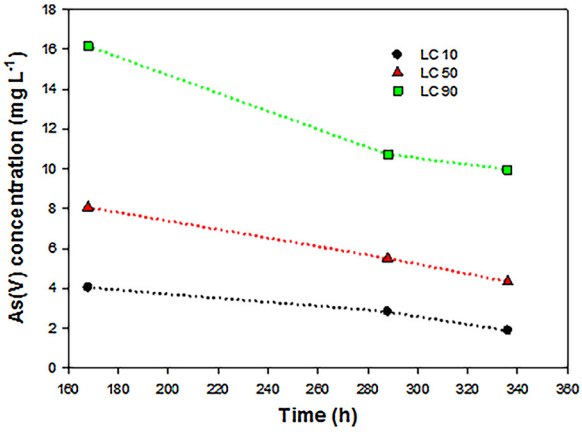

Fig. 4. As(V) Toxicity Profile (TOP) curves based on LC 10, 50 and 90 for *Rhinella arenarum* larvae (S.25) continuously exposed for 336 h.

3.2.2. Larvae

Fig. 2B shows the survival curves of *R. arenarum* larvae exposed to different As(V) concentrations. TOP curves were also obtained for larvae and are shown in Fig. 4. Compared with embryos, *R. arenarum* larvae exposed to As(V) (2–10 mg L^{-1}) solutions showed a more pronounced survival decline even at chronic exposure, reaching higher mortality than embryos (cf. Figs. 2A–B, 3 and 4). In this case, toxicity data were appropriate for Probit analysis from 168 h of exposure onwards, with a 168 h-LC_{50} of 8.07 mg As L^{-1}, which gradually decreased to values as low as 4.32 mg As L^{-1} at 336 h. LC_{90} and LC_{10} also showed a decrease over time, reaching values of 9.97 and 1.88 mg As L^{-1} at the end of the bioassays.

3.3. Survival curves of R. arenarum embryos and larvae exposed to As(V) solutions without and with treatment with nZVI

Fig. 5A and B show the comparative survival curves of *R. arenarum* embryos and larvae exposed to different experimental conditions.

Fig. 5A shows that the exposure of *R. arenarum* embryos to 10 mg L^{-1} As(V) (no nZVI treated) resulted in no significant lethal effects during the first 120 h. However, from this time on, there was a dramatic survival decrease to 33% and then the survival stayed constant up to the end of chronification (336 h). The embryos exposed to 10 mg L^{-1} As(V) solutions treated with nZVI and followed by removal of the nanoparticles (nZVIR) during different times had a better survival, reaching the best value with solutions treated during 60 min, with no significant differences with the control. The survival of embryos in contact with nZVIR 60 (no As) and further removal of the nanoparticles was also similar to the absolute control. In contrast, when nZVI were not removed from the suspension (no As) after the contact for 60 min, embryos were severely affected, showing a marked survival decrease from 48 h exposure onwards and decreasing to 13% during the chronic exposure.

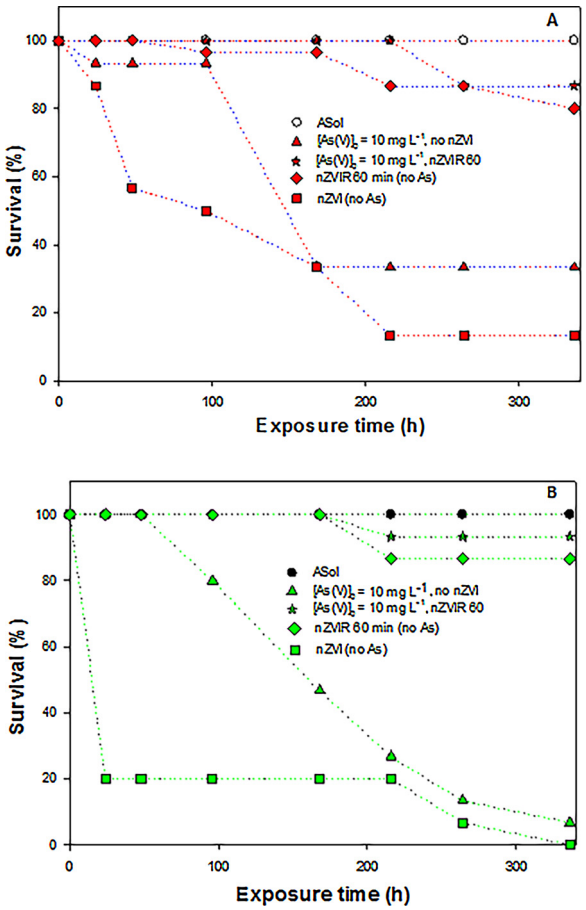

Fig. 5. (A) Survival curves of *Rhinella arenarum* embryos exposed to As(V) solutions without and with treatment with nZVI. 10 mg L^{-1} As(V) was selected for monitoring changes in the toxicity of As(V) solutions submitted to treatment with nZVI. (B) Survival curves of *Rhinella arenarum* larvae exposed to As(V) solutions without and with treatment with nZVI. 10 mg L^{-1} As(V) was selected for monitoring changes in the toxicity of As(V) solutions submitted to treatment with nZVI.

On the other hand, Fig. 5B shows that larvae treated with 10 mg L^{-1} As (no nZVI treatment) did not show lethal effects up to 96 h (acute period) but then the survival decreased continuously up 7% at the end of the chronification. The survival of larvae in contact with nZVIR 60 min (no As) was also similar to the absolute control. In contrast, when nZVI particles were not removed from the suspension after 60 min contact (no As), larvae showed lethality already at 24 h, the survival being reduced to 20% up to 216 h, with 100% lethality at the end of the chronification.

Fig. 5A and B clearly show that larvae are more affected than embryos by exposure either to As(V) solutions (no nZVI treatment) or to nZVI suspensions (absence of As and no removal of the nanoparticles). In both cases, the treatment with nZVI for 60 min (further removal of the nanoparticles) avoided toxicity.

3.4. Sublethal effects produced by As(V) on Rhinella arenarum embryos and larvae

Although there was no mortality during the first days of exposure of embryos to 10 mg L^{-1} As, serious sublethal effects were observed from

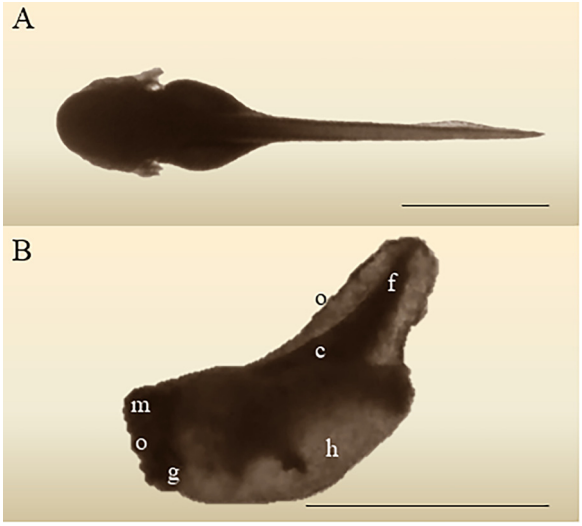

Fig. 6. Sublethal effects of As(V) on *R. arenarum* embryonic development. Embryos fixed at 96 h. A) Control (ASol). B) Embryo exposed to 10 mg L^{-1} As (V). Alterations were microcephaly (m), agenesis or underdevelopment of gills (g), tail curvature (c), irregular outline of the body (o), hydropsy (h), underdeveloped fin (f), delayed development and reduced body size. Note that the embryo arrested the development maintaining the aspect of an embryo at S.19, while the control continued the normal development (S.23). *Scale bars* A and B 3 mm.

a few hours after starting the bioassays. Therefore, embryos arrested their development at 96–120 h from the start of the bioassays, being still alive. They maintained the embryo aspect at S.19 (heartbeat stage) while control embryos (Fig. 6A) continued normally their development at S.23 (opercular fold stage) at regular rate forward the larval period. The main alterations were microcephaly, agenesis or underdevelopment of gills, tail curvatures, irregular outline of the body, hydropsy, underdeveloped fin, developmental delay and reduced body size (Fig. 6 B, cf. with Fig. 6A, control). Ethological disorders such as spasmodic contractions, narcosis, erratic swimming, lethargy and equilibrium loss were also observed.

Fig. 7 shows embryos exposed to solutions with 10 mg L^{-1} As(V) treated with nZVIR during different times (5, 30 and 45 min). These embryos also arrested their development at 96–120 h having an aspect similar to embryos at S.19, while controls were at S.23 (cf. Fig. 7B, C and D with A, control). These individuals showed sublethal effects similar to those of embryos exposed to 10 mg L^{-1} As(V). Fig. 7B illustrates an embryo exposed to 10 mg L^{-1} As(V) solution treated with nZVIR only during 5 min. It shows microcephaly, underdeveloped gills, hydropsy, tail curvature, epidermal bubbles, irregular contour of the body and underdeveloped fin. Fig. 7C displays an embryo exposed to the As solution treated by nZVIR for 30 min, which exhibits overall serious malformations including marked hydropsy, a conspicuous 90-degree curvature and heavily pigmented and thickened tail. Fig. 7D shows an embryo exposed to the As solution treated with nZVIR during 45 min, revealing reduced body size, microcephaly, prominent abdomen, edematized cardiac cavity and general underdevelopment. Embryo surfaces with tumors, hyperkeratosis and altered pigmentation pattern are also visualized in all these experimental embryos. The ethological disorders described above for embryos exposed to 10 mg L^{-1}As(V) were also registered in these embryos.

However, when enough As(V) was removed from solution (i.e., 77%, 60 min nZVIR treatment), the aspect and behavior of embryos were similar to the controls (cf. Fig. 8A and B), almost without toxicity signs. Fig. 8A exhibits a control embryo at the end of development (S.25). Fig. 8B shows an embryo exposed to a solution with 10 mg L^{-1} As(V) treated by nZVIR for 60 min, the time necessary to remove enough As to a concentration that allows an almost normal embryo development. On the other hand, embryos exposed to nZVI (no As) not removed before the bioassays revealed overall deleterious status (microcephaly, hydropsy, important desquamation, tail curvature, underdeveloped fin, irregular outline of the body, underdevelopment of gills and overall serious deterioration) added to ethological disorders such as narcosis (Fig. 8C). In contrast, when embryos were exposed to solutions treated with nZVIR (no As), embryos were similar to the control (cf. Figs. 8A and D).

EC_{50} evaluated by Probit was 4 mg L^{-1}. Therefore, the 168-h TI calculated for *R. arenarum* embryos treated with As(V) from the LC_{50}/EC_{50} ratio (9.43 mg L^{-1}/4 mg L^{-1}) in the present work was 2.36; this value is over the threshold ($\geq$1.5) to consider a substance as a teratogen (ASTM, 1993).

On the other hand, sublethal effects were also observed in larvae exposed to both 10 mg L^{-1} As(V) solutions without nZVI treatment and with nZVIR during 5, 30 and 45 min, evidenced by edema, alterations in the pattern of pigment, irregular outline of body, waving fin, and tail curvatures. However, As(V) caused less dramatic sublethal effects on larvae than on embryos. Larvae also suffered ethological alterations such as spasmodic contractions, weakness, narcosis, lethargy, equilibrium loss, numbness and non-feeding behavior. In contrast, and as in the case of embryos, sublethal effects were almost absent in larvae exposed to As solutions after their treatment with nZVIR for 60 min.

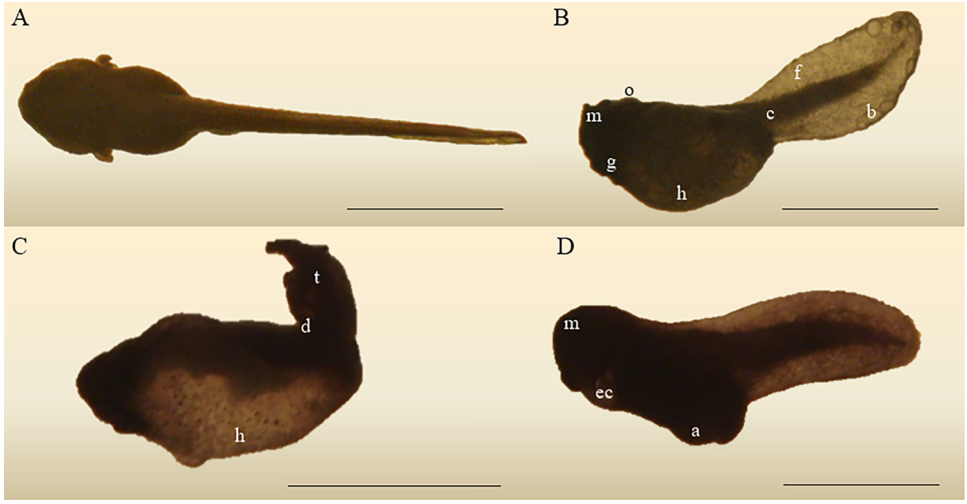

Fig. 7. Sublethal effects of As(V) in embryos exposed to 10 mg L^{-1} As(V) solutions treated with nZVIR during different times. A) Control (ASol, S.23). B) After exposure to As solution treated during 5 min. Note microcephaly (m), underdeveloped gills (g), hydropsy (h), tail curvature (c), epidermal bubbles (b), irregular contour of the body (o) and underdeveloped fin (f). C) After exposure to As solution treated during 30 min. Observe serious hydropsy (h), conspicuous 90-degree curvature (d), heavily pigmented and thickened tail (t). D) After exposure to As solution treated during 45 min. Note the reduced body size, microcephaly (m), prominent abdomen (a) and edematized cardiac cavity (ec). All experimental embryos exhibited an important delayed development resembling embryos at S.19 in contrast with the control that continued the normal development (S.23). *Scale bars* A–D 3 mm.

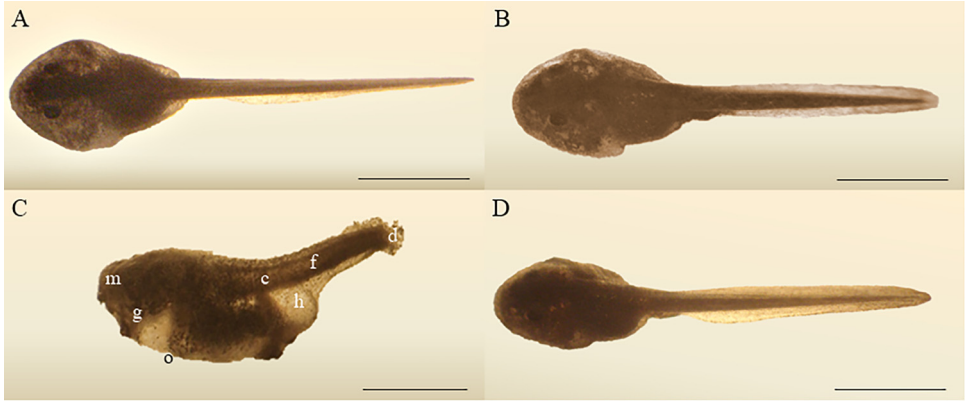

Fig. 8. Sublethal effects of As(V) and nZVI on *Rhinella arenarum* at S.25. A) control embryo. B) Embryo exposed to 10 mg L^{-1} As(V) solution treated with nZVIR during 60 min. Note the almost normal appearance of the embryo. C) Embryo exposed to nZVI without removal (no As). Observe microcephaly (m), hydropsy (h), important desquamation (d), tail curvature (c), underdeveloped fin (f), irregular outline of the body (o), underdevelopment of gills (g) and overall serious deterioration. D) Embryo exposed to solution with nZVI removed by centrifugation (nZVIR). Observe that embryo from picture C) arrested the development maintaining an aspect of an embryo at S.19, while the control continued the normal development (S.25) into larva. *Scale bars* A – D 3 mm.

4. Discussion

In this study, both lethal and sublethal effects of As(V) on *R. arenarum* embryos and larvae have been demonstrated. There is no available information concerning As(V) toxicity in *R. arenarum* development; however, recent data were obtained for lethal and sublethal effects, absorption and excretion of As(III) on this species (Brodeur et al., 2009; Mardirosian et al., 2015; Mardirosian et al., 2017a, b, c, d).

In the present results, As(V) lethal effects on *R. arenarum* development were only chronically expressed. Thus, it was necessary to extend the exposure time beyond 96 h to reveal the lethal effects of As(V) in both *R. arenarum* embryos and larvae (Figs. 2–4). This result highlights the relevance of chronifying exposure to not underestimate the lethal toxicity.

A stage-dependent sensitivity of *R. arenarum* to As(V) was also demonstrated. Comparing TOP curves (Figs. 3 and 4), the lethal effect of As(V) on *R. arenarum* embryos was much less drastic than the mortality of *R. arenarum* larvae, which began at 96 h and was almost complete after 336 h. Therefore, considering LC_{10}-336 h = 6.37 mg L^{-1} for embryos and the same parameter for larvae (1.88 mg L^{-1}), it can be said that there was 3.4 times more sensitivity in larvae than embryos. This can also be seen in survival plots, where in 35% resistant embryos, As toxicity reached a plateau and mortality became time-independent after 168 h, but larval toxicity continued to increase dramatically with exposure time (Fig. 5). Only a few works have evaluated the comparative effects of a xenobiotic among different periods of the development of a species. In contrast with our results, Li et al. (2009) reported that, at concentrations lower than 37.5 mg L^{-1}, As did not affect the survival rate in zebrafish during the embryonic stages (4–120 h post fertilization). However, recently, Sun et al. (2016) observed a differential susceptibility to As(V) and As(III) between embryos and larvae of bighead carp larvae (*Hypophthalmichthys nobilis*), highlighting a higher sensitivity of larvae compared with embryos, similarly as that it has been found in the present work. They exposed the developing organisms to low As(V) and As(III) concentrations (10–150 µg L^{-1}) only during 78 h, obtaining a slight decrease of embryo survival, but a great decrease of larvae survival, with As(III) showing a higher toxic effect than As(V). Concentrations of As lower than 150 µg L^{-1} adversely influenced the development of bighead carp larvae and disturbed their thyroid hormone homeostasis. Mortality values reported for microinvertebrates like *D. carinata* (He et al., 2009) and *D. pulex* (Shaw et al., 2007) reflect also their high sensitivity to As, as the LC_{50} values reported for these microinvertebrates are in the order of the toxicity values obtained for the amphibian (either embryos or larvae) studied in the present work.

Results on As(III) toxicity on *R. arenarum* development informed were unexpectedly low: Brodeur et al. (2009) reported LC_{50}s of 46–50 mg L^{-1} for larvae (S.25) from acute to chronic exposure, while Mardirosian et al. (2015) informed an acute LC_{50} of 24.3 mg L^{-1} for embryos exposed from fertilization up to S.25. Because toxicity is inversely proportional to LC values, those high values indicate a low toxicity for As(III), and then are controversial regarding the general evidence of the higher toxicity of As(III) in relation to As(V) as our present results show. It is important to note that both papers on *R. arenarum* report very low As toxicity, but in none of these papers was the As concentration followed by a direct measurement.

With respect to sublethal effects, As(V) solutions caused a diversity of malformations in embryos of *R. arenarum* as the present results show (Figs. 6 and 7). The mechanisms of action through which As causes toxicity are mainly the inhibition of DNA replication or repair enzymes, interference with tissue respiration and oxidative stress (Hughes et al., 2011; Liu et al., 2008; Obinaju, 2009). In previous works, musculoskeletal disorders similar to those found here for *R. arenarum*, as tail curvatures in fish larvae exposed to As(V), have been described (Sun et al., 2016). Also, flexures, tail kinking, facial deformities and abnormal bending of As(V) on developing salamanders, *Ambystoma maculatum*, were recently informed by Gardner et al. (2017). Gornati et al. (2002) reported acaudia (loss of the tail) in *X. laevis* embryos exposed to As(V). Edema, an osmoregulation failure, was expressed after our experiments in *R. arenarum* development. Also, embryos of *X. laevis*, exposed to As(V), developed ventral edema (Gornati et al., 2002), but compared with other amphibians, the sensitivity to As(V) was lower. This As(V) effect was also reported for *A. maculatum* larvae (Gardner et al., 2017), while Sun et al. (2016) informed pericardium edema in larvae fishes exposed to the metalloid. The As-induced nephron damage and the disruption of Na / K ATPase (the "sodium pump") as underlying biochemical mechanism of As could be related to this particular adverse effect (Mingai et al., 2009). Epidermal lesions including tumors, hyperkeratosis and hyperpigmentation changes, reported for other living organisms such as fish, amphibians and humans (Shannon and Strayer, 1989; Ahmed et al., 2013) after exposure to As solutions, were also observed in *R. arenarum* embryos and larvae in the present study. In general, all these biological structures are targets for As toxic effects. Also neuropathies, expressed as lethargy, seizures, numbness, loss of equilibrium, and general weakness, were similar to those observed in other amphibians. For example, *Rana pipiens* larvae exposed to very low As(V) concentrations (10 to 1000 µg L^{-1}) showed ethological alterations such as reduced swimming performance (Chen et al., 2009).

Concerning teratogenicity, the TI calculated for *R. arenarum* embryos in the present work was 2.36, a value over the threshold (≥ 1.5) to consider a substance as a teratogen (ASTM, 1993). In contrast, As(V) was not found to be teratogenic for *X. laevis*, as the LC_{50} for this amphibian was 470.5 mg L^{-1}, and the EC_{50} was 1086 mg L^{-1}, giving a TI of 0.43 (Gornati et al., 2002). However, in line with our results, Gardner et al. (2017) informed a rather high TI value (1.64) for *A. maculatum*.

As indicated in the Introduction section, the use of nZVI is a very efficient method to remove As from water. For this reason, it seemed

interesting to study the changes in toxicity of As(V) solutions after treatment with nZVI at different contact times; the AMPHITOX test was also a useful bioassay for this purpose. It has been observed that the treatment performed for solutions in contact with nZVIR for 45 min or less was not enough to prevent the c for 60 min.

5. Conclusions

The lethal and sublethal effects of As on embryo and larval development of *R. arenarum*, and the utility of the AMPHITOX test for monitoring changes in the toxicity of the As(V) solutions by nZVI treatment were provided in the present work. Regarding effects of nZVI particles themselves, some effects on *R. arenarum* have been shown here, but more profound studies are needed and are underway. However, it is relevant to point out the need to eliminate nZVI particles from the system for avoiding adverse effects on *R. arenarum* embryos and larvae.

It should be emphasized that studies of this type with other vertebrate groups are scarce and that there are few references about the toxicity of As for amphibians compared with the number of studies with mammals, mainly humans. This work highlights the relevance of assessing not only the lethal toxicity but also the sublethal effects of a physicochemical agent as As, whose teratogenic action was proved. These effects allow the survival of markedly failed adults, and represent a risk for amphibian populations because organisms became more vulnerable to contract diseases, parasites, predation, etc., representing an indirect extinction risk for the species. The present results alert on the conservation of *R. arenarum*, the common South American toad, widely distributed in the Chaco-Pampean plain of Argentina, one of the largest regions with high As occurrence.

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