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Nickel tissue residue as a biomarker of sub-toxic exposure and susceptibility in amphibian embryos

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ABSTRACT

Although low level exposure to physicochemical agents is the most common environmental scenario, their effects on living organisms are very controversial. However, there is an increasing need to integrate low level exposures from risk assessment to remediation purposes. This study focus on the possibility to employ Ni tissue residue values as biomarkers of sub-toxic exposure and susceptibility to this metal in a range of almost pristine to sub-toxic concentrations for *Rhinella arenarum* embryos. For that purpose, three batches of amphibian embryos were pretreated during 10 days with three increasing concentrations of Ni starting in 2, 8 and 20 μg Ni^{2+} L^{-1} and ending in 16, 64 and 160 μg Ni^{2+} L^{-1} (in natural fresh waters this value ranges from 2 to 10 μg L^{-1}; the LC_{50}-24 h for *R. arenarum* is 26.2 mg Ni^{2+} L^{-1}). For the experimental conditions, the Ni tissue residue values at 360 h post exposure were 0.5, 2.1 and 3.6 μg Ni g^{-1} embryo w/w, respectively, corresponding to BCFs of 31, 33 and 23. The susceptibility to Ni in those experimental embryos was evaluated by means of challenge exposures to three lethal concentrations of this metal (10, 20 and 30 mg Ni^{2+} L^{-1}), registering survival during the following 10 days of treatment. As a general pattern, the lower, intermediate and higher pretreatments with Ni resulted in enhanced, neutral and adverse effects on embryonic survival, respectively. Thus, sub-toxic exposure to Ni could modify the resistance of the amphibian embryo to this metal and Ni tissue residue values could be considered as biomarkers of both, exposure and susceptibility.

1. Introduction

Although Ni is not considered a global contaminant, near Ni-emitting sources several ecological changes such as a decrease in the number and diversity of species was reported (IPCS, 1991; Papachristou et al., 1993). Ni toxicity was studied in a wide range of aquatic biota such as microorganisms (Evdokimova and Mozgova, 2003), algae (Spencer and Greene, 1981), aquatic invertebrates (Pane et al., 2003), fishes (Birge and Black, 1980) and amphibian embryos (Herkovits et al., 2000; Fort et al., 2001; for a review see NAVFAC, 2004). In the case of *Rhinella arenarum* embryos, Ni toxicity was reported by means of Toxicity Profiles curves (TOPs), obtained by plotting the LC10, 50 and 90 from 24 to 168 h with their confidence intervals. Those curves exhibit a diminution in the LC50 values from 26.2 mg Ni^{2+} L^{-1} at 24 h to 1.8 mg Ni^{2+} L^{-1} at 168 h of exposure with a maximal declination within the acute period (Herkovits et al., 2000).

There is a significant current interest to quantitatively associate exposure and toxicity to tissue residue as a powerful approach that integrates exposure and kinetics addressing directly a variety of uncertainties in terms of contaminant bioavailability. Thus, the tissue residue-based approach could provide a more accurate prediction of dose and hence, effects of contaminants on aquatic organisms (McCarty, 1991). In a profuse survey on tissue residue database for aquatic organisms treated with inorganic and organic chemicals most of the studies with Ni focus on toxic or sub-toxic exposure conditions in bivalves and some fishes (Jarvinen and Ankley, 1999). The BCFs for Ni sub-toxic concentrations range from 6 to 26 depending on the specie and exposure conditions. In amphibians, even in very low concentration exposures, it was demonstrated influx–efflux of this metal already at early cleavage stages (Sunderman et al., 1990). However, the information available for the uptake of essential metals could be seen as controversial. For instance, a reduction in metal uptake as a physiological mechanism for metal-resistance has been reported for a wide diversity of organisms from bacteria to mammalian cells (Ashida, 1965; Chopra, 1971; Tsuchiya and Ochi, 1994; Gale et al., 2003; Xie and Klerks, 2004).

Low level concentrations of chemicals are the most frequent exposure scenarios and it is well known that low level exposure

Abbreviations: AS, AMPHITOX solution; BCF, bioconcentration factor; LC, lethal concentration; NOEL, no-observable-effect-level; NOEC, no observable effect concentration.

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to physical or chemical agents may exert a beneficial effect on certain parameters such as longevity (Sonneborn, 2005), cell division rate (Morré, 2000), regeneration processes (Weis and Weis, 1986) and an enhanced resistance to a subsequent challenge to the same or even different physico-chemical agent at toxic doses/concentrations (Van Straalen and Vaal, 1993; Herkovits and Pérez-Coll, 1995, 2007; Piantadosi, 2003; McGeer et al., 2007). Low level exposures were considered also relevant for the calculation of acceptable or safe levels of hazardous chemicals in the risk assessment and cleanup criteria (Paustenbach et al., 2006). The response to low level exposure conditions could be modulated by other parameters such as food availability (Hashemi et al., 2008), age (Sellin et al., 2005) and exposure to other environmental agents, e.g., metals (Herkovits and Pérez-Coll, 1990; McGeer et al., 2007) and estrogenic disruption (Herkovits et al., 2005).

In a search for studies on low level effects, from 3776 articles referring to this range of concentrations as sub-lethal, sub-toxic or sub threshold only 67 were considered potentially relevant publications in relation to hormesis, that is associated to the Arndt–Schulz law which predicts a dose–response β curve with a low dose stimulation-high dose inhibition effect all within two orders of magnitude below the no-observable-effect-level (NOEL), (Calabrese and Baldwin, 1998). In a previous study, pre-exposure to Ni concentrations in the order of ng L^{-1}, that is several orders of magnitude below the Arndt–Schulz law range of concentrations, resulted in an increased resistance to this metal (Pérez-Coll et al., 2006). The main aim of this study was to evaluate the possibility to employ Ni tissue residue values as a biomarker of exposure and susceptibility to this metal in a range of concentrations from almost pristine to sub-toxic for *R. arenarum* embryos. The implications of the results for a better understanding of low level exposures for environmental and human health protection purposes will be considered from an epigenetic perspective.

2. Materials and methods

2.1. The amphibian specie

R. arenarum, formerly *Bufo arenarum* (Anura, Bufonidae), named the common South American toad, is found from the coastal southern Brazil and also from Bolivia east of the Andes south to Chubut Province, Argentina. *R. arenarum* has a terrestrial habitat during its adult life but depends on freshwater for reproduction and early life stages. Its preferential habitats are sandy soils, grasslands, ponds and low mountains (Gallardo, 1964).

2.2. Obtention of embryos

R. arenarum adult females weighing 200–250 g were obtained in Moreno (Buenos Aires province). Ovulations were induced by means of i.p injection of homologous hypophysis (Herkovits and Pérez-Coll, 1995). After *in vitro* fertilization, embryos were maintained in AMPHITOX solution, AS, (Herkovits and Pérez-Coll, 2003) until the complete operculum stage (stage 25) that is the end of embryonic development according to Del Conte and Sirlin (1951).

2.3. Solutions and reagents

The composition of AS: NaCl: 36 mg L^{-1}; KCl: 0.5 mg L^{-1}; $CaCl_2$: 1 mg L^{-1}; $NaHCO_3$: 2 mg L^{-1}; The pH was 6,1 and the conductivity 59,4. Nickel solutions were prepared from a stock solution of 1 g L^{-1} of $NiCl_2 \cdot 6H_2O$ (Mallinckrodt) in AS. All experimental Ni solutions employed for the sub-toxic and challenge exposures were measured with a Perkin–Elmer atomic absorption spectrophotometer employing for the calibration curve a standard solution for atomic absorption spectroscopy (Riedel-de-Haen). The difference between nominal and measured concentrations did not exceed 20%. Due to the absence of any chelating compound and as the pH of the solutions employed were below 7, almost the totality of nickel (99.4% calculated by MINEQL+, 4.0) was in the form of free divalent cation.

2.4. Experimental design

The protocols for sub-toxic exposures were selected based on previous studies conducted with *R. arenarum* embryos for Ni toxicity (Herkovits et al., 2000) and the normal levels of this transitional metal in natural fresh waters which range from 2 to 10 μg L^{-1} (IPCS, 1991). Three groups each containing 500 *R. arenarum* embryos at stage 25 were exposed to three different sub-toxic nickel concentrations during 14 days as follows: the treatments started with 2 (G1); 8 (G2) and 20 (G3) μg Ni^{2+} L^{-1} and concentrations were gradually increased as it was reported by Herkovits and Pérez-Coll (1995, 2007) up to final concentrations of 16 (G1); 64 (G2) and 160 (G3) μg Ni^{2+} L^{-1}, respectively – A fourth group (G0) with 500 embryos was simultaneously maintained in AS without additions. The maintaining media were changed every other day coincident with the increase of the nickel concentration in the solution. Experiments were carried out at 20 ± 1 °C. At day 15, batches of embryos providing from the experimental and control groups were selected to conduct the following studies:

(i) *Challenge experiments*: Challenge tests were conducted following the standardized conditions of the short-term chronic toxicity test of AMPHITOX (Herkovits and Pérez-Coll, 2003). Batches of 10 embryos (by triplicate) from each group were challenged with the following lethal concentrations (LC) of nickel: 10 (B1), 20 (B2) and 30 mg Ni^{2+} L^{-1} (B3) in Petri dishes with 40 mL of solution at 20 ± 1 °C. For control conditions batches of 10 embryos by triplicate were maintained in (a) AS without additions, (b) in the last pre-treatment Ni concentration (G1–G3) and (c) exposed to the different LCs of Ni^{2+} employed: 10, 20 and 30 mg $Ni^{2+}L^{-1}$ without Ni pre-treatment. The experimental solutions were changes every other day. The survival of the embryos was evaluated each 24 h up till 10 days.

(ii) *Nickel contents*: Ni contents were quantified in embryos at 48, 96, 168 and 360 h post exposure to sub-toxic Ni concentrations (G1, G2 and G3). 50 embryos (by triplicate) from each group and control (G0) were processed as follows: the embryos rinsed three times with 150 mL of AS were digested with 3 mL of sulphonitric acid 1:1 (v/v) until complete mineralization (Herkovits and Pérez-Coll, 1995). Digested samples were diluted to 5 mL with bidistilled water, and Ni contents were quantified with a Perkin-Elmer atomic absorption spectrophotometer with graphite furnace. In order to report the BCF the mean between the initial and final exposure concentrations for each sub-toxic condition were employed.

2.5. Statistical analysis

Lethal Times 10, 50 and 90, in h, were estimated by means of Kaplan–Meier method and then, comparative analysis among control and each experimental condition by means of Cox-Mantel test for two independent samples were done.

3. Results

Figs. 1a–1c show the changes in the susceptibility of pre-exposed embryos (conditions G1–G3) evaluated by means of challenging them to three lethal concentrations of Ni (B1–B3). In all cases the pre-exposure conditions did not produce lethality in the experimental embryos. The treatment of control and Ni pre-treated embryos with the three lethal concentration of Ni resulted in lethality of the embryos from 72 h onwards with adverse effects

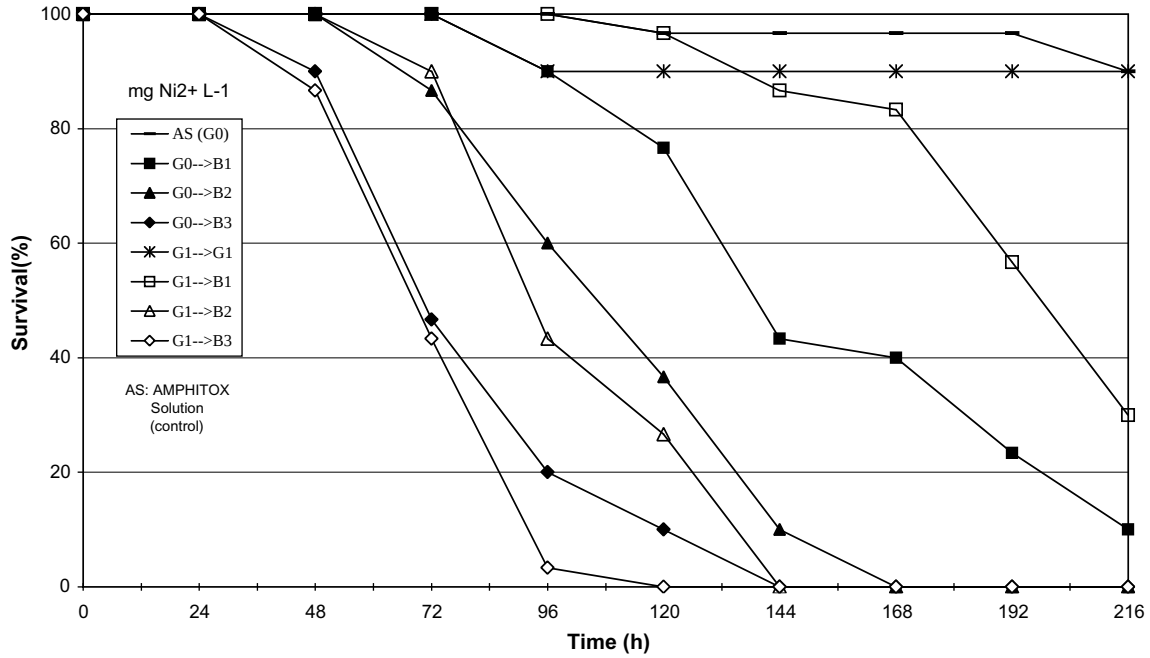

Fig. 1a. Effects of the exposure to sub-toxic nickel concentrations (condition 1) on the survival of *Rhinella arenarum* embryos challenged to different Ni LCs.

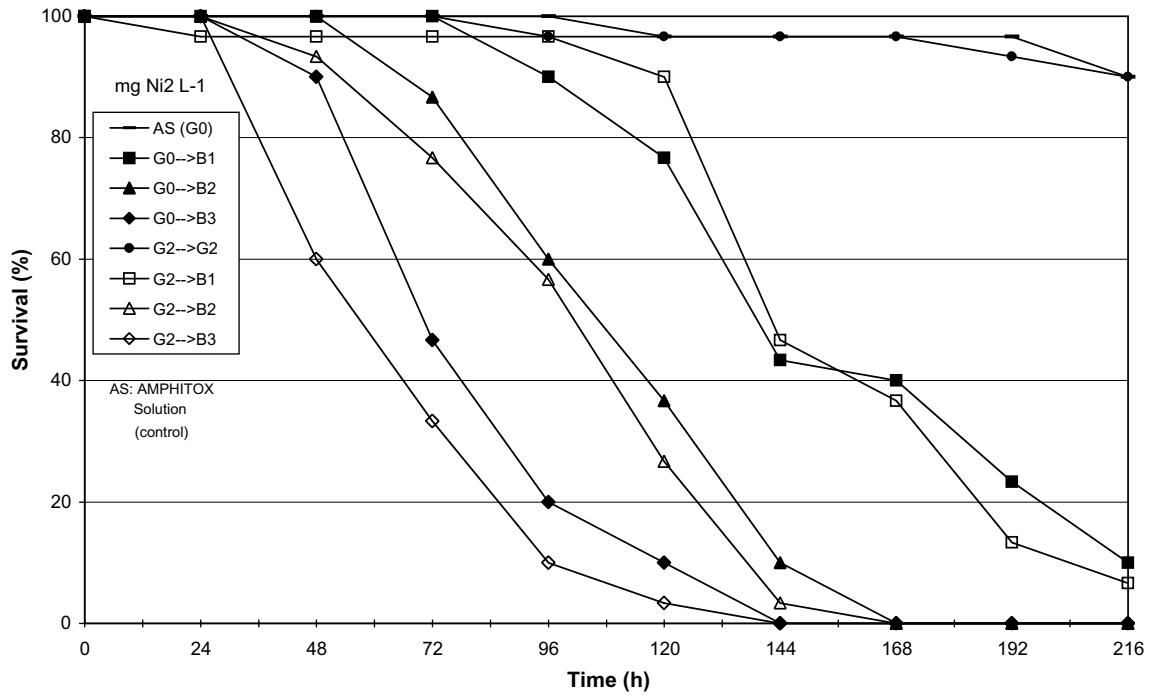

Fig. 1b. Effects of the exposure to sub-toxic nickel concentrations (condition 2) on the survival of *Rhinella arenarum* embryos challenged to different Ni LCs.

proportional to its concentration in the maintaining media. In all cases, the time for lethal effects was inversely proportional to the Ni concentration. Table 1 shows the statistical analysis between pre-exposure and challenge conditions and their respective controls. As a general pattern, the lower Ni pre-exposure condition (G1) could result in a beneficial effect against the toxicity of this metal, the intermediate pre-exposure (G2) did not modify the susceptibility of the embryos while the higher pre-exposure (G3) resulted in all cases in an increased susceptibility to Ni compared with no pre-exposed embryos.

Fig. 2 shows the Ni tissue residue in the amphibian embryos during the exposure period to sub-toxic concentrations of this metal from 48 to 360 h. Up till 240 h the Ni uptake was proportional to its concentration in the maintaining media and the time of exposure. At 360 h, Ni contents were 0.5, 2.1 and 3.6 μg Ni g^{-1} wet weight for G1, G2 and G3, respectively. The BCFs taking into account the mean exposure to Ni in each experimental condition resulted in 31; 33 and 23, respectively. No significant additional increases in Ni contents were found in the embryos by expanding the exposure up to 15 d.

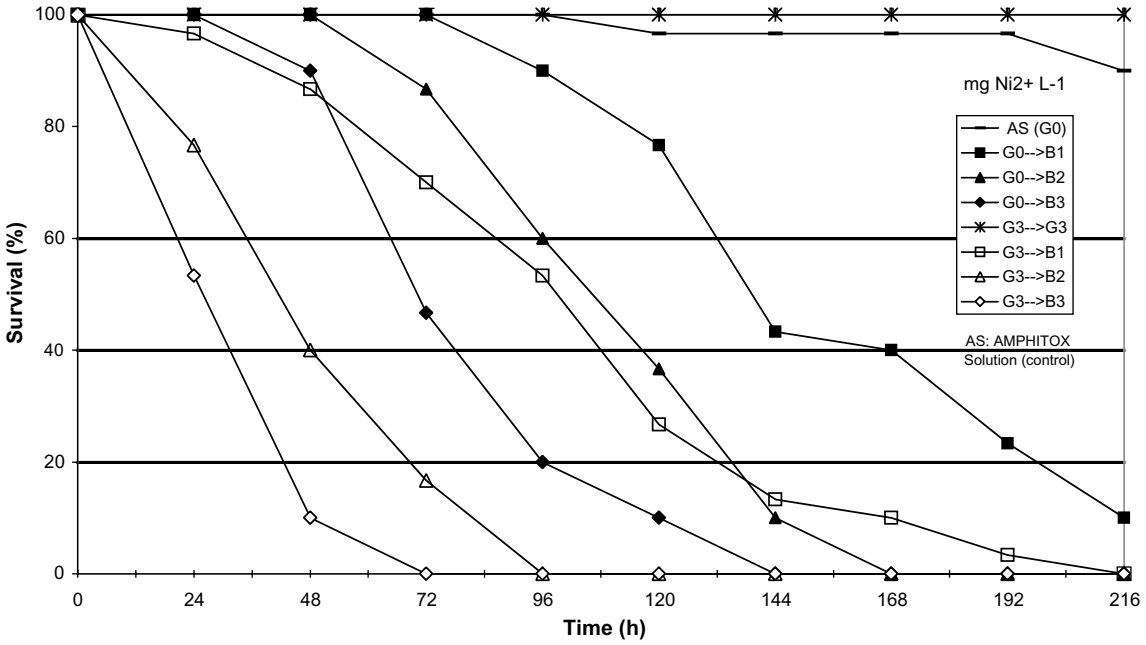

Fig. 1c. Effects of the exposure to sub-toxic nickel concentrations (condition 3) on the survival of *Rhinella arenarum* embryos challenged to different Ni LCs.

Table 1
Lethal times (LTs) in h for *Rhinella arenarum* embryos exposed to 3 different ranges of sub-toxic Ni conditions: G1 (from 2 to 16 μg Ni^{2+} L^{-1}), G2 (from 8 to 64 μg Ni^{2+} L^{-1}), G3 (from 20 to 160 μg Ni^{2+} L^{-1}) and their control (G0), and subsequently challenged to 10 (B1), 20 (B2) and 30 mg Ni^{2+} L^{-1} (B3)

	B1 (10 mg $Ni^{2+}L^{-1}$)			B2 (20 mg $Ni^{2+}L^{-1}$)			B3 (30 mg $Ni^{2+}L^{-1}$)		
Condition	LT10	LT50	LT90	LT10	LT50	LT90	LT10	LT50	LT90
G0	96	164	>216	72	120	164	44	72	120
G1	164	216	>216	72	96	164	48	72	96
G2	120	164	216	72	120	164	44	72	96
G3	44	120	168	24	48	96	24	44	48

Statistical analysis between three exposure conditions and their respective controls. For G1, only B1 condition had a beneficial effect ($p < 0.002$) while for G3, all challenge conditions resulted in adverse effects ($p < 0.0001$).

4. Discussion

This study confirms the Ni toxicity reported for *R. arenarum* embryos (Herkovits et al., 2000) and provides additional information on the time required to exert lethal effect in a range of concentrations between 10 and 30 mg $Ni^{2+}L^{-1}$. Although all those concentrations produce 100% of lethality, as it could be expected, the increase in Ni concentration results in a significant reduction in the time required for lethal effects (Table 1). The fact that even in the case of the higher Ni concentration evaluated, lethality was observed only from 72 h onwards contrasts with other metals such as Cd (Herkovits and Pérez-Coll, 1995), Cu (Herkovits and Helguero, 1998) and Al (Herkovits et al., 1997a), in which this maximal adverse effect could occur within the initial 24 h of exposure. This result confirms the advantage to customize the evaluation period to the toxicity of the agent or environmental sample (Herkovits and Pérez-Coll, 2003). As pre lethal effects in the case of embryos challenged to the higher Ni concentrations we found reduced motility, loss of epithelial cells in the tail and stunted appearance. In mammals, among sub-lethal effects related to nickel toxicity skin allergies, lung fibrosis, variable degrees of kidney and cardiovascular system poisoning and stimulation of neoplastic transformation, were reported (Denkhaus and Salnikow, 2002) reflecting the diversity of adverse effects produced on cells and tissues by this metal. The molecular mechanisms of nickel toxicity include oxidative stress and its effects on heterochromatin, gene silencing induction, DNA hypermethylation, inhibition of histone acetylation, interference with base and nucleotide excision repair, resulting in Ni-induced genetic damage, mutations and carcinogenesis (Costa, 2002).

Low level exposures are increasingly considered as relevant for human health and environmental protection purposes. Ni tissue residues of 0.5, 2.1 and 3.6 μg Ni g^{-1} w/w correspond to 16, 64 and 160 μg Ni^{2+} L^{-1} in the external media corresponding to BCFs ranging from 23 to 33, that is within the range of BCFs for sub-toxic exposure reported for fishes (Sreedevi et al., 1992). As it could be expected the higher BCF is related to the lower exposure condition. It is noteworthy that the tissue residue values did not change by extending the exposure period from 10 to15 days reflecting that a dynamic equilibrium between influx and efflux of Ni was achieved for each exposure condition. Considering that those tissue residue values correspond to enhanced resistance, neutral effect, or increased susceptibility, respectively, they could be employed as biomarkers of both exposure and susceptibility to this metal.

As beneficial or adverse effects could be related to alterations in detoxification processes, it is noteworthy that Ni detoxification is predominantly bound to α-glutamyl of glutathione (Zaroogian and Yevich, 1993), a mechanism that is also utilized among others for Ag, Hg, Pb, Cd and Se. These metals influence xenobiotic metabolizing enzymes such as glutathione-*S*-transferase (GST) and reduce glutathione (GSH) (Iscan et al., 1994). On the other hand, it is well known that other modifications in the environment, including caloric restriction or sub-lethal levels of stress can substantially affect the life-span of organisms (Calabrese and Baldwin 1998) which seems to be closely related to epigenetic mechanisms for gene regulation (Uchida et al., 2005). Nickel has been shown to

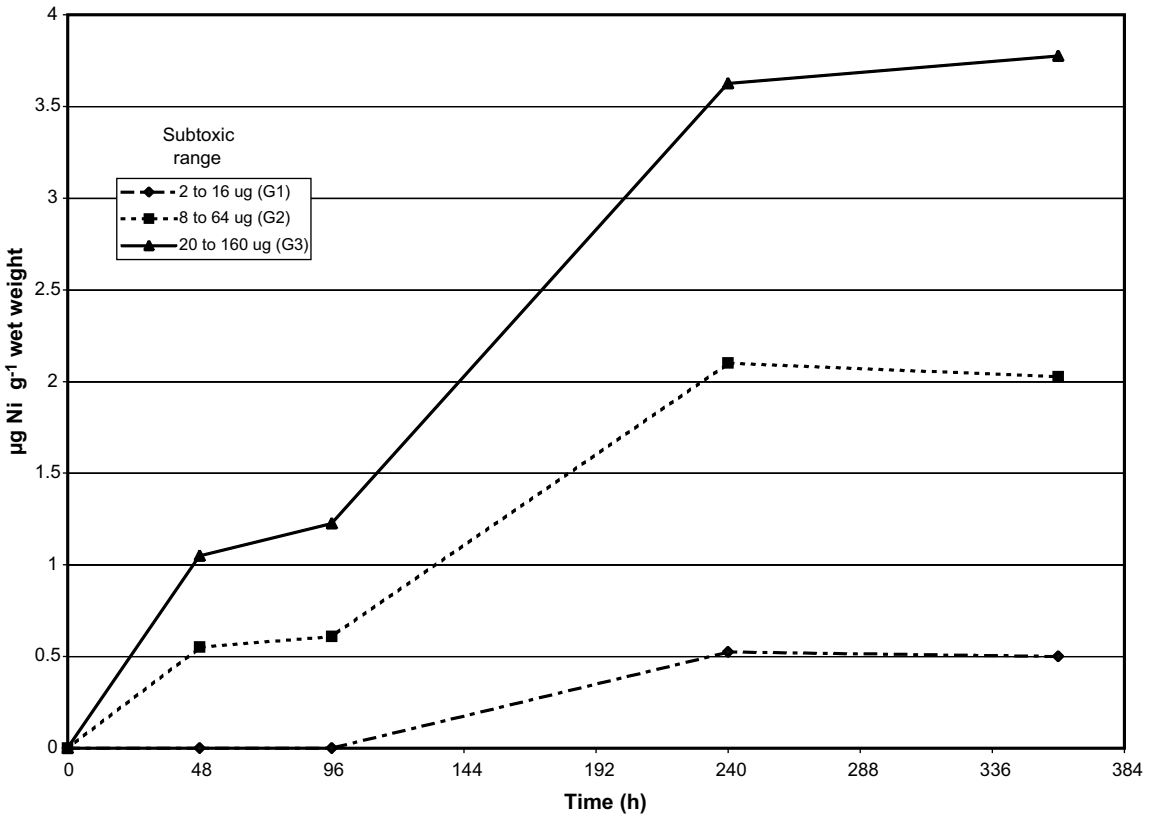

Fig. 2. Ni contents in *Rhinella arenarum* embryos exposed to sub-toxic concentrations of this metal.

alter DNA methylation patterns and histone acetylation status and by these means affect gene expression even in a heritable manner without directly altering the genome. As a whole, a possible explanation of the beneficial, neutral or adverse effects due to different pre-exposure conditions reported in this study could be that the low level exposure initiate defense mechanisms like reduction in the uptake of Ni and/or the induction of protective molecules against Ni toxicity, the intermediate condition result in a balance between those beneficial effects and the toxicity produced by Ni and finally the adverse effect of the higher pre-exposure to Ni is the result of its predominantly adverse effect. It is noteworthy that low level exposure conditions were evaluated in risk assessment for human and environmental health including the establishment of clean up criteria for contaminated scenarios as it was suggested by Paustenbach et al. (2006). The recognition of individual susceptibility by means of low level exposure to chemical stress could be of high value for human health protection purposes as well as for the selection of resistant or susceptible organisms for production or environmental purposes.

The results reported in this study support to some extent the Arndt–Schulz law which predicts within two orders of magnitude below the no-observable-effect-level (NOEL) values, the obtaining of a dose–response β curve with a low dose stimulation-high dose inhibition effect referred sometimes as hormesis (Calabrese and Baldwin, 1998). *R. arenarum* embryos were exposed to three ranges of Ni concentrations, from similar to pristine environmental conditions (2–10 µg $Ni^{2+}L^{-1}$) up to one order of magnitude below its NOEC values. As a general pattern, it can be concluded that the lower pre-exposure condition resulted in an enhanced resistance to Ni, the intermediate treatments in neutral effects, while the higher pre-exposure in adverse results.

It is noteworthy that the exposure of *R. arenarum* embryos to very low Ni concentrations (up to 4 orders of magnitude below the NOEC) results in a significant increase in the resistance of those embryos in case of challenges to lethal concentrations of this metal (Pérez-Coll et al., 2006). Therefore, the response capability of the amphibian embryo to low level concentrations of Ni is by far more complex than anticipated by the Arndt–Shultz law. Moreover, the stage-dependent susceptibility during ontogenesis (Herkovits et al., 1997b) and the wide range of low level concentrations that may have a biological effect (Herkovits and Pérez-Coll, 1993), point out that it is meaningful to revisit the low level exposure/effects concept. As the amount of metal that binds to living organisms is determined by a competition for metal ions between the biotic ligand and the other aqueous ligands, particularly dissolved organic matter (Di Toro et al., 2001), for environmental conditions the biotic ligand model (BLM) could be of high value in order to assess exposure conditions.

Adaptation to metal concentrations in field populations was interpreted as the dynamic interaction between the selective pressure of elevated pollutants and gene flow (Brandon, 1990; Groenendijk et al., 2002). Thus, from an evolutionary perspective, the fact that the response capability of the amphibian embryo to Ni (Pérez-Coll et al., 2006) is far below its values in natural fresh waters that range from 2 to10 µg L^{-1} (IPCS, 1991), could be considered as a reminiscence of ancient environmental conditions with very low bioavailable Ni concentration in fresh water bodies. This assumption is in line with the concept that changes in susceptibility as well as in the embryonic metabolism during ontogenesis could be considered as biomarkers of environmental changes during the evolutionary process (Herkovits, 2006).

5. Conclusion

The resistance to Ni in *R. arenarum* embryos could be modulated by means of sub-toxic exposures to this metal. By means

of subsequent challenge exposure to lethal concentrations of Ni, the lower, intermediate and higher sub-toxic concentrations of Ni resulted in enhanced, neutral and adverse effects on embryonic survival, respectively. Ni tissue residue values could be considered as biomarkers of exposure and susceptibility of the amphibian embryos to this metal.

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