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Efeitos sub-letais do efluente de uma mina de urânio abandonada em *Rana perezi* Seoane

dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia, realizada sob a orientação científica da Doutora Ruth Maria Oliveira Pereira, Investigadora Auxiliar do CESAM da Universidade de Aveiro e da co-orientação do Doutor Fernando José Mendes Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave *Rana perezi* Seoane; Mina de urânio; Metais; Genotoxicidade; Histopatologia

resumo

Nas últimas décadas a preocupação com o impacto ambiental causado por minas abandonadas tem vindo a aumentar de forma significativa. Tal facto deve-se principalmente à produção de efluentes ácidos, mesmo após o fim da exploração mineira, responsáveis pela diminuição da qualidade da água de recursos adjacentes e subsequente comprometimento das comunidades biológicas locais e da saúde humana. Estes problemas agravam-se quando existem elementos radioactivos, como o urânio, presentes no efluente, sendo um destes casos a mina de urânio da Cunha Baixa (Viseu, Centro de Portugal). Desta forma, com o objectivo de proceder a uma avaliação de riscos, específica para o local procedeu-se à avaliação dos impactos do efluente, produzido e acumulado em lagoas (M), na fauna indigena, de água doce. Com este objectivo foram utilizadas massas de ovos, larvas e adultos de rã-verde (*Rana perezi* Seoane) por ser um organismo bastante sensível cujos estádios iniciais de vida são inteiramente aquáticos e por surpreendentemente habitar o local de estudo. As massas de ovos foram recolhidas num troço do rio Vouga (VR), considerado como local de referência (VR), a norte da cidade de Viseu. Após a exposição aguda das massas de ovos e das larvas a uma gama de concentrações do efluente, com o pH ajustado para um nível neutro verificaram-se alterações, principalmente nas concentrações mais elevadas, a nível de crescimento, pigmentação, reacções a estimulos externos e acumulação de metais. Desta forma é possível concluir que o efluente estudado mesmo com um pH neutro exerce efeitos tóxicos sub-letais nas fases iniciais de desenvolvimento deste anfíbio.

De modo a compreender como podem ser afectados os adultos de *Rana perezi* residentes nas lagoas do efluente, um número ética e estatisticamente aceitável de organismos foi capturado em M eVR. Efectuou-se a avaliação de metais bioacumulados e de alterações histopatológicas no figado, rim, pulmão, baço e gónada dos animais. Simultaneamente recolheram-se amostras de sangue para avaliar danos genéticos. Nos organismos capturados na mina foram registadas concentrações significativamente mais elevadas de Be, Al, Mn, Fe e U no figado, bem como Pb e U nos rins. Estes organismos revelaram ainda alterações histopatológicas, nomeadamente um ligeiro aumento de centros melanomacrofágicos (MMC) no figado, rim e pulmão; dilatação to lumen dos túbulos renais associada a necrose, e uma ligeira hiperplasia do epitélio alveolar em conjunto com um discreto espessamento do septo alveolar. Concomitantemente foi registado um número significativamente mais elevado de anomalias nucleares nos eritrócitos dos animais capturados na mina.

keywords *Rana perezi* Seoane; Uranium mine; Metals; Genotoxicity; Histopathology

abstract

In the last decades there has been a growing concern with the environmental impact caused by abandoned mines. Such fact results mainly from the local production of acidic effluents, even after the end of the mining activity, responsible for the impairment of the environmental quality of nearby freshwater resources with subsequent effects on biological communities. These problems worsen when radioactive elements, like uranium, are present in the effluent. Such is the case of Cunha Baixa uranium mine (Viseu, Central Portugal). Hence it is necessary to study the impact of the effluent produced and accumulated in small ponds (M), in the freshwater fauna. For such purpose Iberian green frog (Rana perezi) egg masses, larvae, and adults were used, since this organism is very sensitive to environmental contaminants, the early life stages are entirely aquatic and its presence has been recorded in the study site. The egg masses were collected from a section of Vouga river (VR) located in the north part of the city of Viseu, which was considered as a reference site. Egg masses and larvae were exposed to a range of effluent concentrations, with pH adjusted to a neutral value. Alterations were recorded in the higher concentrations mainly for larvae, in growth, pigmentation, external stimuli reaction and metal accumulation. It was possible to conclude that the uranium mine effluent, even with a neutral pH, exerts sub-lethal toxic effects on these amphibian early life stages.

To bring a better understanding on how *Rana perezi* adults inhabiting the mine ponds might be affected, an ethical and statistical acceptable number of green frogs was captured in M and VR. Bioaccumulation of metals and histopathological alterations were evaluated in the liver, kidney, lung, spleen and gonad of animals. Simultaneously, blood samples were collected for the evaluation of genetic damages. Animals from the mine showed significant higher levels of Be, Al, Mn, Fe and U in the liver, as well as Pb and U in the kidney. Some animals from M showed histopathological alterations, namely: a slightly increase in melanomacrophagic centers in liver, kidney and lung; dilatation of the renal tubule lumen associated with tubular necrosis, and a slight hyperplasia in the alveolar epithelium along with a discreet thickening of the alveolar septa. A significant higher number of erytrocytic nuclear abnormalities was recorded, as well, in frogs from the mine.

“Serious problems cannot be dealt with at the level of thinking that created them”
(Albert Einstein)

Contents

General Introduction

General Introduction

When a mineral deposit is explored, fragmentation and grinding processes of its minerals take place. These processes involve several physical, chemical and technological procedures to extract the element of interest. The instability in the chemical balance of minerals may increase greatly, leading to the release of metallic elements from their structures and, their entrance in secondary geochemical cycles. Thus, they can become available in soil, air and water compartments, being the latter usually the most affected through leaching from mines and mine tailings (Lefcort et al., 1998; Oliveira et al., 1999; Lopes et al., 1999; Pereira et al., 2004).

Usually, and like the great majority of mining activities, the exploration of uranium gives rise to large amounts of solid residues enriched with metals and, in this particular case, with decay-chain radionuclides (e.g. ^{226}Ra and ^{222}Rn), which are persistent in the environment or have long half-life (Neves and Matias, 1998; Araujo et al., 2002). Human populations, along with most wildlife animals, breeding cattle, native flora and local cultivated fruits and vegetables may become exposed to low levels of such contaminants by inhalation, through the diet and drinking water supplies (Cothern and Lappenbusch, 1983; Cothern et al., 1983) or through roots adsorption (Dushenkov et al., 1997). Nonetheless the dispersion, bioavailability and bioaccessibility of contaminants depend on physical, chemical and biological factors (Araujo et al., 2002; Caussy et al., 2003). Moreover, in uranium mine areas when the ore was poor in its metal content, *in situ* leaching with sulphuric acid, was performed (Dias and da Costa, 1980). As a consequence, effluents originated from uranium mines are rich in metals, but also very acidic even in the case of inactive mines (Oliveira et al., 1999). The acidic mine drainages can affect the surrounding aquatic ecosystem due to their high content in several toxic metals as well as to their low pH, which in turn increases the dissolution of toxic elements, transporting them to considerable distances from their source (Oliveira, 1997) and, making them bioavailable for aquatic organisms. These problems may reach higher concern levels in inactive or abandoned mines, mainly through the lack of control and monitorization of the processes involved in the effluent production (Oliveira and Ávila, 2001). Even when this monitorization occurs is frequently a problem of difficult resolution. Consequently, the study of the toxicity of such effluents, and their mixtures of contaminants, becomes of paramount importance, to assess the risks posed to surrounding water resources and their living organisms.

For such purpose, and since they are an integrative measure of combined toxicity of chemicals, the use of whole effluent toxicity (WET) tests is almost of imperative use.

Nonetheless it is necessary to ensure the use of a sensitive species in these tests, along with chemical analyses to determine the effluent chemical composition and to identify the main chemicals potentially responsible by the effects on biotic communities (de Vlaming et al., 2000).

Amphibians, particularly frogs, are especially appropriate to conduct such studies since they have both aquatic and terrestrial life stages and a semi-permeable skin. (McDiarmid and Mitchell, 2000). This phased development makes amphibians susceptible to chemical exposure as eggs, larvae and adults, and even target of exposures from multiple sources in a single season (Henry, 2000). Some of the possible exposure pathways in the egg phase, include maternal deposition, yolk absorption, surface transportation and biocconcentration from surrounding waters. In a subsequent life-stage the possible pathways of exposure might include gaseous exchange through gills or buccopharingeal, pulmonary or body skin and pre or post metamorphosis dietary sources (Henry, 2000). Due to the variety of exposure pathways, the aquatic and terrestrial phases, the position in higher levels of food chains and, the variety of exposed tissues, which allow absorption of a great variety of chemical (eg. lipophilic, hydrophilic, ionic) and physical forms, amphibians also contribute to the transference of contaminants among habitats and trophic levels (Henry, 2000)

The conjugation of many intrinsic and extrinsic factors, some already mentioned and others, like the input of great amounts of noxious substances drained to amphibians natural habitat from surroundings (Vogiatzis and Loumbourdis, 1998) has been responsible by a decrease in many amphibian species around the world (Blaustein and Wake, 1990). Nonetheless the use of amphibians to assess contamination seems very important, if we want to advice more protective measures for the reclamation of contaminated sites.

Specifically, tadpoles are useful indicators. This relates to the fact that tadpoles feed both off the substrate and attached algae, continuously process water for respiration and to their semi permeable skin that readily absorbs substances from the environment (e.g. Schuytema and Nebeker, 1998). Also, tadpoles may occupy a central place when considering trophic levels, by predating macroinvertebrates, even if in a small scale. Consequently, their tissues are potentially exposed to a variety of environmental pollutants, including dissolved toxins, sediment-bound contaminants, and bioconcentrated metals (Freda, 1991; Horne and Dunson, 1995; Berzins and Bundy, 2002). As a result several sublethal responses that include changes in growth, development rate, pigmentation, and expression of morphological deformities are a common feature and may arise in a lesser time when compared to other biota as well as humans (Berzins and Bundy, 2002). Similarly, since their behaviour is easily monitored they can be excellent

models for detecting subtle effects that may occur when contamination is below the dose, at which physiological effects are noticed (Lefcort et al., 1998). Moreover, the ecology and physiology of amphibians, especially as larvae makes them ideal organisms for monitoring levels of contaminants and investigating long-term patterns of natural and anthropogenically induced changes in aquatic ecosystems (Harfenist et al., 1989).

Many studies have already been done with amphibians, especially with tadpoles exposed to metals (e.g. Calevro et al., 1998; Lefcort et al., 1998; Berzins and Bundy, 2002). A wide range of alterations that may include physical and behavioural responses might be recorded when assessing the effects of heavy metals (e.g. Demichelis et al., 2001). Nevertheless the effects of metals in tadpoles will depend of metal relative abundance and bioavailability (Lefcort et al., 1998). Even though many studies have already focused the effect of such contaminants in amphibians, there are still many questions to be answered if we consider mining sites, since the great majority of studies only tested metal solutions, under laboratorial conditions assessing the effects of single metals (e.g. Calevro et al., 1998; Vogiatzis and Loumbourdis, 1998; Loumbourdis and Vogiatzis, 2002; Chen et al., 2006). These laboratorial solutions did not mimic mine effluents in terms of metal composition and pH, which makes difficult to predict responses of organisms under environmental exposures to wastewaters.

Based on the ecotoxicological data gathered, as part of the ecological risk assessment that is being carried out in the area, the presence of Iberian green frog (*Rana perezi* Seoane) in the effluent ponds of Cunha Baixa uranium mine area was unexpected. Hence, our study aimed to assess the real lethal and sub-lethal effects, yielded by short-term (laboratorial) and long-term (*in situ*) exposures to the metal-rich uranium mine effluent in the hatching, early life stages and adults of this species. In addition we also aimed to assess the recovery ability of larvae, when exposure stops. In order to meet these main objectives, two studies were performed simultaneously and are described in the two chapter of this thesis. Both chapters are manuscripts which were already submitted for publication in international journals. Thus:

- Chapter I describes the evaluation of lethal and sub-lethal effects on Iberian green frogs hatching and early life-stages undergoing short-term exposures (96h) to the Cunha Baixa, uranium mine effluent, after pH adjustment, and under laboratorial conditions. The recovery of endpoints was assessed, following larvae for more 96h, after removing them from the effluent;
- Chapter II aimed to perceive the effects derived from in-situ long-term exposures to the effluent, describes the metal contents bioaccumulated and the histopathologies recorded in different organs of Iberian green frog adults, captured

on the mine, as well as genotoxic effects evaluated through an erythrocyte nuclear abnormalities (ENA) assay.

Both chapters were preceded by a general introduction, focusing the problematic of contamination in mining areas; and followed by a general conclusion which integrates the main conclusions of both studies.

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Chapter I

Effects of a uranium mine effluent in the early-life stages of *Rana perezi* Seoane

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Effects of a uranium mine effluent in the early-life stages of *Rana perezi* Seoane

Abstract

Amphibians have been reported as sensitive organisms whose survival has been impaired by several environmental factors. However, sometimes amphibians are found inhabiting in very extreme environments. Thus, in order to perceive the presence of Iberian green frogs (*Rana perezi* Seoane) in the ponds of an uranium mine (Central Portugal) this study aimed to assess the ecotoxicological effects promoted by the mine effluent in the early-life stages of this species. To attain this objective, eggs (collected in a nearby reference river) and laboratory hatching larvae were exposed during 96h to different dilutions of the effluent. All the effects on the hatch success were recorded. The highest concentration of the effluent produced a significant decrease in body length of larvae, as well as a decrease in stimulus reactions and an increase in pigmentation along with tail deformities. A recovery assay showed an increased bioaccumulation of metals, uranium included, resulting from increased effluent exposure.

Introduction

Degradation of freshwater resources quality is a world-wide growing concern. Many are the causes of such degradation that go from agricultural practices to industrial activities such as mining. One of such cases is the abandoned uranium mine in Cunha Baixa (Mangualde, Central Portugal). Uranium mining activity in this area has contributed since the beginning of the extraction and even after the activity has ceased for the production of toxic metal rich-effluent (Antunes et al., 2007a; Antunes et al., 2007b). This kind of effluent usually presents a serious menace to the local fauna (Jarvis et al., 1997) frequently compromising the survival, growth and behaviour of organisms (Lefcort et al., 1998; Antunes et al., 2007a) especially in aquatic systems.

In Cunha Baixa mine, besides the presence of metals in the effluent there, are additional concerns resulting from the presence of radioactive elements such as uranium and their daughter radionuclides (Oliveira and Ávila, 2001). As Sheppard et al. (2005) reviewed, uranium may exert both chemical and radiological toxicity on terrestrial and aquatic organisms. Nonetheless, the main concern regards the aquatic compartment (Antunes et al., 2007a), where the bioavailability and toxicity of U for freshwater species

depends of several physical and chemical parameters of which the pH, alkalinity and hardness are the most important (Sheppard et al., 2005).

Amphibians are, in many cases top predators, playing an important role in the aquatic community and bioaccumulating contaminants (Loumbourdis et al., 1999). In addition they are some of the most sensitive vertebrates to environmental changes, due, in most cases, to an early aquatic-dependent development stage and a highly permeable skin (Duellman and Trueb, 1994). Several studies have shown the toxic effects of metals in this kind of animals. Among the most common deleterious effects of metals on amphibians, reduction of immune functions (Linzey et al., 2003), limb, mouth and tail malformations and other kind of abnormalities (Calevro et al., 1998; Rowe et al., 2001; Linzey et al., 2003), behaviour alterations, growth reduction and survival decrease (Lefcort et al., 1998) were recorded. Even though much information is available on the effects of metal in amphibians, few studies have focused on the effects of radioactive metal rich effluents in these organisms.

Due to the unexpected presence of the Iberian green frog (*Rana perezi* Seoane) in the effluent ponds of Cunha Baixa uranium mine area, our study intended to assess lethal and sub-lethal effects, yielded by short-term exposures to the metal rich-effluent in the hatching and early life stages of this species. In order to do so, egg masses collected from a contaminant-free nearby freshwater resource were used. In addition we also aimed to assess the recovery ability of these animals, regarding parameters impaired and bioaccumulated metals, comparing larvae hatched in the 100% treatment (embryos assay) and submitted to a continued exposure, during more 96h, with those that were transferred to FETAX medium, after the first 96h exposure.

Materials and methods

Study site

The study site was an abandoned uranium mine located in the small village of Cunha Baixa (Mangualde, Central Portugal). The mining area is included in the designated uraniferous belt of the Iberian Peninsula and the ore extraction occurred between 1967 and 1993 (Santos Oliveira and Ávila, 1998). After the operation period the mine pit was filled with low-grade ore and it was flooded with sulphuric acid to extract uranium through an *in situ* leaching process (Santo et al., 1983). Presently, there are 3 small ponds in this area which have a variable water volume depending on the uprising of the underground acidic effluent which, in turn, is determined by fluctuations in the underground water level.

This effluent, because of its origin, has a complex mixture of metals and an extremely low pH (Pedrosa and Martins, 1999). Despite the proximity of the ponds they showed very different chemical characteristics, being the largest (the M pond) the one that provided extremer conditions on account of the direct connection with the underground tunnels of the mine pit and consequently with the mine effluent.

The reference site chosen for this study was an unpolluted segment of the Vouga River (VR) located in the north part of the city of Viseu, a few kilometres from Mangualde, where the presence of the Iberian green frogs has been recorded.

Water sampling

The water samples for the acute assays were collected in both VR and M sites in 20 L plastic containers. These flasks were previously filled with nitric acid (50%, v/v) left overnight and after this period thoroughly rinsed with distilled water. Afterwards the samples were immediately transported to the laboratory where they were stored for no longer than 48h in the dark at 4ºC until further usage in the assays. Water samples for chemical analyses were also collected in the same locations in 0.5 L plastic bottles, washed following the procedure described above and, acidified with *pro analysis* nitric acid (65%), MERCK® to a pH below 2, to prevent metal adsorption. These samples were then stored at 4ºC until chemical analyses was possible.

Test organisms

Egg masses from the Iberian green frog (*Rana perezi* Seoane) with less than 36h were collected in the Vouga River (VR) for the 96h embryo test. For the larvae acute toxicity test (96h) the organisms were obtained from the remaining egg masses, maintained in VR water with aeration, at a constant temperature ($24^{o}C \pm 2$) and photoperiod ($14h^{L}:10h^{D}$), until hatching occurred.

Acute assays

The bioassays were performed according to ASTM recommendations for conducting acute toxicity assays with effluents (ASTM, 1997). The VR water was used to obtain the different effluent dilutions (6.25%, 12.5%, 25%, 50%, 75%, 100%) for both assays. Both effluent and dilution water were filtered by cellulose nitrate ALBET® filters with a 47 mm diameter and a 0.20 µm pore to reduce possible bacteria and parasite contamination. A FETAX (Dawson and Bantle, 1987) control and a VR control were considered to ascertain the quality of the water from the reference site. For each concentration the pH was

adjusted to 8, with 5M NaOH, to eliminate the effect of the effluent acidity. Animals were not fed during the larvae assay.

For the 96h embryo toxicity test the jelly coat of the egg masses was removed by gently stirring for 2-3 minutes in a 2% cistein (Sigma®) solution diluted with FETAX solution and with a 8.1 adjusted pH (Mann et al., 2000). Afterwards, for each of the 2 replicas of effluent concentrations and each of the 4 replicas of the controls, 20 eggs from the same *R. perezi* egg mass were placed, with plastic pipettes, in plastic Petri dishes with 20 ml of the respective effluent dilution and of the controls. This assay was carried out at constant temperature (24±2ºC) and photoperiod ($14h^{L}$:$10h^{D}$) conditions. Embryos mortality, as well as the dissolved oxygen and pH were checked every 24h during the test. At the end of the assay larvae were checked for abnormalities and the body length measured using an Olympus SZX9 stereoscope.

For the acute larvae toxicity test the organisms were obtained from the laboratory hatching eggs. In this assay larvae from the same egg mass with less than 6 days were placed in 500ml plastic vessels in groups of 5 *per* each of the 5 replicas for every effluent concentration and controls (FETAX and VR water). The test was performed in the conditions already described. The body size was recorded at the beginning and at the end of the assay, and the pH, the dissolved oxygen and the mortality were registered every 24h. The dead larvae found were removed from the vessels in each observation. Larvae movement was checked daily for any alterations by gentle prodding using a plastic pipette and at the end of the test the existence of abnormalities in the organisms was verified.

An extra 96h assay (recovery assay) was carried out using the larvae obtained from the embryo test. In this assay the larvae obtained from the FETAX control were kept in FETAX and were also used as a control. The 20 larvae were divided in 4 replicas of 5 organisms placed in 500ml plastic vessels and maintained at the above described laboratory conditions. Of the total 40 larvae obtained from the 100% effluent concentration 20 were maintained in the same effluent concentration using, as mentioned for the control, 4 replicas of 5 organisms. The remaining 20 larvae were placed in FETAX solution. The parameters measured were the same as for the larvae toxicity test with the additional evaluation of the whole-body metal concentrations. For this purpose 5 randomly selected larvae exposed to each assay condition (FETAX-FETAX; 100% effluent concentration-FETAX; 100% effluent concentration-100% effluent concentration) were stored at -20^{o}C until metal quantification was possible.

Chemical analyses

The total concentrations of Ca, Mg, Be, Al, Mn, Fe, Ni, Zn, Sr, Cd, Pb, and U were quantified, in the VR water, in the FETAX solution, in the mine effluent (M) and in the larvae, from the recovery assay, by inductively coupled plasma mass spectrometry (ICP/MS) (APHA, 1995). Hardness values based on total content of Ca and Mg were determined by the following equation:

Hardness, mg equivalent $CaCO_3$/L = 2.497 [Ca, mg/L] + 4.118 [Mg, mg/L] (APHA, 1995).

For the quantification of metals bioaccumulated in larvae a previous wet-digestion was required. Hence, the organisms were oven-dried at 105ºC until a stable weight was achieved. After having been dried, the weight of the larvae was recorded, to the nearest 0.1mg, and they were digested, in closed teflon flasks, with 3ml of nitric acid suprapur Merck®, 65%, in a 60ºC sand-bath. After the solution became free of solid fragments, aliquots of 0.5ml of suprapur hydrogen peroxide (30%) MERCK® were added. The final volume of the solution was made up to 5ml with Milli-Q® water (18.2Ω). Since the dry mass of each larva was extremely reduced, all the larvae from each assay condition were digested jointly.

Statistical analyses:

The results obtained from the acute assays did not allow the determination of an EC_{50}. Thus these results obtained for the endpoints in the embryo and in the larvae assays were analysed for differences between organisms exposed to the VR water and those exposed to the different effluent concentrations, using a parametric one-way analysis of variance (ANOVA) followed by a Dunnet test (Zar, 1996). Significant differences among the organisms exposed to the VR water and those exposed to the FETAX medium were also analysed. Finally the recovery assay had the same statistical treatment but the comparisons were made between the FETAX control and the two other tested conditions.

Results

The chemical characterization of the FETAX solution, VR and M water samples, maximum recommended values (MRVs) and maximum admissible values (MAV) for water for human consumption, available on Portuguese legislation (MA, 1998) are described on Table 1. Since these legal values may be considered overprotective, other benchmark

values, for metals in surface waters, were obtained from EPA databases and are also reported on Table 1.

Table 1 – Chemical and Metal concentration data for VR (reference) and M (effluent). Metal concentration analysed by inductively coupled plasma mass spectrometry (ICP/MS) and hardness calculated for water samples by ICP-MS determination of Ca and Mg concentration.

	EC20 -SB	EPA R4-SB	SW EPA R5-SB	SW EPA R6 FW - SB	**MRV**	**MAV**	**FETAX**	**VR**	**M**
Hardeness $CaCO_3$ (mg/l)					NLV	500	109.7	3.0	27.8
Dissolved O_2 (mg/l)					NLV	NLV	6.9	7.48	6.6
Conductivity (µS/cm)					400	NLV	---	45	23000
pH					6.5-8.4	NLV	7.9	7.6	4.25
Metal (µg/l)									
Be	NLV	0.53	3.6	5.3	NLV	NLV	<1	<1	**23**
Al	75	87	NLV	87	50	200	<10	36.2	---
Mn	NLV	NLV	NLV	120	20	---	<0.5	9.7	**7450**
Fe	NLV	1000	NLV	1000	50	200	<10	99.3	**3260**
Ni	11	87.7	28.9	87.4	---	50	<0.5	<0.5	**154**
Zn	21	58.91	65.7	58.1	5	NLV	30	15.1	**451**
Sr	NLV	NLV	NVL	1500	NLV	NLV	5.46	11.8	65.3
Pb	0.35	1.32	1.17	1.0	NLV	50	<0.5	<0.5	0.69
U	NVL	NVL	NVL	700	NLV	NLV	<0.1	0.26	**1750**

MRV and MVA stands for Maximum recommendable values and Maximum admissible values of waters for human consumption (MA, 1998).
EC20 – Sensitive species surface water screening benchmark. Available on http://rais.ornl.gov/cgi-bin/eco/bench_select_(9/12/2007);
EPA R4 Chronic Surface water screening benchmark. Available on http://rais.ornl.gov/cgi-bin/eco/bench_select_(9/12/2007);
SW EPA R5 ESL Surface water screening benchmark. Available on http://rais.ornl.gov/cgi-bin/eco/bench_select_(9/12/2007);
SW – EPA R6 FW Surface water screening benchmark. Available on http://rais.ornl.gov/cgi-bin/eco/bench_select_(9/12/2007);
NLV stands for No Legal Values established.
The values exceeding the different benchmark values available were written in bold letter.

The water from the M pond revealed an acidic pH, being below the corresponding Portuguese MRV, in opposition to the VR site where a neutral pH value was recorded.

Conductivity was extremely high in the M pond being well above the value recorded in the VR water course as well as the MRV. Regarding total metal concentrations, higher values were recorded in the M pond for all the elements analysed. Beryllium, manganese, iron, nickel, zinc and uranium total concentration even surpassed the corresponding MRV or the benchmark values for surface waters provided by EPA databases. As for Al, due to apparatus interferences, it was not possible to measure its concentration in the water sample from the mine. However, previous metal determinations on water samples from the same pond revealed values that ranged from 495 to 9070 µg/L, in spring and autumn respectively (Antunes et al., 2007a). Therefore this element was certainly present in the mine water. In spite of the U total concentration in the M pond have greatly exceeded (6730x), the one recorded at VR water course and the benchmark values proposed by EPA, it was below the predicted no-effect concentration value for freshwater fishes (2.8mg U/L) determined by Sheppard et al. (2005).

The embryo acute toxicity assay revealed that the hatching success was not affected by any concentration of the effluent. Nonetheless, at the end of the assay a significant increase ($F=9.591$; $d.f.=6$; $p<0.001$) in the body length was registered between the larvae from the VR and from the 100% effluent concentration (Figure 1). No significant difference was observed among the two controls, which validated VR as natural reference water. Additionally, no abnormalities were recorded on hatching larvae from all the tested effluent concentrations.

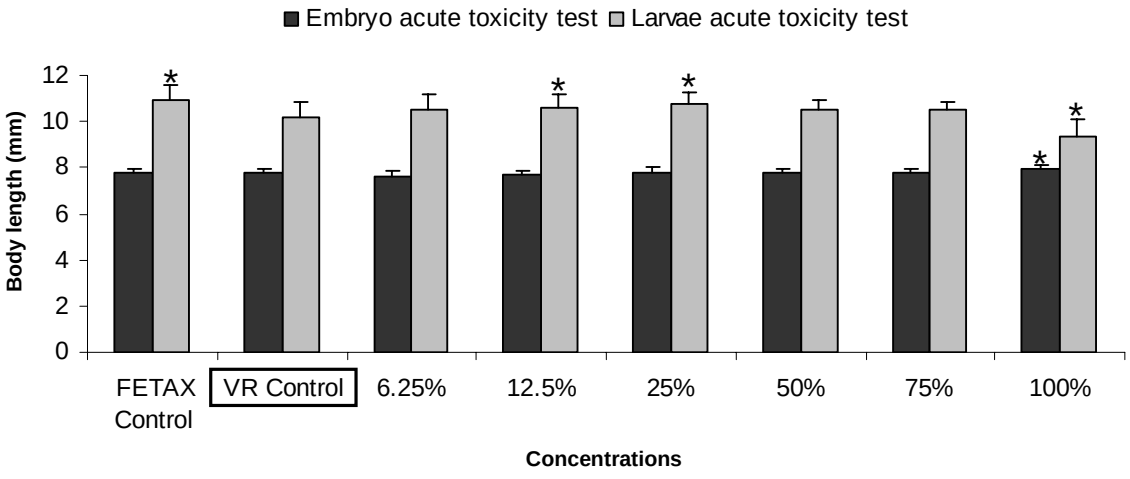

Figure 1. Larvae body length at the end of both the embryo acute toxicity assay and the larvae acute toxicity assay. *: represents a statistical significant difference ($p<0.05$) between VR control and the respective treatment. Error bars represent standard deviation.

For the larvae acute toxicity test a significant mortality was recorded only for the 100% effluent concentration with a 28% mean ($F=12.250$; d.f.=6; $p<0.001$). Furthermore, a significant decrease ($F=14.959$; d.f.=6; $p<0.001$) in body size of larvae exposed to this effluent concentration was observed (Figure 1). Nonetheless, larvae from the 12.5% and 25% effluent concentrations revealed a significantly higher ($F=14.959$; d.f.=6; $p<0.001$) body length when compared with those exposed to the VR control. Even though FETAX solution control presented larvae with a significantly higher body length, ($F=15.201$; d.f.=7; $p<0.001$) due probably to higher mineral content (Table 1), both controls exhibit organisms with similar physical appearance while larvae from the 100% effluent concentration had a darker pigmentation (Figure 2, 3). Adding to this, tail abnormalities were also observed (Figure 2, 3) as well as differences in response to mechanical stimulus not only for 100% effluent concentration but for every dilution of the effluent above 50%. Larvae response to movement was delayed and sometimes reaction was only obtained after gentle prodding with a plastic pipette. Larvae mainly remained at the bottom of the vessels. In the lower concentrations no prodding was necessary to observe movement due to a rapid response of larvae to water disturbance by the pipette.

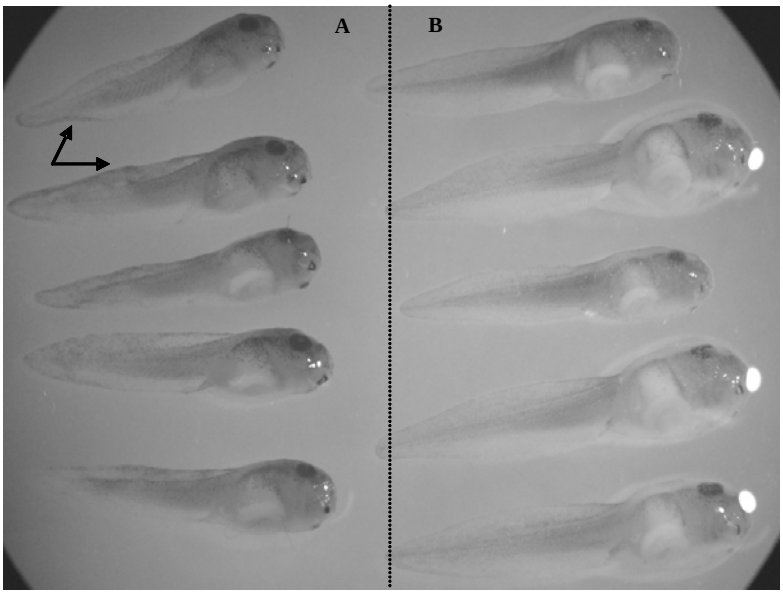

Figure 2. A –*R. perezi* larvae after a 96h exposure to 100% in the larvae acute toxicity test revealing darker pigmentation. Arrows point to tail abnormalities.
B – *R. perezi* larvae after a 96h exposure to VR control in the larvae acute toxicity test. (Amplification 8X)

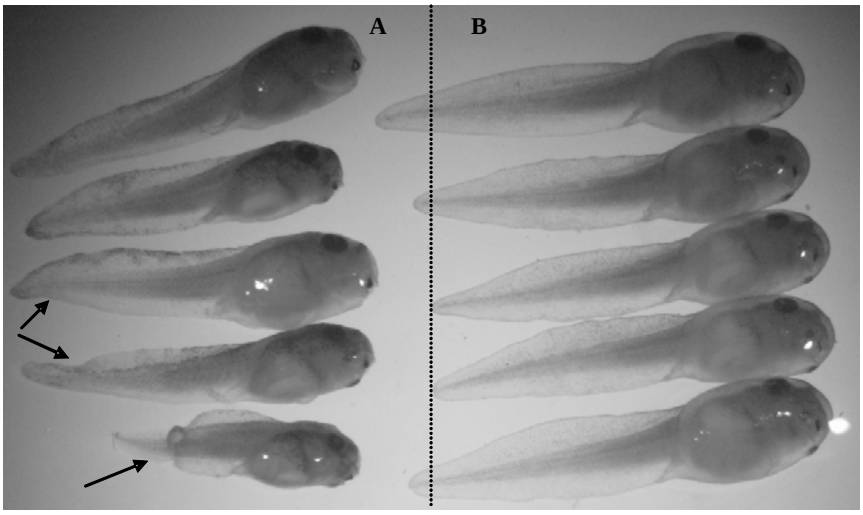

Figure 3. A –*R. perezi* larvae after a 96h exposure to 100% in the larvae acute toxicity test revealing darker pigmentation. Arrows point to tail abnormalities.
B – *R. perezi* larvae after a 96h exposure to FETAX control in the larvae acute toxicity test.
(Amplification 8X)

Mortality in the recovery assay was negligible, however a significant decrease ($F=41.464$; $d.f.=2$; $p<0.001$) in body length was recorded for larvae that continued their exposure in the 100% effluent concentration for another 96 hours (Figure 4).

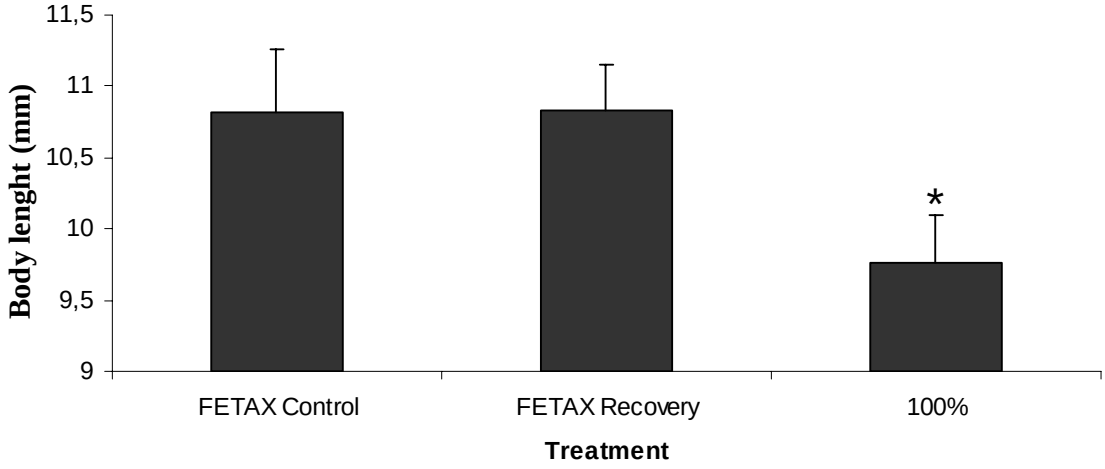

Figure 4. Larvae body length at the end of the recovery assay. *: represents a statistical significant difference ($p<0.05$) between FETAX control and the respective treatment. Error bars represent standard deviation.

The larvae that were removed from the effluent to the FETAX medium were not statistically different from the larvae that were always in the FETAX solution, in terms of body length. As observed, for the larvae acute toxicity assay, movement was also affected in the 100% effluent concentration larvae after an exposure during more 96h. As expected, the bioaccumulated metal concentrations (Table 2) revealed higher values for larvae with a greater exposure to the effluent.

Table 2 – Metal concentrations bioaccumulated in larvae (n=5 each sample) from the recovery assay analysed by ICP/MS. Values correspond to dry weight. Total time exposure to effluent is also presented.

Metal	FETAX larvae (mg/kg) 0 h	FETAX Recovery larvae (mg/kg) 96 h	100% larvae (mg/kg) 192 h
Be	-------	0,05	1,40
Al	34,06	40,71	603,01
Mn	2,54	22,16	118,63
Fe	117,14	115,70	321,48
Ni	2,83	2,62	5,36
Zn	195,23	225,86	237,97
Sr	0,73	1,11	2,83
Pb	0,56	1,02	2,27
U	0,08	0,78	113,37

Discussion

Metals present in metal contaminated freshwater systems have proved to be toxic to many aquatic organisms, from macrophytes (Charles et al., 2006), to invertebrates (Lopes et al., 1999; Pereira et al., 2000; Antunes et al., 2007a, Antunes et al., 2007b; Marques et al., submitted) fish (Labrot et al., 1999) and amphibians (Mitchell et al., 2005; Lefcort et al., 1998). The latter group revealed to be quite sensitive to water contaminants due to the high permeability of their skin and the presence of gills in the earlier life stages. Together these two structures provide the major routes for metal uptake (Vogiatzis et al., 1997). In our embryo acute toxicity assay (Figure 1) the apparent absence of any toxic effect and, in opposition, the stimulation of growth in the highest concentration might be owed to the fact that in such early development stage gills are rudimentary, the mouth is not functional and

the embryo is protected by the inner jelly coat that remains after the removal of the outer jelly coat, reducing thus the contact with the test solution. Even though the jelly coat reduces chemical uptake by the embryos (Edginton et al., 2007) a small portion remains available, which may stimulate growth. Sagan (1987) has reported that in small concentrations radionuclide may be responsible for such growth stimulation, and although these elements were not analysed in this study, their presence in the mine effluent is unquestionable. Despite this enhancement of growth and the possible chemical protection barrier provided by the inner jelly coat, our recovery assay suggested a great increment in the bioaccumulated metal concentrations (mainly for Al, Mn, Fe, Sr and U) with an increasing period of exposure (Table 2). These results might also imply that the metals present in the effluent only influence *R. perezi* development after organogenesis (Haywood et al., 2004). Mitchell et al. (2005), when testing depleted uranium in *Xenopus laevis,* obtained relatively similar results for the 96 hour embryo assay, observing no differences between controls and treatments but revealing toxicity for longer exposures, strengthening thus the hypothesis that the metals present in the effluent only have deleterious effects after organogenesis.

As larvae continue developing, their mouth becomes functional, gills develop and the jelly coat disappears making them more susceptible to water contaminants (Mitchell et al., 2005). Such was the case of the larvae in the 100% treatment in both the recovery assay and in the larvae acute toxicity assay, whose body length was significantly smaller than in the corresponding controls (FETAX and VR water, respectively). Growth retardation is a very common effect yielded by exposures to metals (Haywood et al., 2004; Lefcort et al., 1998) due to metabolic costs of detoxification mechanisms (Rowe et al., 1998). A higher risk of predation for tadpoles is also expectable, because they spend more time in more vulnerable stages (Loumbourdis et al., 1999), as well as an increasing possibility of spending the winter as larvae, reducing even further the probability of survival (Carey and Bryant, 1995). Nonetheless in our work the results for the larvae acute toxicity assay showed a small growth stimulus in the 12.5% and 25% effluent concentrations, when compared with the VR control. A similar result was obtained with cladocerans (*Daphnia* spp.), which were exposed to effluent from the same pond and have showed a stimulation of measured sub-lethal parameters in the lowest dilutions (Antunes et al., 2007b). As it was mentioned above it is possible that the existent radionuclides in small concentrations in this effluent might have acted as a stimulus for growth. In fact this stimulus usually results from biosynthetic correction mechanisms or

even, in aquatic organisms, derives from excess water accumulation which is not related with a normal growth (Stebbing, 1982).

The recovery assay showed that an acute embryonic exposure to the 100% treatment and the subsequent transference to the FETAX solution apparently produces no short-term morphologic or physiologic effects. Nonetheless, when comparing with the FETAX control a higher metal bioaccumulation occurred. In the recovery assay larvae exposed continuously to the 100% effluent also showed an increased uptake of metals, in parallel with a decrease in growth, and changes on movement and on pigmentation. Tail abnormalities were also observed probably contributing to the lower reaction of organisms to mechanical stimulus. Both movement and pigmentation were similarly affected in the larvae acute toxicity assay above the 50% concentration. Regarding the increase in pigmentation, such result might be related with the ability of melanin from pigmented cells to sequester metals, pointing this pigment as a powerful antioxidant, as it has been reported by other authors (Korytowski et al., 1995). In addiction, other studies have also reported the increase of pigmented cells, called melanomacrophage, with the increase of contaminants in the liver of amphibians (Fenoglio et al., 2005; Loumbourdis and Vogiatzis, 2002). Such is consistent with the antioxidant action mentioned above for melanin as well as with the increase in pigmentation of larvae exposed to effluent dilutions above 50%, recorded in our study.

In this study, from all the observed metals in the effluent one that particularly rises concern is U since it is not only chemically toxic but it is also associated with radiations released by its decay to lower energetic levels. This metal is present in the Cunha Baixa mine effluent in considerable concentrations; however other toxic metals such as aluminium have proved to be bioaccumulated by amphibian larvae. Nonetheless the toxicity of metals, in this study, might have been milded by the pH adjustment since several studies have shown the dependence of the toxicity and bioavailability of metals from this factor (Charles et al., 2002; Sheppard et al., 2005).

Conclusions

Acute effects should not be the only biological response considered in the evaluation of adverse results of complex mixtures of metals from mine effluents. Sub-lethal effects, yielded by such exposures are probably more significant in evaluating meaningful ecological impacts. Our study revealed effects on Iberian green frogs mainly occurring at higher effluent concentrations and after the hatching of the egg masses. Reduced growth,

small tail deformities, darker pigmentation and reduced movement in response to a stimulus were some of the verified abnormalities. These results were consistent with the absence of amphibian larvae on the mine effluent pond under evaluation, which is used by adults mainly as a shelter. Some authors reviewed by Rowe and Freda (2000) have demonstrated the ability of adult amphibians to avoid potentially toxic breeding sites. Hence, in this mining contaminated environment, field surveys with the assessment of several molecular biomarkers, tissue residue analysis and histopathological effects are required and are in course in order to assess if these apparent mild effects recorded in laboratory assays persist and evolve as result of an *in situ* bioaccumulation of metals.

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Chapter II

Histopathological Changes and Erythrocytic Nuclear Abnormalities in Iberian Green Frogs (*Rana perezi* Seoane) from an Uranium Mine Pond

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Histopathological Changes and Erythrocytic Nuclear Abnormalities in Iberian Green Frogs (*Rana perezi* Seoane) from an Uranium Mine Pond

Abstract

In spite of their sensitivity to anthropogenic stressors, adults of *Rana perezi* Seoane were found inhabiting effluent ponds from a uranium mine. Due to the presence of such organisms on this environment, it becomes of paramount importance to assess the damages induced by local contamination on these aquatic vertebrates, in order to integrate this information on a site-specific risk assessment that is being carried out on the area. To attain this purpose an ethical and statistical acceptable number of green frogs were captured in the mine pond (M) and in a pristine river (VR), a few kilometres from the mine. Bioaccumulation of metals and histopathological alterations were evaluated in the liver, kidneys, spleen, lungs and testes of animals. Simultaneously, blood samples were collected for the evaluation of genotoxic damage on erythrocytes. Animals captured on the M pond showed significant higher levels of Be, Al, Mn, Fe and U in the liver, as well as Pb and U in the kidney. The liver was the main target organ for the bioaccumulation of Be, Al, Fe and U. However, renal histopathologies were more severe. The main tissue alterations recorded in animals from the mine were: a slightly increase in melanomacrophagic centers (MMC) in liver, lung and kidneys; dilatation of the renal tubule lumen associated with tubular necrosis. A significant higher number of erythrocytic abnormalities (lobed, notched and kidney shaped nuclei and micronuclei) was recorded in frogs from M, along with a significant lower frequency of immature erythrocytes. Both observations suggested that the removal of abnormal blood cells may be compromised.

Introduction

The mining activity is a source of physical, chemical, biological and landscape alterations. Usually large amounts of solid residues are produced and accumulated in the vicinity of the mines (Oliveira et al., 2001; Araújo et al., 2002). These solid wastes exposed to atmospheric conditions as well as the processes carried out to treat pore ore, usually give rise to the production of metal-rich acidic effluents (e.g. Lopes et al., 1999; Antunes et al., 2007a). As a consequence of low pH values, and of the high persistency of metals in the environment, fauna and flora may suffer alterations, causing a decrease in the populations density or even the total disappearance from the site (Linzey et al., 2003). Furthermore

the situation is worsened when radioactive elements, like uranium, are extracted as they exert both chemical and radiological toxicity (Domingo, 2001). Despite the hazard posed by this kind of contamination there are few studies assessing its impact on indigenous species (e.g. Antunes et al., 2007a; Antunes et al., 2007b), and frequently they did not cover all the trophic levels, or main functional groups, of food chains. Regarding vertebrates, several studies have already shown that chronic exposures to metals both in laboratory (e.g. Donnadieu-Claraz et al., 2007) and in field conditions (e.g. Pereira et al., 2006) might damage internal organs of vertebrates. Mainly owed to their role in detoxification and homeostasis (Kerr, 1999) liver and kidneys are usually target organs and may accumulate metals and display damages (Cooley et al., 2000a; Cooley et al.,2000b; Loumbourdis, 2005; Pereira et al., 2006). These organs may undergo alterations in their enzyme activities (e.g. Fenoglio et al., 2005) and, either mild (e.g. tissue swelling) (Linzey et al., 2003; Pereira et al., 2006) or severe (e.g. necrosis) (Cooley et al., 2000a) histopathological damage. Besides these two organs, others, that play different roles in the organism, such as gonads or lungs, may accumulate metals as well, and/or display alterations in the activity of several enzymes (Cooley et al., 2000b; Periyakaruppan et al., 2007).

Beyond histological alterations metals (e.g. Cd, Zn, Be, Sr, Ni and Pb) may also present genotoxicity (Banu et al., 2001; Gordon and Bowser, 2003; ATSDR, 2004; ATSDR, 2005; Monleau et al., 2006; ATSDR, 2007). Regarding uranium, few studies are available describing its toxicity to genetic material in humans and other animals (e.g. Monleau et al., 2006). When these effects were reported for uranium miners it was not possible to establish direct cause-effect relationships, since these workers were simultaneously exposed to a panoply of other elements (ATSDR, 1999a). A direct relationship was established more recently by Monleau et al. (2006), which showed the induction of DNA damage in bronco - alveolar and in kidney cells of rats undergoing repeated acute exposures to UO_2, through inhalation.

With respect to aquatic vertebrates, amphibians are particularly sensitive to changes since they have both aquatic and terrestrial life stages and a semi permeable skin (McDiarmid and Mitchell, 2000). This kind of organism has shown to be a good bioindicator of aquatic contamination when compared to other aquatic vertebrates (e.g. Selvi et al., 2003; Marques et al., submitted) mainly because of the highly permeable skin that readily absorbs substances from the environment (Schuytema and Nebeker,1998; Demichelis et al., 2001; Mitchell et al., 2005). Such vulnerability has been responsible as well by the decline in amphibian populations worldwide (Linzey et al., 2003; Stuart et al.,

2004). However, in spite of their widely recognized sensitivity, adults of green frogs (*Rana perezi* Seoane) were found inhabiting effluent ponds from an abandoned uranium mine Cunha Baixa (Mangualde, Central Portugal).

Hence, as part of a risk assessment that is been carried out in this contaminated mine area, this study aimed to assess the impacts of the locally produced acidic and metal rich effluent on green frogs inhabiting the mine ponds. In order to attain this goal a histopathological evaluation of liver, kidney, lung, testes and spleen of animals was performed, along with an erythrocytic nuclear abnormalities assay (ENA) to assess genotoxic effects. The micronuclei (MN) assay, is a simple and one of the preferred methods to evaluate genotoxic damage because it enables the measure of chromosome loss and breakage (Fenech, 2000). Micronuclei are expressed in dividing cells containing chromosome breaks and/or chromosomes unable to travel to the spindle poles during mitosis (Fenech, 2000; Stoiber et al., 2004). They can result from an interaction of chemical, physical or biological agents with nongenomic structures such as the mitotic spindle. Besides MN some authors have interpreted other nuclear abnormalities as resulting from analogous damage (Ayllón and Garcia-Vazquez, 2000; Serrano-Garcia and Montero-Montoya, 2001). Thus, nuclear abnormalities such as, kidney shaped, lobed and notched nuclei (Carrasco et al., 1990) along with micronuclei can be counted in an ENA assay to assess the exposure to genotoxic contaminants (Ayllón and Garcia-Vazquez, 2000; Pacheco et al., 2005; Guilherme et al., 2007). The histopathological evaluation and the ENA assay were performed both in mine pond frogs and in frogs captured in a nearby unpolluted water course in order to compare the results obtained.

Material and Methods

Study site

The present study was carried out in an abandoned uranium mine located in the small village of Cunha Baixa (Mangualde, Central Portugal) where ore extraction occurred between 1967 and 1993 (Santos Oliveira and Ávila, 1998). After the exploration period the mine pit was filled with low-grade ore and it was flooded with sulphuric acid to extract uranium through an *in situ* leaching process (Santo et al., 1983). Presently, there are three small ponds in this area which have a variable water volume, depending on the uprising of the underground acidic effluent which, in turn, is determined by fluctuations in the level of the aquifer. This effluent, because of its origin, has a complex mixture of metals and an extremely low pH (Antunes et al., 2007a; Antunes et al., 2007b). Despite

the proximity of the ponds they revealed very different chemical characteristics, being the largest (the M pond) the one with extremer conditions on account of the direct connection with the underground tunnels of the mine pit and consequently with the mine effluent. Iberian green frogs (*Rana perezi* Seoane) are frequently found on the three ponds; however no mass eggs were recorded on the M pond.

The reference site chosen for this study is an unpolluted segment of the Vouga River (VR) located in the north part of the city of Viseu, a few kilometres from Mangualde, where frogs from the same species have been recorded.

Organisms and water sampling

Iberian green frogs were captured from both VR and M sites using a hand net, and then taken alive to the laboratory where they were anaesthetised, proceeding with the measurement of body weight and length and sex determination. Animals were then sacrificed and blood was collected by cardiac puncture with heparinised syringes. Blood smears were immediately prepared (please see below). Lung, spleen, testes, liver and kidney were dissected for histopathological evaluation and weighted to the nearest 0.1mg. Sections of liver and kidney were taken and stored at -20ºC until metal quantification was possible

Water samples for chemical analyses were also collected from the VR water course and from the M pond in 0.5 L plastic bottles acidified with *pro analysis* nitric acid MERCK® (65%), to a pH below 2, to prevent metal adsorption. The bottles were previously filled with nitric acid (50%, v/v) left overnight and after this period thoroughly rinsed with distilled water. Conductivity, as well as pH values were measured onsite before water sampling using a LF 330/SET conductivity meter and a WTW 330/SET-2 pH meter.

Tissues preparation for light microscopy

Small sections of liver, kidney, spleen, lung and testes were collected and fixed for 24h in Bouin's fluid. Sequentially dehydration in graded concentrations of ethanol and sample inclusion in paraffin wax was performed. Sections (5-7 µm thick) were obtained and stained with hematoxylin-eosin for light microscopic examination. Observations were made and photographs taken using an Olympus BX41 microscope equipped with an automatic photomicrographic system.

Genotoxicity and immature erythrocyte frequency

In order to evaluate genotoxic effects the erythrocytic nuclear abnormalities assay was performed in mature peripheral erythrocytes (Carrasco et al., 1990). For such purpose three blood smears were performed per animal. Blood smears were fixed with methanol for 10 minutes and stained with Giemsa during 30 minutes. For each slide 1000 erythrocytes were scored under a 1000x magnification in order to determine erythrocyte nuclear abnormalities. According to the classification of Carrasco et al. (1990) the observed nuclear alterations were divided in the following categories: kidney shaped, notched, micronucleus and lobed. Additionally the immature erythrocyte (IE) frequency was determined for each of the 1000 erythrocytes per slide using the following expression:

$$\text{IE frequency (‰)} = (IE/(ME+IE))x1000$$

IE = immature erythrocyte; ME = mature erythrocyte

Chemical analyses

The total concentrations of Be, Al, Mn, Fe, Ni, Zn, Sr, Cd, Pb, and U were quantified, in the VR water and in the mine effluent (M) , as well as in the liver and kidney of animals from both sampling sites, by inductively coupled plasma mass spectrometry (ICP/MS) (APHA, 1995). Furthermore for water samples Ca and Mg were also determined by ICP/MS and hardness values, based on the total content of Ca and Mg, were determined by the following equation:

Hardness, mg equivalent $CaCO_3$/L = 2.497 [Ca, mg/L] + 4.118 [Mg, mg/L] (APHA, 1995).

For the quantification of metals in the liver and kidney a previous wet-digestion was required. Hence, the tissues were oven-dried at 105ºC until a stable weight was achieved. After having been dried, the weight of the tissue was recorded, to the nearest 0,1mg, and they were digested, in closed teflon flasks, with 3ml of nitric acid suprapur Merck®, 65%, in a 60ºC sand-bath. After the solution became free of solid fragments, aliquots of 0.5ml of suprapur hydrogen peroxide (30%) MERCK® were added. The final volume of the solution was made up to 5ml with Milli-Q® water (18.2Ω). Sample blanks were obtained following the same procedure described for wet digestion.

Statistical analyses:

To test statistical significant differences for body measurements (weight and length), erythrocytic nuclear abnormalities and immature erythrocyte frequency between

organisms from VR and M sites, results were analysed using a parametric one-way analysis of variance (ANOVA). The same statistical procedure was used to analyse differences in metal content, for each element analysed, between livers and kidneys of animals from VR and M sites. Since only the assumption of normal distribution of data was not accomplished, in some situations, ANOVAs were performed with raw data (Zar, 1999; ATSDR, 1999a). Pearson's correlation coefficients (r) were calculated to investigate, for each tissue (liver and kidney), associations among the concentrations of metals recorded.

Results

Water samples chemical characterization

The water quality of both VR and M sampling sites was evaluated by comparing the results of chemical parameters (Table 1) with the existent maximum recommended values (MRVs) and maximum admissible values (MAV) for water for human consumption available on Portuguese legislation (MA, 1998). Since these values may be considered overprotective, other benchmark values, for metals in surface waters, were obtained from EPA databases and are also reported on Table 1.

The water from the M pond revealed an acidic pH, being below the corresponding Portuguese MRV, in opposition to the VR site where a neutral pH value was recorded. Conductivity was extremely high in the M pond being well above the value recorded in the VR water course as well as the MRV. Regarding total metal concentrations, higher values were recorded in the M pond for all the elements analysed. Beryllium, manganese, iron, nickel, zinc and uranium total concentration even surpassed the corresponding MRV or the benchmark values for surface waters provided by EPA databases. As for Al, due to apparatus interferences, it was not possible to measure it's concentration in the water sample from the mine. However, previous metal determinations on water samples from the same pond revealed values that ranged from 495 to 9070 µg/L, in spring and autumn respectively (Antunes et al., 2007a). Therefore this element was certainly present in the mine water. In spite of the U total concentration in the M pond has greatly exceeded the one recorded at VR water course, as well as benchmark values proposed by EPA, it was below the predicted no-effect concentration value for freshwater fishes (2.8mg U/L) determined by Sheppard et al. (2005).

Table 1 – Chemical and Metal concentration data for VR (reference) and M (effluent). Metal concentration analysed by inductively coupled plasma mass spectrometry (ICP/MS) and hardness calculated for water samples by ICP-MS determination of Ca and Mg concentration.

	EC20 - SB	EPA R4-SB	SW EPA R5-SB	SW EPA R6 FW -SB	**MRV**	**MAV**	**VR**	**M**
Hardeness $CaCO_3$ (mg/l)					NLV	500	3.0	27.8
Dissolved O_2 (mg/l)					NLV	NLV	7.48	6.6
Conductivity (µS/cm)					400	NLV	45	23000
pH					6.5-8.4	NLV	7.6	4.25
Metal (µg/l)								
Be	NLV	0.53	3.6	5.3	NLV	NLV	<1	**23**
Al	75	87	NLV	87	50	200	36.2	---
Mn	NLV	NLV	NLV	120	20	---	9.7	**7450**
Fe	NLV	1000	NLV	1000	50	200	99.3	**3260**
Ni	11	87.7	28.9	87.4	---	50	<0.5	**154**
Zn	21	58.91	65.7	58.1	5	NLV	15.1	**451**
Sr	NLV	NLV	NVL	1500	NLV	NLV	11.8	65.3
Pb	0.35	1.32	1.17	1.0	NLV	50	<0.5	0.69
U	NVL	NVL	NVL	700	NLV	NLV	0.26	**1750**

MRV and MVA stands for Maximum recommendable values and Maximum admissible values of waters for human consumption (MA, 1998).
EC20 – Sensitive species surface water screening benchmark. Available on http://rais.ornl.gov/cgi-bin/eco/bench_select_(9/12/2007);
EPA R4 Chronic Surface water screening benchmark. Available on http://rais.ornl.gov/cgi-bin/eco/bench_select_(9/12/2007);
SW EPA R5 ESL Surface water screening benchmark. Available on http://rais.ornl.gov/cgi-bin/eco/bench_select_(9/12/2007);
SW – EPA R6 FW Surface water screening benchmark. Available on http://rais.ornl.gov/cgi-bin/eco/bench_select_(9/12/2007);
NLV stands for No Legal Values established.
The values exceeding the different benchmark values available were written in bold letter.

Tissues residues analysis

All of the captured Iberian green frogs were males, with exception of two from the M pond, reducing the influence of sex, in metals bioaccumulation and on the effects recorded. Individuals sampled in the uranium mine showed, in average, a significantly higher body length ($F=5.386$; $d.f.=1$; $p<0.032$) however, no statistically significant differences were recorded in body weight (Table 2).

Table 2 – Average body length and body weight of the captured organisms in VR (n=5) and M (n=15) with the corresponding standard deviation values.

Site	Body Lenght (cm)	Body Weight (g)
VR	6.4 ± 0.62*	24.65 ± 11.06
M	7.5 ± 0.90*	34.68 ± 13.16

*: represents a statistical significant difference ($p<0.05$) between VR water course and the M pond.

Metal concentrations were recorded in the liver and in the kidney (Table 3) of organisms from both sites. The results gathered for the liver showed significant higher values of Be ($F=7.091$; d.f.=1; $p<0.016$), Al ($F=6.182$; d.f.=1; $p<0.023$), Mn ($F=5.546$; d.f.=1; $p<0.030$), Fe ($F=16.145$; d.f.=1; $p<0.001$) and U ($F=12.411$; d.f.=1; $p<0.002$) in the contaminated site organisms when compared with frogs from the VR water course. The same trend was recorded for the kidney, since, in general, higher concentrations of metals were recorded in animals from the mine pond. Nonetheless statistical significant results were obtained only for Pb ($F=5.924$; d.f.=1; $p<0.038$) and U ($F=13.725$; d.f.=1; $p<0.005$), being the higher values of both metals recorded in frogs captured in the mine effluent.

Statistical significant correlations, between metals, in the liver and kidney of *Rana perezi* are described in Table 4. Positive associations, with statistical significance, between essential and non-essential elements were recorded in the liver (Fe/Be: $r = 0.626$, $p = 0.00315$; Fe/Al: $r = 0.724$, $p <0.001$; Zn/Be: $r =0.666$, $p = 0.00136$; Zn/Al: $r = 0.603$, $p = 0.00492$; Fe/U: $r = 0.653$, $p = 0.00180$; Zn/U: $r = 0.520$, $p = 0.0188$). Nonetheless, associations of the same kind were stronger in the kidney (Mn/Be: $r =0.999$, $p <0.001$; Mn/Al: $r =0.984$, $p =0.001$; Fe/Be: $r = 0.612$, $p =0.0455$; Ni/Sr: $r =0.700$, $p = 0.0164$; Mn/U: $r = 0.699$, $p = 0.0166$; Fe/U: $r =0.714$, $p =0.0136$). In both organs very strong significant positive associations were recorded between two non-essential elements Al and Be (Liver Al/Be: $r =0.933$, $p<0.001$; Kidney Al/Be: $r = 0.984$, $p<0.001$).

Table 3 – Concentration of metals on *Rana perezi* from VR and M sites.

		Liver		Kidney	
		Average ± SD	n	Average ± SD	n
Be					
	VR	0.005 ± 0.010*	5	0.000 ± 0.000	4
	M	0.379 ± 0.309*	15	0.024 ± 0.065	7
Al					
	VR	35.478 ± 33.795*	5	19.996 ± 6.119	4
	M	141.890 ± 92.219*	15	31.049 ± 40.380	7
Mn					
	VR	8.405 ± 3.122*	5	9.868 ± 2.228	4
	M	16.185 ± 7.059*	15	45.201 ± 83.059	7
Fe					
	VR	356.670 ± 192.955*	5	213.186 ± 80.329	4
	M	2005.384 ± 895.063*	15	306.947 ± 72.390	7
Ni					
	VR	0.450 ± 0.223	5	5.507 ± 4.345	4
	M	1.910 ± 4.170	15	4.514 ± 2.045	7
Zn					
	VR	52.478 ± 18.381	5	84.630 ± 32.369	4
	M	64.787 ± 21.794	15	92.717 ± 27.542	7
Sr					
	VR	0.372 ± 0.187	5	1.281 ± 0.680	4
	M	0.369 ± 0.203	15	0.834 ± 0.336	7
Pb					
	VR	0.124 ± 0.067	5	0.577 ± 0.120*	4
	M	0.124 ± 0.116	15	0.430 ± 0.082*	7
U					
	VR	0.123 ± 0.056*	5	0.213 ± 0.073*	4
	M	90.197 ± 56.141*	15	11.163 ± 5.775*	7

Concentrations are expressed in µg/g, dry weight.
*: represents a statistical significant difference ($p<0.05$) between VR water course and the M pond.

Table 4 – Pearson correlation coefficients for the relationship between elements in the organs of *Rana perezi*.

Liver		Kidney	
Al/Be	0.933***	Al/Be	0.984***
Fe/Be	0.626**	Mn/Be	0.999***
Fe/Al	0.724***	Mn/Al	0.984***
Zn/Be	0.666**	Fe/Be	0.612*
Zn/Al	0.603**	Fe/Mn	0.613*
Zn/Fe	0.515*	Zn/Fe	0.659*
U/Be	0.903***	Ni/Sr	0.700*
Fe/U	0.653**	U/Be	0.679*
Zn/U	0.520^	U/Al	0.660^
		Mn/U	0.699*
		Fe/U	0.714*

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Histopathological analysis

Animals from both sampling sites did not show any gross pathologies on the different organs. The livers of frogs from both M and VR sites revealed no morphological structure alteration (Fig. 1a) with exception of the liver of a frog captured in the M pond, which had lost the integrity of the natural arrangement of its hepatocytes (Fig. 1b), along with an unusual quantity of melanomacrophagic centers (MMC) with high levels of pigment. In general, frogs from the mine appeared to have higher quantities of MMC (Fig. 1c) preserving however the morphological structure of the tissue.

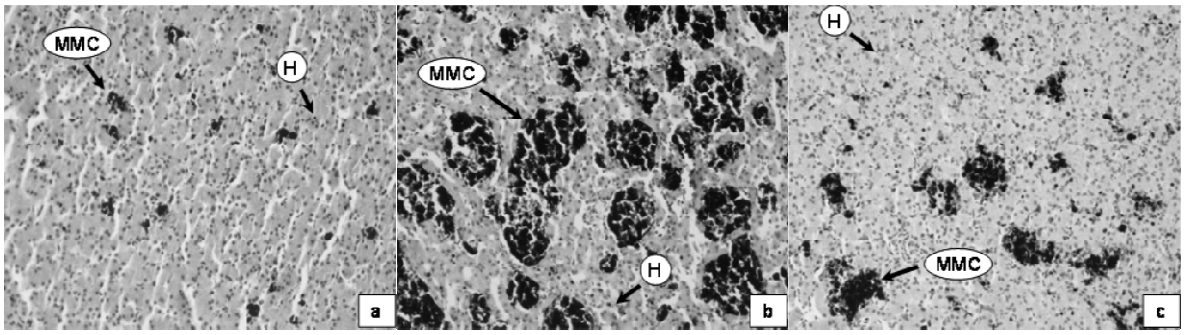

Figure 1. Liver from captured *Rana perezi.* (a) Liver from a VR frog with few MMC and no structural alterations (400x); (b) Liver from a M frog with extremely high quantities of MMC and loss of structural architecture(400x); (c) Liver from a M frog with abundant MMC and no structural alterations (400x); Arrows show hepatocytes (H) and melanomacrophagic centers (MMC).

With respect to kidneys, frogs from the VR site had no perceptible structural alterations (Fig. 2a), while those from the mine showed dilatation of the renal tubule lumen associated with tubular necrosis (Fig. 2b). Other two animals from the M pond revealed the accumulation of melanic pigment in their structure as well.

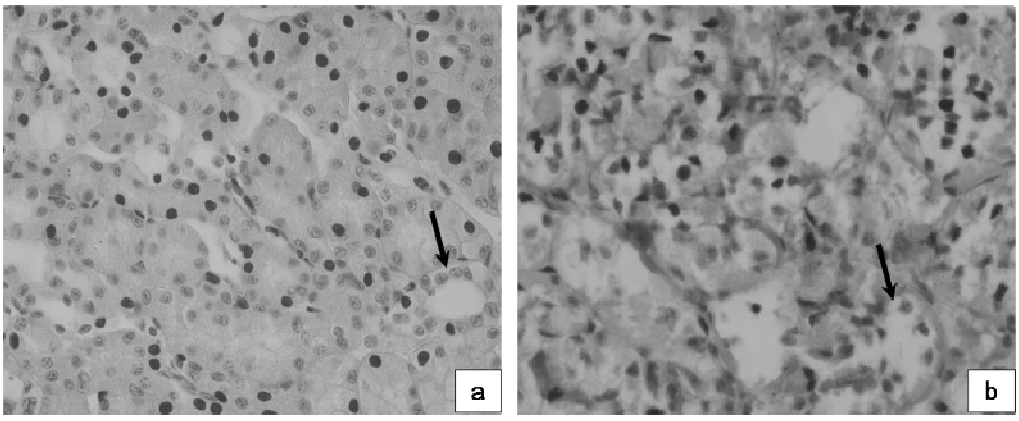

Figure 2. Kidney from captured *Rana perezi.* (a) Kidney from a VR frog with no structural alterations of the renal tubules(400x); (b) Kidney from a M frog with structural alteration of the renal tubules, revealing dilatation of the renal tubule lumen (400x) associated with tubular necrosis. Arrows show renal tubules.

The observation of testes revealed similar results for animals from both sampling sites, showing a normal structure with intense mitosis and spermatogenesis (Fig. 3). No alterations to the normal expected structure were recorded in spleen histological sections in all the captured frogs.

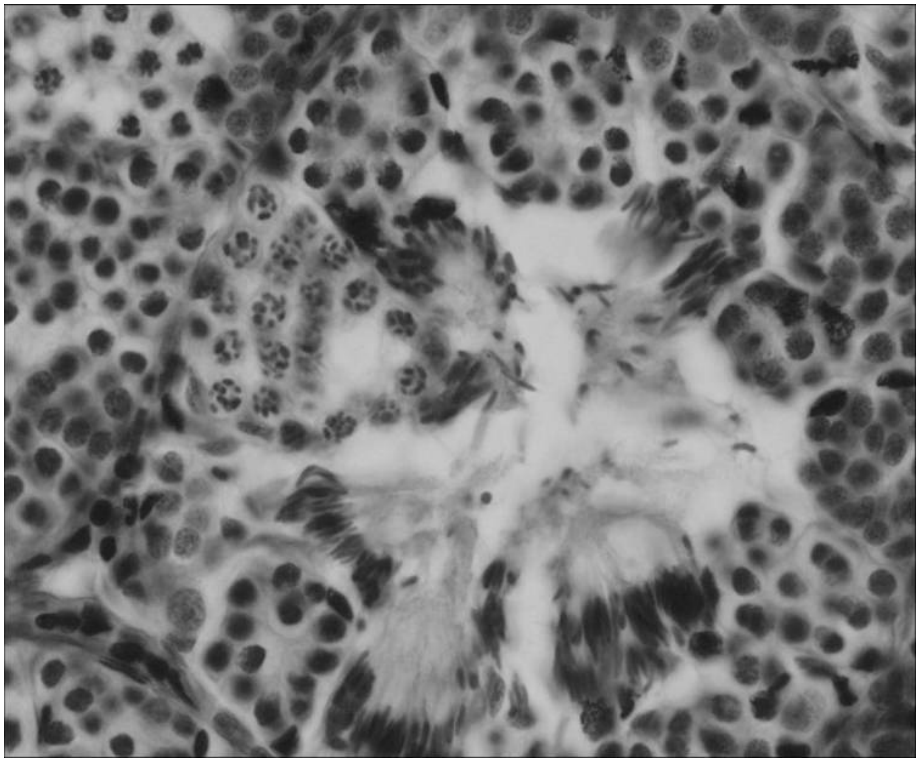

Figure 3. Testes from a VR frog exhibiting intense espermatogenesis (400x).

In opposition to the low number of organisms with no parenchymal alterations in the lungs (Fig. 4a), almost every animal showed a slight hyperplasia in the alveolar epithelium (Fig. 4b) along with a discrete thickening of the alveolar septa (Fig. 4b) and slight hipoplasia of the goblet cells (Fig. 4c). The presence of MMC was also registered (Fig. 4d) especially in M animals, as almost all the frogs presented this feature, while for the VR sampling site it was observed only in one. Parasites were also recorded in the histological sections of this organ (Fig. 5), in several frogs from the mine pond, having none been recorded for VR frogs.

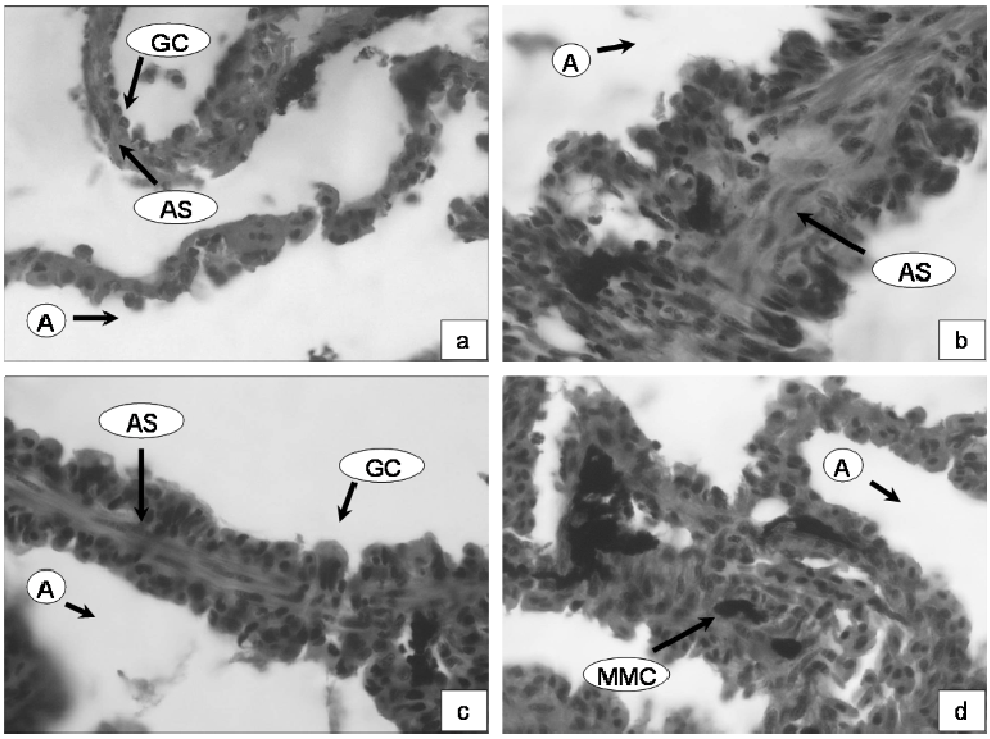

Figure 4. Lung from captured *Rana perezi.* (a) Lung from an VR frog with no parenchymal alterations (400x); (b) Lung from a M frog with a slight hyperplasia in the alveolar ephitelium and discrete thickening of the alveolar septa (400x); (c) Lung from an M frog with slight hipoplasia of the goblet cells (400x); (d) Lung from an M frog with the presence of MMC (400x). Arrows show Alveola (A), Alveolar septa (AS), Goblet cells (GC) and melanomacrophagic centers (MMC).

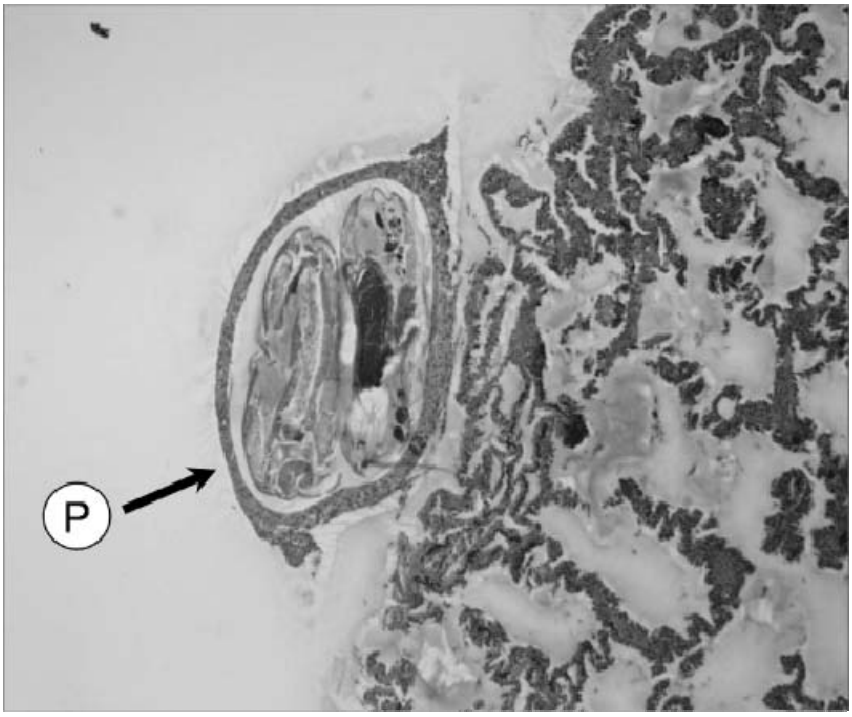

Figure 5. Lung from a frog captured in M revealing the presence of a parasite nematode (40x). Arrow shows a parasite (P).

Genotoxicity score

Four kinds of abnormalities were observed in the erythrocytic nuclear abnormalities assay (ENA). The abnormalities considered were: lobed nuclei (Fig. 6.a), notched nuclei (Fig. 6.b), micronuclei (Fig. 6.c) and kidney shaped nuclei (Fig. 6.d).

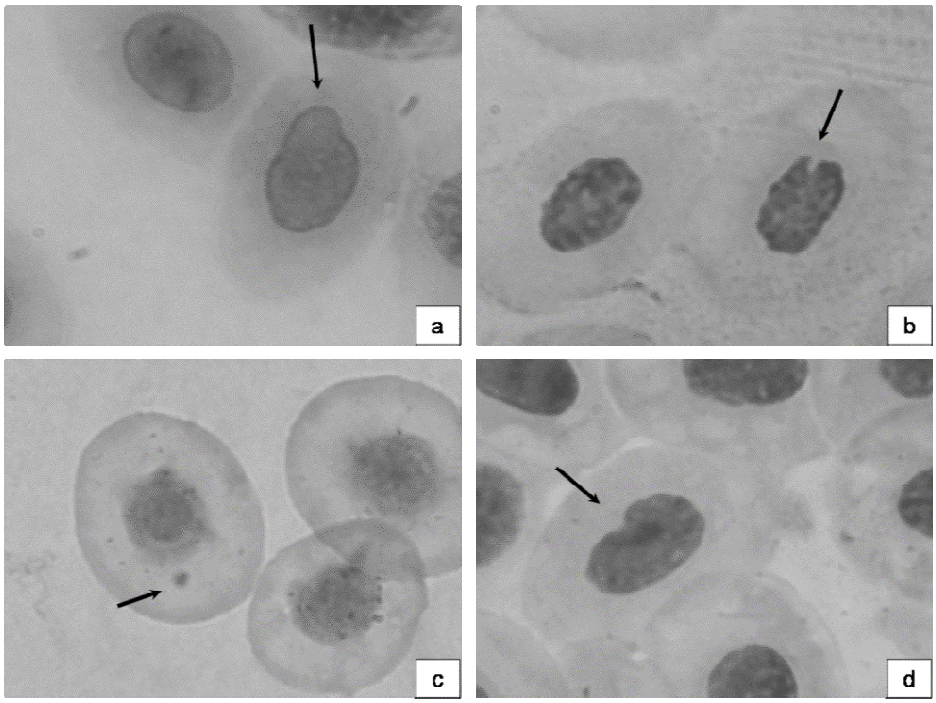

Figure 6. Micronucleus and nuclear alterations identified in erythrocytes of *Rana perezi* from M and VR: (a) Lobed nuclei; (b) Notched nuclei; (c) Micronuclei; (d) Kidney shaped nuclei. Arrows show ENA (1000x).

The results obtained for the ENA assay, when considering total nuclear abnormalities, and for the immature erythrocyte frequency (Table 5) revealed statistical significant differences when comparing organism from both sites (Fig. 7).

Table 5 – Mean immature erythrocyte frequency and mean frequency (‰) of each nuclear abnormality category (±SD) in peripheral blood erythrocytes of *Rana perezi* from VR and M.

	VR	M
IE Frequency (‰)	617.3 ± 183.25*	235.43 ± 139.92*
Lobed (L)	1.60 ± 3.37*	7.68 ± 6.05*
Notched (N)	0.80 ± 1.14	2.14 ± 2.56
Micronuclei (MN)	0.00 ± 0.00	0.64 ± 1.13
Kidney Shaped (KS)	0.00 ± 0.00	0.36 ± 0.62
Total (L+N+MN+KS)	2.40 ± 4.12*	10.82 ± 7.38*

*: represents a statistical significant difference ($p<0.05$) between VR and M.

The IE frequency was significantly lower in M organisms ($F=46.557$; $d.f.=1$; $p<0.001$), while the opposite was observed in the ENA assay results. The frogs from the mine had a significantly higher level of abnormalities ($F=11.580$; $d.f.=1$; $p<0.002$), especially erythrocytes with lobed nuclei, which also displayed significantly higher levels ($F=8.988$; $d.f.=1$; $p<0.005$) when analysed separately from the other abnormalities.

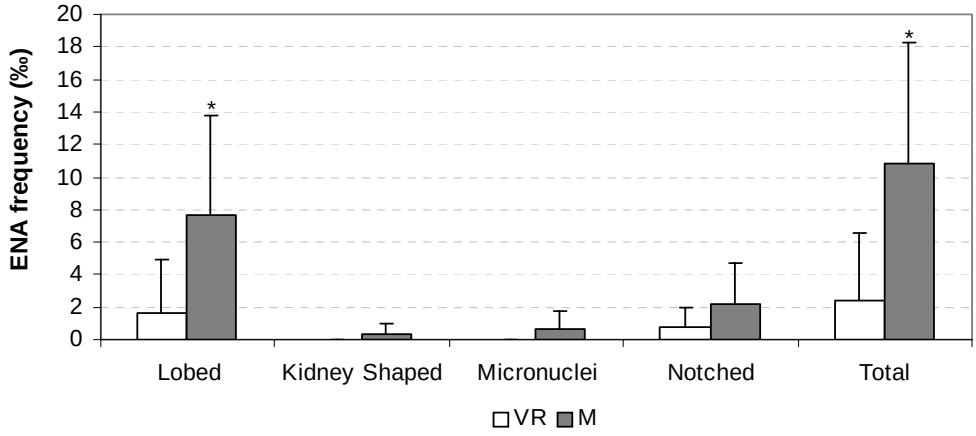

Figure 7. Mean frequency of each nuclear abnormality and total nuclear abnormalities in *Rana perezi* from VR and M.
*: represents a statistical significant difference ($p<0.05$) between VR and M. Error bars represent standard deviation.

Discussion

Even though the study area has originated some ecotoxicological studies (e.g. Antunes et al., 2007a; Antunes et al., 2007b; Antunes et al., in press b; Marques et al., submitted) and the effluent as been thoroughly analysed, it has showed a great variability in terms of their chemical composition. This variability has been recorded at least between seasons (Antunes et al., 2007a). Nonetheless, this effluent has always revealed high levels of metals, specially Mn, Fe, Ni, Zn and U, and low pH values, which were responsible for acute toxic effects on aquatic species of invertebrates (Antunes et al., 2007a; Antunes et al., 2007b). Hence, the Iberian green frog population inhabiting the M pond was chronically exposed to a complex mixture of metals and to a low pH value not only by direct contact with the effluent but probably also with the mine tails spread in the surrounds. In fact amphibians might become exposed to metals through several pathways. The possibilities might go from direct absorption from water through the skin, ingestion either of soil or sediment (Linder and Grillitsch, 2000) and, ingestion of contaminated food (Lefcort et al., 1998). Regarding sediments, from this pond in particular

Antunes et al., (in press b) observed that sediment bound contaminants seemed to be present in non-bioavailable forms and subsequently that sediments are not a meaningful source of contaminants to the water column. With respect to the mine pond surrounding soil, Pereira et al. (in press) recorded it's contamination, mainly with high concentrations of U and Al, whose mobility (Pereira et al., in press) and potential bioavailability to induce toxic effects on edaphic species (Antunes et al., in press a) was also demonstrated. Therefore, even considering that water adsorption may be the main exposure pathway of frogs captured on the mine, due to their behaviour, the soil compartment cannot be excluded as a significant source of contaminants for these animals. Thus, all of the site specific data, regarding soil and water chemical and ecotoxicological characterization, gathered in the first tier of the ecological risk assessment that is being carried out in the area, supported the need to proceed to higher tiers of the process. In this context, the aim of the tier 2, in particular, is to get helpful information in deciding if ecological receptors of concern are actually at risk of harm (Weeks and Comber, 2005).

Hence, as it was recorded in this study, performed to meet the above described purpose, the total metal contents recorded, both in liver and kidneys, showed significant differences in several elements, being the higher values recorded in frogs from the M pond, except for Pb levels bioaccumulated in the kidney, when compared with animals from the reference site (VR water course). Uranium was the only metal showing significant higher values bioaccumulated in both organs (Table 3). In fact it has been observed for other species, freshwater fishes included, that once in the body, uranium preferentially distributes to bone and kidney, and then in the liver, being the kidney the main responsible by it's excretion and subsequent maintenance of body homeostasis (ATSDR, 1999a; Kerr, 1999; Cooley and Klaverkamp, 2000b).

Moreover, uranium as well as Be were the only non-essential elements showing a positive and significant association in both organs with the same essential element – iron (Table 4). Although 60% of the uranium forms (mainly uranyl ion) are carried out in body fluids as soluble bicabornate complexes the correlation between uranium and iron concentrations bioaccumulated in both organs may be explained by the additional transport of the uranyl ion bound to plasma proteins such as transferrin and ferritin which are responsible by the delivery of the iron ion (Cooper et al., 1982; Hainfeld, 1992; Goyer and Clarkson, 2001). A competition scenario between U and Fe, due to the share of plasma transporters, would imply an expectable negative correlation between their accumulation profiles. However, it seems that in a situation with concomitant exposure to a high concentration of both metals, this process favours the bioaccumulation of elements

in the same organs, Moreover, high positive and significant associations were recorded in both organs between non-essential metals (U, Be and Al) which were recorded in higher concentrations in the effluent, suggesting similar metabolic pathways involved in the bioaccumulation of these elements.

Histopathological analysis of the liver revealed, in general, no morphological or structural alterations. However, in frogs from the M pond the liver appeared to have a higher frequency and size of MMCs. In the same manner, well developed MMCs were observed in the lungs, and granules of melanic pigment were found in the kidney of contaminated site organisms. MMC are macrophage like pigmented cells containing melanin, lipofuscin and hemosiderin (Wolke, 1992). Melanin can act as a powerful free radical scavenger, protecting the pigmented cell against free radical damage (Różanowska et al., 1999). Thus the higher number and size of MMC in M frogs may be considered as a response to metal exposures and accumulation to provide antioxidant protection. Our results were corroborated by Fenoglio et al. (2005) who registered wider MMC in the liver of *Rana esculenta* from a polluted site, relating the results with reactive oxygen species (ROS) and by Loumbourdis and Vogiatzis (2002) that observed the same trend in *Rana ridibunda* after a chronic exposure to Cd. Periyakaruppan et al. (2007) also reported the occurrence of oxidative stress on lungs of rats promoted by exposures to uranium.

The damage observed in the kidneys of organisms from the M pond was in agreement with the results obtained by Loumbourdis (2005) with *Rana ridibunda* exposed to Cd (200mg/L) for 30 days. Moreover, Cooley et al. (2000b) recorded the occurrence of similar histopathologies (proximal tubule necrosis and increased abundance of MMC) in lake whitefish exposed to a range of U concentrations (0-10mg/g) provided through a contaminated diet. Kidney uranium toxicity is usually explained by the filtration of the uranium bicarbonate complex through glomerulus and subsequent reabsorption of bicabornate in the proximal tubules with release of the uranyl ions, that are responsible by local damages (Goyer and Clarkson, 2001). Hence, strengthening what as been stated by Hinton et al. (1992), renal histopathologies, not only in fishes, but also on amphibians, are candidates to be considered a good biomarker of metal contamination. However and since the Iberian green frogs analysed in this study were exposed to an environmental mixture of metals, it is difficult to establish a direct cause-effect relationship with a specific element.

Cases have been reported in which environmental pollution resulted in immunosuppression, increasing the susceptibility to infections (Bernier et al., 1995)

inclusively of parasitic origin. Therefore, it is possible that the parasites observed in the lung of frogs from the mine pond might have been related with a possible immunosuppression caused by the mixture of metals.

The ENA assay showed the existence of a significant higher value of nuclear abnormalities in erythrocytes of *R. Perezi* from the mine when compared with reference animals. Even though MN did not reveal a significant difference between sites, the existence of other anomalies can indicate, as well, the presence of genotoxicity (Pacheco and Santos, 1996; Ayllón et al., 2001). Moreover, according to Serrano-Garcia and Montero-Montoya (2001), budding cell nuclei, which include lobed nuclei and bi-nucleated cells, have a similar origin as MN. Taking this into consideration and since that the lobed nuclei frequency and the sum of all the scored abnormalities, showed a similar pattern, our results point out for genotoxic effects on blood erythrocytes, most probably derived from the exposure to the mine effluent.

The occurrence of nuclear lesions may be altered by several factors such as erythropoiesis, required time for maturation and lifespan of erythrocytes (Udroiu, 2006). In order to perceive the erythrocyte kinetics in frogs from both sites, the IE frequency was determined. Displayed results showed a significantly lower frequency of IE in the mine pond. If we consider that circulating abnormal cells tend to be removed faster from the organism (Das and Nanda, 1986), our results suggested an alteration of the erythrocyte kinetics in the M frogs, that might be a consequence of erythropoiesis as well as a removal of abnormal cells failure. Nonetheless, no conclusions may be taken, since we must take into consideration that IE frequency reflects the balance between factors such as cell maturation rate, immature cell input and cell removal by spleen or liver.

Conclusions

This study provided additional information to be integrated on a site specific risk assessment, which gives rise to new evidences about the impacts of a locally produced uranium mine effluent on aquatic organisms, amphibians in particular. This effluent with total concentrations of some metals (Be, Fe, Mn, Ni and U) above benchmark values defined for surface waters is responsible by the bioaccumulation of metals, histopathological alterations on tissues (mainly liver and kidneys) and genotoxic effects on Iberian green frogs undergoing an *in situ* chronic exposure. However, the additional exposure of these animals to contaminate mine soils can not be ignored. Significantly higher concentrations of metals were recorded on frogs captured on the mine pond, being

the liver the main target organ for Be, Al, Fe, and U. Nevertheless, renal histopathologies were more pronounced, what may be determined by the role of this organ on metal's excretion. The alterations observed were similar to those reported by other authors for rats and fishes exposed to uranium for long periods of time. This observation reinforced the use of renal histopathologies as a biomarker of chronic exposure to uranium and other metals. Genotoxic effects were also recorded, however since other metals with genotoxic potential were present in the effluent, as well as, on surrounding soils, it is not possible to establish a direct cause-effect relationship with a specific element. The erythrocyte nuclear abnormalities recorded on frogs from the mine, co-occurred with a lower frequency of immature erythrocytes, suggesting that the removal of abnormal cells may be compromised.

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General Conclusions

General Conclusions

The evaluation of ecotoxicological damages on amphibians assumes a crucial role in the risk assessment of metal contaminated environments, when they are local ecological receptors of contamination. Such fact results from their sensitivity to several metals (e.g. Lefcort et al., 1998; Haywood et al., 2004) and to their important role in the transference of contaminants between environments, mainly owed to the existence of two distinct development phases, an aquatic phase and a terrestrial phase, along with a semi-permeable skin (McDiarmid and Mitchell, 2000) which facilitates the absorption of contaminants on both compartments. Hence, the ecotoxicological information gathered, with amphibians, will allow a more realistic site specific evaluation of risks and the development of recommendations more focused on the protection of sensitive species.

Within the first tier of the site specific risk assessment that is being carried out in the Cunha Baixa uranium mine, the contamination of the mine pond water and their ecotoxicological effects on algae and invertebrate species, was already performed (Antunes et al., 2007a; Antunes et al., 2007b). The results obtained give rise to the necessity to proceed with the ecotoxicological evaluation for vertebrate species, from higher throphic levels in the food chains, namely fishes and amphibians. Regarding this last group, it was integrated on species battery, because it was also important to perceive, how such sensitive species was able to survive in the mine effluent, with high concentrations of metals and low pH. Hence our study started with the assessment of the effects promoted by a short-term exposure in the early life stages (egg masses and larvae) of Iberian green frogs. It was possible to observe that short-term exposures to the uranium mine effluent did not compromise, the hatching of eggs. However, if exposure occurs in the early-life stages, mortality of larvae may occur, and the growth of larvae may be significantly compromised. Other morphological and behavioural alterations, qualitatively analysed, such as small tail deformities and reduced movement in response to a stimulus, might jeopardise the survival of these organisms (Lefcort et al., 1998). The recovery bioassay demonstrated that these damages may be worsened if the exposure continues for a long period of time, and they can be attributed to the total concentrations of some metals, probably to those above benchmark values for surface waters (Be, Mn, U and Fe) which were accumulated at higher concentrations in larvae exposed to the effluent. Regarding aluminium, it was not recorded in the effluent analysed by ICP/MS. However, according to previous suspicions, it was present, since it was one of the main elements bioaccumulated by larvae. Moreover, it is important to mention that the effects recorded on the larvae assay can not be attributed to pH, since this parameter was

adjusted to neutrality, before the assay. This procedure was followed because *in situ* and laboratory assays with fish species in course (unpublished data) showed that the pH of the effluent *per si* would be responsible by lethal effects in tadpoles. The results gathered with this first part of the study were in agreement with *in situ* observations, according to which, Iberian green frogs use this mine pond (the M pond), mainly as shelter but not for mating and laying eggs masses. In fact egg masses were recorded only in the other two ponds, with neutral pH values and low total metal contents (data not published). The avoidance of contaminated pond waters by females, mainly of acidic environments, was a behaviour already reported for amphibians, aimed to reduce their exposures to contaminants and to protect their early-life stages (Rowe and Freda, 2000).

With respect to adults, from the Iberian green frogs population, inhabiting the mine ponds, where they undergo long-term exposures to the effluent and to the surrounding contaminated soils, our work demonstrated the bioaccumulation of metals in their liver and kidneys, through the comparison with animals captured in a pristine river, used as a reference site. The analysis of histopathologies in several organs also showed that liver, and mainly kidney, were the most impaired organs. Dilatation of the renal tubule lumen associated with tubular necrosis, was recorded in frogs from the mine. In addition the presence of higher numbers of wider MMC, in these frogs, was a common feature among liver, kidney and lungs, probably originated as a antioxidant response to the high levels of metals (Różanowska et al., 1999; Loumbourdis and Vogiatzis, 2002). Genotoxicity was also recorded with clear differences in the ENA assay between *R. perezi* from both sites. As it was stated by other authors this evidence confirmed once more the usefulness of this assay for assessing aquatic genotoxicity (e.g. Guilherme et al., 2007).

The present study is of great relevance since it provides a first insight on the sub-lethal effects of short-term and long-term exposures to a metal contaminated effluent, from a uranium mine, on an indigenous amphibian population. Although it was not possible to derive cause-effect relationships with specific elements, this information was extremely useful to assess site-specific risks posed by this mine wastewater. It was evident that in a worst case scenario, if this effluent is released to surrounding watercourses, even with an increase of pH, promoted by its dilution, sub-lethal effects on the early-life stages of resident amphibians will be expectable.

However, it still is unexplained how these organisms tolerate such contaminated environment, where radiological contamination (not assessed in this study) occurs in parallel with chemical contamination, and if the effects recorded at the individuals' level are translated in effects on population. Thus future works should focus on the possible existence of differential genetic expression in organisms exposed in this mining

environment leading to the development of mechanisms of resistance as well on the analysis of endpoints at the population level.

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