Universidade de Aveiro Departamento de Biologia
Ano 2011

Sérgio Miguel Reis Luís Marques

Expressão genética na compreensão de mecanismos de toxicidade em anfíbios

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Ruth Maria de Oliveira Pereira, Professora Auxiliar Convidada da Faculdade de Ciências da Universidade do Porto, do Doutor Fernando José Mendes Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro, e da Doutora Sandra Isabel Mourinha Lopes Chaves, Investigadora no laboratório de Microbiologia e Biotecnologia da Faculdade de Ciências da Universidade de Lisboa.

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o júri

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palavras-chave

efluentes mineiros, *Pelophylax perezi*, expressão genética diferencial, metais, pH, biomarcadores.

resumo

De uma forma geral os anfíbios são conhecidos como organismos que apresentam uma grande sensibilidade a vários tipos de contaminantes. Contudo existem casos, como o de *Pelophylax perezi* (rã-verde), em que estes organismos habitam áreas extremamente contaminadas. Este facto verifica-se na mina de urânio desactivada, da Cunha Baixa (Viseu, centro de Portugal), em que uma população destas rãs habita na lagoa de efluente ácido mineiro (M). Estudos ecotoxicológicos anteriores com estes organismos revelaram apenas efeitos de toxicidade ténues levantando algumas questões.
Com o objectivo de elucidar quais os mecanismos que permitem a *P. perezi* permanecer neste local, sem sofrer aparentemente efeitos perniciosos, encetamos este trabalho.
Numa primeira abordagem, avaliámos o sistema de defesa antioxidante de rãs adultas, bem como o conteúdo em metais de alguns órgãos. Desta forma verificámos alterações enzimáticas, principalmente no pulmão e acumulação de metais nos vários órgãos. Posteriormente foi realizado um estudo de expressão genética diferencial, também em organismos adultos e desta feita foram sugeridos alguns mecanismos de protecção basal que estarão por detrás da capacidade de suportar este ambiente extremamente contaminado.
Numa etapa seguinte abordámos os efeitos em fases larvares, fazendo inicialmente uma exposição *in situ*, a vários efluentes, caracteristicamente diferentes, do complexo mineiro. Avaliámos o crescimento, a acumulação de metais e a actividade de alguns biomarcadores de stress oxidativo. Como resultado pudemos constatar que nas fases larvares para além de alguma mortalidade existe acumulação de metais bem como algumas alterações a nível de biomarcadores de stress oxidativo. Numa última abordagem realizamos uma exposição crónica dos girinos a efluente da mina com diversos níveis de pH para distinguir os efeitos da toxicidade do pH, dos efeitos da toxicidade pelo conteúdo de metais. Para tal avaliámos novamente biomarcadores de stress oxidativo, crescimento, acumulação de metais e efectuamos ainda um estudo de expressão genética diferencial. Esta última aproximação permitiu verificar que a toxicidade do efluente resulta primariamente do pH ácido, assumindo a contaminação por metais um papel secundário. Contudo o crescimento dos girinos de *P. perezi* apresenta-se estimulado por pHs mais baixos. São apontados ainda alguns mecanismos, em girinos, para lidar com o stress causado pela contaminação por metais.

De uma forma geral pôde-se constatar que quer anfíbios adultos quer girinos expostos ao efluente apresentam valores altos de metais acumulados. Os biomarcadores de stress oxidativo na sua maioria não apresentaram respostas coerentes mediante as várias exposições. Este trabalho apresenta-se como um contributo importante para a ecotoxicologia de anfíbios, aumentando os níveis actuais de conhecimento sobre o efeito de contaminação proveniente de efluentes mineiros, sugerindo ainda mecanismos de resistência quer em larvas, quer para adultos.

keywords

mine effluents, *Pelophylax perezi*, differential gene expression, metals, pH, biomarkers.

abstract

Amphibians are generally known as organisms that present a great sensitivity to several kinds of contaminants. However, there are some cases as the one of *Pelophylax perezi* (green frog), where these organisms inhabit extremely contaminated areas. This fact has been recorded, in the deactivated uranium mine of Cunha Baixa (Viseu, Centre of Portugal) where a frogs' population inhabits the acid mine effluent (M). Previous ecotoxicological studies carried out with these organisms have revealed only mild toxic effects. This has raised some questions on these organisms ability to endure extreme conditions, gaining certainly some advantage in these places in terms of refuge. Aiming to uncover the mechanisms that allow the presence of *P. perezi* in this area without apparent deleterious effects, this work was carried out. A first approach was to assess the antioxidant defence system in frogs as well as metal content in various organs. Thus enzymatic changes, mainly in lung, and metal accumulation were reported. Afterwards a differential gene expression study was also performed in adult organisms and basal protection mechanisms were suggested as supporting the ability of enduring extremely contaminated environment. These mechanisms encompass the possible increase of the albumin and fibrinogen antioxidants. In the next stage effects on larvae were addressed with an *in situ* exposure to several mine complex effluents with different characteristics. Growth, metal accumulation and oxidative stress biomarkers were assessed. As a result we can point that in the larval stages, beyond mortality, metal accumulation exists, as well as some oxidative stress enzyme responses. In a final approach, to distinguish metal toxicity effects from pH toxicity effects, tadpoles were exposed chronically to the mine effluent at various pH values. With this purpose, oxidative stress biomarkers, growth, metal accumulation and differential gene expression were performed. This approach allowed verifying that the effluent toxicity results primarily from acidic pH, with metal contamination assuming a secondary role. However *P. perezi* tadpoles' growth seems to be stimulated by lower pH values. Some mechanisms in tadpoles are pointed as a way to cope with metal contamination stress. These mechanisms include an increase in osmotic regulation and energy acquisition investment. Overall, it could be noted that both adult amphibians and tadpoles exposed to the effluent have higher metal accumulation. Also the majority of the oxidative stress biomarkers did not present consistent responses throughout the various exposure conditions. This work presents as a new contribution for amphibian ecotoxicology, increasing current knowledge on mine effluent contamination effects and suggesting resistance mechanisms both in larvae and adults.

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Chapter I

General Introduction

1. General Introduction

1.1. Metals as freshwater contaminants: main sources and environmental fate

Freshwater environments are narrow when compared, for instance, with marine environments. Nonetheless, they are centers of biodiversity and their water is essential for many aquatic and land dwelling animals. However these environments have become more precious since many are increasingly impacted by various sources of contamination (Cunningham et al., 2005). Some of the oldest contaminants in our planet, with extremely deleterious effects on freshwater environments are metals. Metals are widely spread in the environment sometimes in trace levels and their natural cycle is permanently renewed in a balanced flux between the geosphere, hydrosphere and atmosphere, through processes like erosion and volcanic eruptions. However, these elements may become harmful when they occur at high concentrations and become bioavailable in any of the environmental compartments (air, soil, water). Overall, the aquatic environment is usually the final recipient of metal contaminants and the risks for this environment, posed by metal contamination, independently of the origin, are increased by the fact that metals cannot be degraded. Thus, they become incorporated in sediment, water or biota (Linnik and Zubenko, 2000) with a panoply of subsequent direct or indirect effects (e.g. Fleeger et al., 2003; Marques et al., 2009).

There are many sources of metal contamination, both natural and anthropogenic. As far as natural causes are considered, high concentrations of metals in the environment may result from many different sources as natural forest fires (Larocque and Rasmussen, 1998), physical and chemical weathering of ore bearing rocks (Cunningham et al., 2005) and volcanic eruptions. The anthropogenic sources include runoff from urban areas, which comprise industrial and vehicle trafficking runoff (Marsalek et al., 1999), agricultural contamination due to the use of pesticides and fertilizers (Järup, 2003), industrial and domestic untreated wastewater discharges (Bu-Olayan et al., 2001) and mining activities (Milu et al., 2002).

Throughout human history the exploitation of mineral resources has been an important economic activity, as it allows the acquisition of raw materials with probably the widest range of applications. The extraction of geologic materials can be performed by various methods, such as open-pit mining, strip-mining and underground mining. Nevertheless, all share a common trait, which is contamination of the surrounding environment (Cunningham et al., 2005). Besides the resulting soil contamination (Pereira et al., 2008) a far worse problem results from mining: the production of acidic effluents, usually designated by acid mine drainage (AMD). One of the main factors influencing the hazardousness of AMD is the site mineralogical composition (Williams, 2001). The mineralogical background can influence both acidity and chemical composition of AMD. One of the main reactive components in metal extracting mines are sulfide minerals that are mainly stable in anoxic and dry conditions. However, once exposed through the mining process to oxidation by air, water or microorganisms, the dissolution occurs and the formation of acidic waters takes place (Johnson, 2003). Taking pyrite (FeS_2) as an example, the stoichiometry of the reaction may be resumed as (Edwards et al., 2000):

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$$

Furthermore, acid liquors formed by this process or by the intentional release of acids for heap leaching of poor ore, a procedure that worsens this problem, are usually so strong that they can degrade minerals such as aluminosilicates, leading to the release of more metal elements to the AMD.

Despite AMDs being rich in a complex mixture of metals, once reaching freshwater resources, the bioavailability of these components will be dependent from several factors such as pH, hardness, alkalinity, complexing ligands and temperature. Nonetheless, pH is probably the most relevant factor ruling bioavailability, since it affects metals speciation, transport and solubility (John and Leventhal, 1995). Usually a decrease of pH leads to an increase of metal ions in solution, since free protons will compete with bound metal ions for negatively charged sites in both organic and inorganic ligands. Hardness, which is usually

determined by Ca^{2+} and Mg^{2+} ionic concentrations, decreases bioavailability of metals by competing with them for organisms binding sites influencing in this way the ability to uptake potentially toxic elements. When considering alkalinity, its influence on bioavailability is analogous to hardness. Nonetheless, it reduces bioavailability by forming metal-carbonate complexes. Relatively to complexing ligands, these might be either organic or inorganic, and one of the possible consequences on the bioavailability of metals is the interference with metal transport across membranes, as well as precipitation of complexes (ICMM, 2007). Regardless of higher or lower biovavailability of AMD metal content, the aquatic ecosystems receiving them have their physicochemical parameters altered and local biota deleteriously effected (Antunes et al., 2007a; Lopes et al., 1999).

1.2. Toxicokinetics and toxicity of metals

In environmental contaminated scenarios, metals enter the blood stream after uptake by animals and become distributed in target organs, binding to sulphydryl, amino and phosphate groups among other functional groups of biomolecules (Harford and Sarkar, 1997; Quig, 1998; Sood et al., 1998). Of the formed complexes the most stable involve metal binding to sulfur or nitrogen (Seiler and Sigel, 1988).

When considering distribution and mobilization of metal in the body, there are some factors that must be regarded. The first is the metal form (*i.e.* organic or inorganic), then there is the fact that they are not often susceptible to metabolic detoxification mechanism, mainly inorganic forms (van der Oost et al., 2003), being harder to be confined and controlled by the organism. Furthermore, small metal ions or inorganic metal compounds can circulate easily among body tissues and fluids, being allowed to persist in some tissues where they bound more tightly, increasing thus their biological half-lives (Seiler and Sigel, 1988). Nonetheless, there are rare exceptions where inorganic metal compounds can be converted to organic compounds and then metabolized (e.g. ATSDR, 2007). Depending on the element, metals can present distinct patterns of accumulation in the organism. For example, since uranyl ion (UO_2^{2+}) exchanges with Ca^{2+} on the surfaces of mineral

crystals (ATSDR, 1999), uranium accumulates preferably in mineralized tissues such as bones or scales (Cooley and Klaverkamp, 2000). On the other hand, metals such as As or Cu may be found accumulating in liver or kidney (e.g. (Farombi et al., 2007; Papadimitriou and Loumbourdis, 2003). Furthermore, the same element may also reveal different accumulation patterns according to the organism (Farombi et al., 2007; Vogiatzis and Loumbourdis, 1998). Overall, metal accumulation patterns are very diverse and tend to be specific both from the species and from the environmental conditions. However, metals entering the organisms are not all accumulated in tissues or maintained in circulation. They can also be eliminated from the organism. There are two main pathways of excretion of inorganic compounds: via kidney and via the gastrointestinal tract. Nonetheless both of these routes of metal elimination are once again dependent on the various metal species in circulation (Seiler and Sigel, 1988).

The toxicological effects of different metals have been thoroughly studied and reported for a wide range of organisms. These effects comprise mortality, malformations, growth decrease, damage in various tissues (e.g. gills, liver, kidney, lungs, testis, etc.) including necrosis, erythrocytic nuclear abnormalities and DNA damage (Calevro et al., 1999; Hansen et al., 2002; Stohs and Bagchi, 1995; Zhang et al., 2007). The toxicity of metals may come from direct metal action through the displacement of elements or through binding to sulfhydryl groups of proteins, causing disruption of their structure and function (Quig, 1998). This action mode may interfere in many important processes such as central metabolic pathways (e.g. glycolysis, Krebs cycle), as reviewed by Strydom et al. (2006), leading to changes in energy production. Nonetheless, metal toxicity, as reviewed by many authors (e.g. Ercal et al., 2001; Galaris and Evangelou, 2002; Halliwell and Gutteridge, 1984; Stohs and Bagchi, 1995), may derive more often from the ability to induce reactive oxygen species (ROS) which can result in lipid peroxidation (LPO), DNA damage, depletion of sulphydryls and alteration of calcium homeostasis. Depending on ROS level, cellular death may occur. There are many different forms of ROS: singlet oxygen ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot -}$) and the hydroxyl radical ($OH^{\cdot}$). Their production is normal in animal systems, being estimated that about 1 to 3 % of the O_2 consumed will

result in ROS (Halliwell and Gutteridge, 1999). However, in normal conditions, the biological systems do not present any deleterious effect promoted by normal ROS production. There are various enzymes with an antioxidant function that cope with normal levels of ROS and help maintain the redox status of cells. ROS levels can be increased by metals, even those that do not enter easily on redox cycling (e.g. Hg, Cd, Pb, As). This increment may result thus from a direct or indirect way. Specifically ROS can be induced directly by metals, mainly those that enter redox cycling (e.g. Fe, Cr, Cu, V), through Fenton like reactions, as the following example for the formation of the highly reactive $OH^{\cdot}$ through Fe^{2+} oxidation:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

$$Fe^{3+} + O2^{\cdot -} \rightarrow Fe^{2+} + O_2$$

The indirect induction of ROS levels results, at least in part, from the depletion of free sulphydryl groups and damage to the antioxidant enzymes (Ercal et al., 2001; Stohs and Bagchi, 1995), allowing ROS yield by the normal respiration process to accumulate. Overall, the presence of ROS is only harmful when an unbalance between their levels and the antioxidant response occurs, leading thus to oxidative stress and eventually deleterious effects on cells and tissues. In order to prevent damage effects of ROS, cells present various levels of protection that consist first of all in prevention of ROS formation, secondly in the termination of ROS by antioxidant enzymes or free radical scavengers and finally in repairing damaged cell components (Storey, 1996).

Considering the mechanisms involved in metal toxicity, a common procedure of assessing effects of metals is to evaluate the activity of enzymes implicated on the antioxidant defense system. This includes, among others, superoxide dismutase (SOD), glutathione reductase (GRed), glutathione peroxidase (GPx) and catalase (CAT) (Livingstone, 2001). All these enzymes exist as a coordinated system to detoxify distinct ROS such as hydrogen peroxide and superoxide (Fig. 1). The complementary analysis of LPO gives an insight of the damage caused to cell membranes giving the information if the cell antioxidant defense system is being responsive and able to deal with exposure.

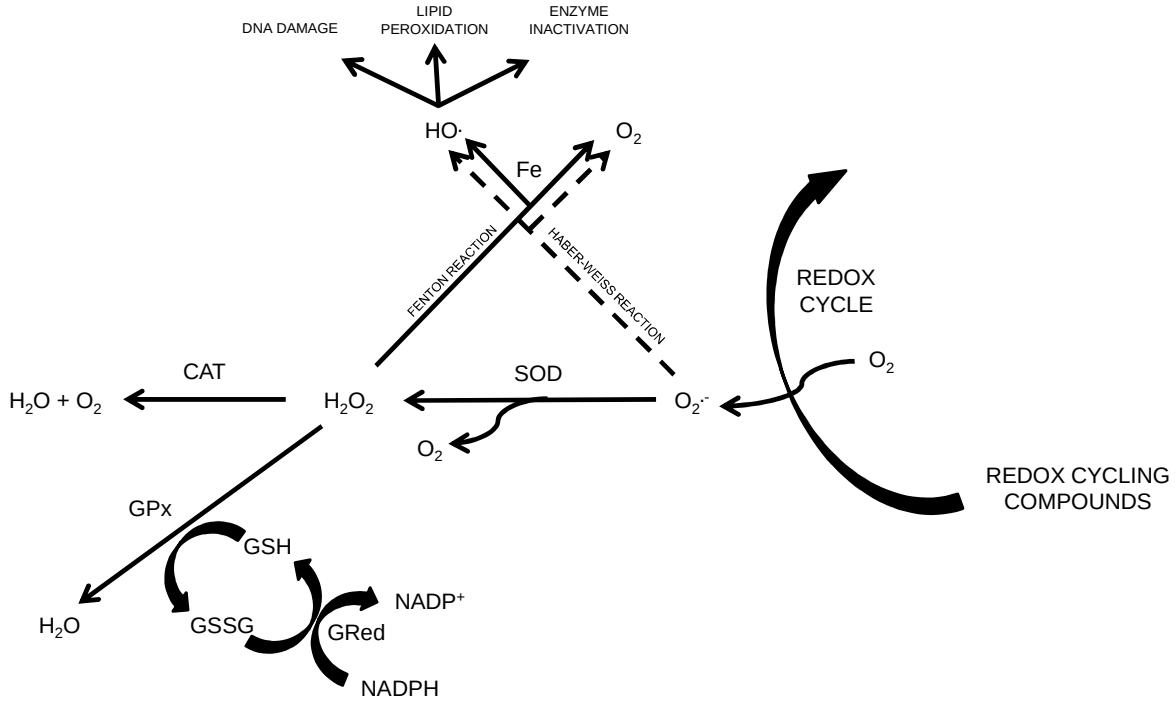

Fig. 1. Schematic representation of the antioxidant defense system and its action to neutralize ROS. (adapted from: Kappus, 1987)

1.3. Amphibians

1.3.1. *Amphibian traits*

The class Amphibia is presently composed by three orders: the Gymnophiona, the Caudata and the Anura (Hickman et al., 1997). In general, amphibians have the singularity of changing between water and land during their life cycle. Usually, the larval stages are spent in water and after metamorphosis most species become terrestrial even though reproduction is still water dependent (Almeida et al., 2001). Since these organisms are ectotherms, they may hibernate during adverse weather conditions, presenting various strategies depending on the species (e.g. burrowing or taking refuge under rocks). Another important characteristic of amphibians is their naked skin which is highly permeable and extremely important in water regulation. This organ is also very important for respiration, mechanical and chemical protection. Their reproduction is oviparous in the majority of the species. Nonetheless one can find also viviparous and ovoviviparous species. In the viviparous and ovoviviparous reproduction the embryo develops inside the progenitor, which allows a higher protection from predators and more efficient

colonization of environments with less water availability (Almeida et al., 2001). As for oviparous species, the eggs are usually covered by a jelly coat to provide protection from predators, pathogens, mechanical and chemical aggressions (Almeida et al., 2001; Edginton et al., 2007). Furthermore, the jelly coat allows water balance and gaseous exchange with the surrounding environment.

Generally, due to their water-dependence, amphibians only have a main ecological restriction, which is water availability or high levels of humidity. Nonetheless, there are some exceptions, were species have developed strategies to endure very dry habitats. Thus, they can be found in many different habitats such as rain forest, desert, savanna and arctic tundra (Almeida et al., 2001; Rödel and Bangoura, 2004). In their habitats, amphibians may be considered both a keystone and a sentinel species (Murphy et al., 2000a) since they have a central role in the ecosystem, being usually both predator and prey. Furthermore, they have proved to be sensitive to changes in the surrounding environment (Davidson et al., 2002) both in aquatic and terrestrial compartments (Hatch and Blaustein, 2000; James et al., 2004). Accordingly, amphibian populations of many species have suffered decreases worldwide (Beebee and Griffiths, 2005; Stuart et al., 2004), being affected by increasing pressure in their environments from many factors. Specifically, we can indicate the increase in UVB radiation due to ozone depletion (Blaustein et al., 1995), salinization (Ortiz-Santaliestra et al., 2010a; Viertel, 1999), acidification (Rowe et al., 1992), human exploration mainly for food and medicine resources (Benítez, 2011), exotic species invasion (Polo-Cavia et al., 2010) and contamination through metal, fertilizers, pesticides and polycyclic aromatic hydrocarbons (PAHs) (Loumbourdis and Wray, 1998; Mann et al., 2009; Marquis et al., 2009; Ortiz-Santaliestra et al., 2010b). All of these factors may have contributed, at least in part, for amphibians presenting, besides reptiles, the highest threat status of all terrestrial vertebrates (≈30% of all amphibian species), with significantly higher number of species at risk than birds or mammals (Vié et al., 2009).

1.3.2. Pelophylax perezi

In Portugal, amphibians occupy a wide variety of biotopes, being found in different habitats, such as costal dunes, mountain, agricultural and deciduous forest zones. In general, they can be found covering all of the Portuguese territory, depending however, on the species and their specific ecological requirements. Nonetheless, their presence, mainly during reproduction periods, is restricted to sites with high humidity and water availability (Almeida et al., 2001).

Pelophylax perezi is commonly known as the Iberian green frog. Until recently it was designated by *Rana perezi*, but due to the inconsistent grouping of the genus *Rana* (Frost et al., 2006) recommended the restriction of *Rana* genus only to brown frogs. Thus, the sub-genus *Pelophylax*, previously encompassing all European green frogs, was adopted as the new genus for green frogs. *P. perezi* is widely spread in Portugal and Spain, being also found in southern France. This species is highly water-dependent and has a high tolerance when considering their ecological requirements (Loureiro et al., 2010). Its reproductive period occurs mainly in spring, but it can also extend to the summer. The egg masses released during reproduction usually have between 800 and 10 000 eggs that hatch in few days (Almeida et al., 2001). The larval development is very slow and may take two to four months to be complete. Presently, there are two main reasons for concern with this species conservation. One is the introduction of other European green frogs, (*i.e. P. ridibunda* and *P. lessonae*), that can originate hybrids, leading to a genetic modification of populations (Pagano et al., 2001). The other is the degradation of aquatic habitats (Loureiro et al., 2010), especially because this species is frequent in permanent man-made aquatic water bodies which are more prone to be impacted (Valera et al., 2011).

1.4. Ecotoxicology and the amphibians

1.4.1. *Overview of amphibians' metal ecotoxicology*

Through history, amphibians have adopted behaviors and strategies that have allowed their survival through the last four mass extinctions periods (Blaustein and Bancroft, 2007). However, more recently, there have been significant decreases in

natural populations, concomitant with the disappearance of some amphibian species all over the world (Stuart et al., 2004). Various causes have been pointed as responsible for this decrease of amphibian numbers (Beebee and Griffiths, 2005; Blaustein and Kiesecker, 2002; Stuart et al., 2004). Some of the pointed causes are direct responsibility of humans (see section 1.2.1.). Due to the awareness of amphibian decrease, ecotoxicologists have recently focused their attention on these organisms, mainly with the objective of understanding how contaminants, many responsible for extreme habitat alteration, affect them. There are many examples of this recent interest in amphibian ecotoxicology (e.g. (Calevro et al., 1999; Loumbourdis et al., 2007; Mouchet et al., 2007) and the main approach consists usually on laboratorial short duration assays (normally until 96h) or chronic assays (ranging from several days to various weeks) (Hatch and Blaustein, 2003; Tong, 1999). To a lesser extent, and as an alternative to laboratorial testing, field assays have also been undertaken, gathering more realistic data (Sparling et al., 2000). The increasing number of toxicological studies, allied to the recognized importance of amphibians, lead to the development of standardized protocols focusing specifically these organisms. These standardized protocols include AMPHITOX (Herkovits and Pérez-Coll, 1999), FETAX (ASTM, 1998) and AMA (OECD, 2009), encompassing in their whole acute and chronic exposures and assessing various parameters such as mortality, growth, malformations and endocrine disruption.

Amphibian larvae and adults, due to their highly dependence of water and skin permeability, are highly susceptible to water contaminants. Based on these traits and on the fact that they can transfer contaminants between water and terrestrial compartments, they have been the subject of many toxicological studies regarding metals (e.g. Calevro et al., 1998; Sparling and Lowe, 1996; Sura et al., 2006; Zhang et al., 2007). Their exposure to metals may occur by direct absorption of water through skin (including soil pore water) or by ingestion of contaminated soil, sediment or food (Lefcort et al., 1998; Linder and Grillitsch, 2000). The assessed endpoints range from mortality to growth, behavior alterations, histological alterations, bioaccumulation, alterations in cells genetic material, oxidative stress enzyme activities and body abnormalities (Lefcort et al.,

1998; Marques et al., 2009; Marques et al., 2008; Zhang et al., 2007). For tadpoles, as reviewed by (Venturino et al., 2003) the results consist in development rate delay, reduced weight and size, depigmentation and abnormalities in the notochord, gills, snout and caudal fin. Furthermore, lethargy has also been registered as a consequence of metal exposure. The great incidence of toxicological studies in tadpoles is related to their greater sensitivity when compared to adults. Nonetheless, toxicological studies have also been carried out in adults, revealing histological alterations in various tissues (mainly liver and kidney), metal accumulation, increased genotoxicity (Marques et al., 2009) and increased metallothionein levels (Loumbourdis et al., 2007). Considering all, there is a great amount of information regarding toxicological effects on amphibians, even so, consistent information gathered with standard ecotoxicological assays still is scarce, making difficult to integrate amphibians' sensitivity on the derivation of water quality criteria. As pointed out by Bridges et al. (2002), tadpoles are very tolerant to some contaminants, but very sensitive to others. That may compromise their protection when benchmark values are obtained based on ecotoxicological data gathered for surrogate species, like fish. Further, a detailed knowledge about mechanisms of toxicity in these organisms is still scarce and new approaches are required to fill the gaps that still remain. The uncovering of toxicity mechanisms would allow understanding how species that are supposedly sensitive to contamination, due to their biological and ecological traits, endure relatively high levels of contaminants (Marques et al., 2009; Marques et al., 2008).

1.4.2. *Ecotoxicogenomics, the new ecotoxicological approach*

One of the challenges facing ecotoxicology is understanding the mechanism of action of toxicants on living organisms (Snape et al., 2004). In the early years much of the work carried out was based mainly in acute assays, assessing effects like mortality (e.g. Baudouin and Scoppa, 1974; Linden et al., 1979; Rehwoldt et al., 1973). Gradually with the intention of clarifying how toxicants affected organisms, chronic exposures to environmental relevant concentrations, assessing sub-lethal parameters such as growth, reproduction, behavior and biochemical

alterations became more usual (e.g. Antunes et al., 2007b; Calevro et al., 1999; Farombi et al., 2007; Zhang et al., 2007). More recently, first toxicology and then ecotoxicology have joined genomics to look for answers on toxicity mechanisms. In a broad range definition, genomics can be described as the study of nucleotide sequences, function and structure of genes in the genome, to determine their interaction and influence in the biological pathways (Pennie et al., 2001). This new "association" between genomic and toxicology/ecotoxicology allowed the study of the complex interactions between environmental factors and genome leading to meaningful differences on organism's expressed genome with a subsequent variability of phenotypic responses triggered by different stress agents. This is usually achieved by studying the genome-scale mRNA expression (transcriptome) after exposure to a stressor (Gatzidou et al., 2007). This gene expression profile is the basis for protein synthesis, and various techniques can be used to assess it. One of the most frequently used techniques is the DNA microarray that is based on the hybridization between two DNA strands. Briefly, various probes based on previous genome knowledge, are designed specifically according to the genes intended to detect and then are attached to a solid surface. Subsequently both the control and exposure cDNA samples (obtained from the reverse transcriptase reaction applied to extracted mRNA), labeled distinctly with different dyes will hybridize with matching probes. Afterwards the array is visualized with a fluorometer obtaining two different images, one from each dye. The images are then superimposed through a software and a pattern based on two colors, one corresponding to activated genes and the other to repressed genes, is obtained (Lettieri, 2006). The patterns of relative induction/repression levels of gene expression provide information in the mechanisms involved in cellular perturbations and responses, elicited by specific stressor exposure. In the most favorable cases specific biomarkers for specific molecular damage can be identified from gene expression profiling (Murata et al., 1999; Poynton et al., 2007; Waring et al., 2001). Nonetheless, there is still a major drawback in array based techniques, since they rely on previous knowledge of the transcripts likely to be present in a sample in order to design in advance the gene probes (Clark et al., 2002). This fact limits the application of this technique in organisms for which

available genomic information is limited. In such cases there are other techniques that may be employed to identify stress-induced genes. Among other options (e.g. representational difference analysis), two PCR-based techniques can be referred: the differential display (DD) PCR and the suppressive subtractive hybridization (SSH) (Snell et al., 2003). The DD PCR was first described by (Liang and Pardee, 1992) and in this technique the extracted mRNA will be bound to anchored primers (oligo-dTG; oligo-dTA; oligo-dTC) which are designed specifically to bind to the 5' boundary of the mRNA poly A tail. After reverse transcription, PCR amplification is performed using all combinations possible between anchored primers and a set of degenerate primers of known nucleotide pattern and number. This will generate a mixture of cDNA fragments of different sizes that will be separated by a polyacrylamide gel. Afterwards, the resulting bands are compared with the control group. The cDNA from the bands can then be excised and sequenced to obtain information about the genes differentially expressed. However, DD-PCR has some shortcomings as the high percentage of false positives (Huang et al., 2007). The SSH technique, described initially by (Diatchenko et al., 1996), also enables the identification of genes that are differentially expressed and that can be involved in a particular function or response. This technique is based in the selective amplification of target cDNA fragments (differentially expressed) and simultaneously suppression of non-target cDNA amplification. It is supported by PCR suppression effect in which long inverted terminal repeats attached to DNA fragments can selectively suppress amplification of undesirable sequences (Diatchenko et al., 1996). SSH also combines normalization and subtraction in a single procedure. This technique allows the screening of the entire genome for differentially expressed genes without being necessary previous knowledge of the genome. In fact, it has been applied with success to a wide range of organisms such as bacteria (Rebrikov and Kogan, 2003), zooplankton (Hansen et al., 2007), fish (Sheader et al., 2004) and plants (Degenhardt et al., 2005). In one particular example, SSH was applied to study differential gene expression within two soil arthropod (*Orchesella cincta*) populations, of different origins (reference and metal contaminated mining site population), exposed to cadmium. Specifically both populations were exposed to two conditions, control and a cadmium concentration.

The results yielded by SSH and the analysis performed by the authors revealed many transcriptional responses with highly significant interactions between Cd exposure and population source (reference or metal contaminated mining site). These results helped the authors to confirm a micro-evolutionary process occurring in soil arthropod populations (Roelofs et al., 2009; Roelofs et al., 2007). Furthermore SSH has proved to be an invaluable tool to obtain low abundance transcripts, as reviewed by Huang et al. (2007), who mentioned the example of the identification of six genes differentially expressed in Jurkat cells treated with phorbol 12-myristate 13-acetate (PMA), which have failed to be detected by other techniques.

Gene expression profiling techniques have been applied to metal toxicity studies to elicit the affected pathways. Among the studied elements we can find essential metals like copper or zinc (González et al., 2008; Yepiskoposyan et al., 2006) or non-essential metals like cadmium (Wang and Crowley, 2005). From the range of studies performed on eukaryotes distinct set of genes involved in different functions have been identified. Among the most commonly expressed genes, we can find metal binding proteins (Armendariz et al., 2004) as the well known metallothionein (Roelofs et al., 2008) and also heat shock and transcription involved proteins (Hamadeh et al., 2002; Koizumi and Yamada, 2003). However other genes can also be found differentially expressed according to the organism and metal involved. In general, the increasing use of gene expression profiling has been able to identify novel biomarkers resulting from different stressors including metals (Koskinen et al., 2004; Perkins et al., 2004; Poynton et al., 2008). Overall genes associated with stress responses can be divided into (Farr and Dunn, 1999):

- genes that respond to the presence of a compound;
- genes that respond to the damage caused by the compound;
- genes that respond to altered levels of crucial metabolites;
- genes that respond to changes in the cellular redox status or pH.

Despite the knowledge gathered by gene profile the use of traditional function base biomarkers targeted for the affected systems is still important to validate the toxic mechanisms of contaminants.

1.5. Cunha Baixa's uranium mine case study

The Cunha Baixa uranium mine is located in the small village of Cunha Baixa (Mangualde, Portugal) and besides the usual contamination resulting from the mining activity it also presents radiological contamination (Carvalho et al., 2009). Furthermore, the AMD produced locally is composed by elements such as uranium (U) and strontium (Sr) which can be extremely toxic (ATSDR, 1999). Presently the mine is deactivated, but when it was active between 1967 and 1993 (Oliveira and Ávila, 1998) the ore extraction was made both by open-pit mining and underground mining. After the exploitation ending, the mine pit was filled with low-grade ore and uranium extraction was carried out by an *in situ* leaching process, flooding the mine pit with sulfuric acid (Santo and Freire, 1983).

Several studies, performed as part of an ecological risk assessment study, have already been carried out in this mine, covering both aquatic and terrestrial compartments. Soil contamination by metals with ecotoxicological effects has been confirmed (Pereira et al., 2008), detected mainly through earthworms avoidance assay (Antunes et al., 2008), plants seed germination and growth assays (Pereira et al., 2009) and through the evaluation of reproduction, genotoxic or citotoxic parameters on earthworms as well (Lourenço et al., 2011). From the assessed soils, toxicity was mainly observed in areas where effluent runoff occurred or where effluent sludge deposition was made (Antunes et al., 2008). Despite this, no acute toxicity was obtained in any of the studied soils. For the aquatic compartment, studies were divided between sediment and water column. Both were assessed in three different ponds with very distinct physical and chemical properties that reflect various processes occurring in the mining complex (*in situ* leaching process, open-pit extraction and effluent treatment). Until now overall results for the aquatic compartment have shown that toxicity is mainly owed to the water column and that the toxicological role of the sediment is

secondary (; Antunes et al., 2007a; Antunes et al., 2007b; Antunes et al., 2007c). Acidity has also been pointed as one of the main causes of the effluent toxicity.

1.6. Goals and thesis structure

Deleterious effects caused by mining activities in natural populations are known (Jarvis and Younger, 1997). Nonetheless, in the Cunha Baixa deactivated uranium mine, a population of *P. perezi* has been reported (Marques et al., 2009) withstanding high levels of contamination in both aquatic and terrestrial compartments (Antunes et al., 2007c; Pereira et al., 2008). Histopathological and chemical analysis in tissues of adult *P. perezi* organisms from the uranium mine revealed increased metal content of Al, Be, Fe and U as well as histological alterations, which included increase in melanomacrophagic centers and dilatation of the renal tubules lumen associated with tubular necrosis (Marques et al., 2009). However, these results were not as pernicious as would be expected by the extreme local conditions. Furthermore, acute exposure of embryos and larvae of *P. perezi* to the pH neutralized mine effluent revealed contradictory results in body size lengths (Marques et al., 2008) and mortality only occurred in larvae. Taking into consideration this background information, the main goal of the present thesis is to increase the understanding of the toxicity/resistance mechanisms involved, both in adults and tadpoles, that are and may become exposed to an acid mine drainage with a composition similar to the one from the deactivated mine of Cunha Baixa. In order to achieve that goal, this thesis presents the following specific objectives:

- Assess the activity of various biomarkers involved in oxidative stress responses in *P. perezi* adults inhabiting the mining site and relate them with accumulated metals.
- Assess potential mechanisms of resistance in *P. perezi* adults inhabiting the mining site through differential gene expression.
- Assess development, antioxidant enzymatic responses and bioaccumulation of metals in *P. perezi* tadpoles exposed *in situ* to different mine effluent ponds.

- Evaluate the influence of pH in effluent chronic toxicity effects on *P. perezi* tadpoles and identify genes possibly involved in triggering physiological responses.

The present thesis is composed of six chapters. The first chapter is of introductory nature, addressing various aspects important for understanding the global perspective of this thesis. Chapters two, three, four and five are concurrent with the above mentioned specific objectives and the final chapter (VI) consists of the final remarks, where all of the results obtained in the previous chapters are discussed providing a global perspective.

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Chapter II

Antioxidant response and metal accumulation in tissues of Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine

2. Antioxidant response and metal accumulation in tissues of Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine

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Abstract

Human mining activities tend often to generate greatly impacted areas which remain contaminated for long periods of time, giving rise to extreme habitats. Mining sites are usually characterized for the production of metal rich effluents with very low pH. In this work we analyzed physical and chemical parameters of water from a deactivated uranium mine pond (M) and a reference site (REF) as well as their metal content. Furthermore, we determined and compared metal accumulation in liver, kidney, bones, muscle and skin of *Pelophylax perezi* from REF with *P. perezi* from M. We also determined the enzymatic activities of glutathione-S-transferases (GSTs), catalase (CAT), glutathione reductase (Gred), and glutathione peroxidase (GPx; both selenium-dependent and selenium-independent) in liver, kidney, lung and heart. Additionally, lipoperoxidation (LPO) was also assessed in the same tissues via thiobarbituric acid reactive substances (TBARS) assay and lactate dehydrogenase (LDH) activity was determined in muscle. Our results revealed that the majority of metals were in higher concentrations in tissues of organisms from M. This trend was especially evident for U whose content reached a difference of 1350 fold between REF and M organisms. None of the organs tested for antioxidant defenses revealed LPO, nonetheless, with exception for liver, all organs from the M frogs presented increased total GPx activity and selenium-dependent GPx. However, this response was significant only for the lung, probably as a consequence of the significant inhibition of CAT upstream and to cope with the subsequent increase in H_2O_2. Lungs were the organs displaying greater responsiveness of the anti-oxidant stress system in frogs from the uranium mine area.

Keywords

Oxidative stress; biomarkers; uranium mine; *Pelophylax perezi*; metals

2.1. Introduction

Mining activities are known to exert great impact not only in the extraction site, but also in its vicinities. Such impact comprises, besides the usual landscape transformation, physical, biological and chemical alterations (Oliveira and Ávila 2001; Marqués et al., 2003). A major contribution for these alterations derives from the accumulation of great amounts of mining tails, which through processes of runoff and leaching generates acidic effluents, normally with high contents of metals. Such effluents have usually a negative impact in local fauna and flora leading to the disappearance of the most sensitive species (Marqués et al., 2003).

Amphibians are usually highly sensitive to contamination (Lefcort et al., 1998; Marques et al., 2008; Ortiz et al., 2004). Such fact is related to some key features like highly permeable skin and both aquatic and terrestrial life stages which increase the array of contaminants to which amphibians are exposed consequently augmenting their susceptibility to them (McDiarmid and Mitchell 2000). Nonetheless Marques and co-workers (2009) registered the occurrence of amphibians in metal contaminated areas, without apparent adverse effects. However, even with no evident consequences, species dwelling in metal contaminated areas tend to present high contents of metals in their tissues (Cooper and Fortin 2010; Marques et al., 2009; Stolyar et al., 2008) which may contribute to an alteration of key physiological functions, such as aging, apoptosis, energy balance, redox homeostasis, conditioning the onset of specific diseases (Franco et al., 2009; Leonard et al., 2004; Valavanidis et al., 2006).

Metals act through the establishment of redox cycles, with concomitant generation of highly unstable, reactive oxygen species (ROS). In the presence of abnormally high levels of metals the reducing environment within the cell tends to unbalance due to the increased production of reactive oxygen species (ROS) such as $O^{\cdot -}$ and $HO^{\cdot}$. This increased production of radicals through metal action may result directly from Fenton-like reactions, or indirectly through the formation of thiyl radicals resulting from labile ligations of metals with thiol groups, which in turn may react with molecular oxygen and generate ROS (Galaris and Evangelou, 2002). Also and since aerobic metabolism involves the production of oxygen radicals, the increase of ROS might result from inhibition of antioxidant defence system due to

metal action, rather than direct action (Stohs and Bagchi, 1995). As Strydom et al. (2006) reviewed, this increased production of ROS may affect among others, central metabolic pathways such as glycolysis, protein and amino acid metabolism. Furthermore ROS have also the ability of inducing lipid peroxidation and damaging DNA (Galaris and Evangelou, 2002) and in an ultimate analysis cell death may result from high levels of these radicals. Even known diseases can be provoked by metal derived oxidative stress, that comes via environmental exposure, as shown by Leonard et al. (2004).

Taking into consideration the severe consequences to living organisms of environmental exposures, to high levels of metals, especially in aquatic environments (Antunes et al., 2007a; Lombourdis et al., 1999; Marques et al., 2008), it is important to use a battery of biomarkers, focused on the antioxidant response, which may give an indication of the condition of the antioxidant defence system of organisms (Stochs and Bagchi, 1995). In addition, it is also important to determine environmental and body levels of contaminants and evaluate possible damages to the cells. The activity of several biomarkers involved directly or indirectly in the antioxidant response, such as catalase (CAT), glutathione-S-transferases (GSTs), glutathione reductase (GRed) and glutathione peroxidase (GPx) may be used to assess the potential oxidative effects elicited by chemical contaminants (Stochs and Bagchi, 1995; Valavanidis et al., 2006). This information is important to provide clues on the organisms' ability to cope with oxidative stress, and may further serve as an early-warning indicator of contamination (Van der Oost et al., 2003).

Previous studies (Marques et al., 2008, Marques et al., 2009) have shown the toxicity of the acidic mine effluent from the deactivated uranium mine of Cunha Baixa in *Pelophylax perezi* (formerly *Rana perezi*), both in tadpoles acutely exposed to the effluent, and in adults inhabiting the mining site. The latter ones exhibited higher number of erythrocytic nuclear abnormalities, histological alterations (liver, kidney and lung) and also the accumulation of metals (liver and kidney). In order to enhance knowledge on the effects of life-time exposure of frogs to this particular contaminated site our work intended to assess the activity of various enzymes involved in oxidative stress response such as GSTs, CAT, GRed

and GPx, in *P. perezi* organs (liver, kidney, lungs, and heart). In addition, lipid peroxidation (LPO) was also assayed in these tissues through the determination of thiobarbituric acid reactive substances (TBARS) and lactate dehydrogenase (LDH) activity was determined in muscle to assess possible alterations in energy acquisition mode (shift of aerobic to anaerobic pathway). Metal content in bones, liver, kidney, skin and muscle tissues was measured as well, to look for possible cause-effect relationships between metal accumulation and biomarker responses.

2.2. Material & Methods

2.2.1. *Study site*

The selected site to carry out this study was the deactivated uranium mine located near the village of Cunha Baixa (Mangualde, Centre of Portugal) where ore extraction occurred between 1967 and 1993 (Oliveira and Ávila, 1998). After the exploration period has ceased, the mine pit was filled with low-grade ore and was flooded with sulphuric acid to extract uranium through an *in situ* leaching process (Santo and Freire, 1983). Presently, there are three small ponds in this area, with variable water volumes that fluctuate according to the uprising of the underground acidic effluent, which, in turn, is determined by variations in the level of the aquifer. This effluent, due to 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. The largest of the ponds (M pond) is the one with the most extreme conditions because of its direct connection with the underground tunnels of the mine pit and, consequently, with the mine effluent. Iberian green frogs (*P. perezi* Seoane) are frequently found on the three ponds. However, no egg masses were recorded on the M pond. Furthermore during their winter lethargic state, they have been found burrowed (authors' personal observation) in the highly metal contaminated sludge that results from the effluent treatment pond (Pereira et al., 2008). The reference site chosen for this study is an unpolluted segment of the Vouga River located in the north part of the city of Viseu, a few kilometers from Mangualde, where frogs from the same species have been recorded (Marques et al., 2009).

2.2.2. *Animal capture and tissue sampling*

Adult animals were captured in spring from both REF and M sites using a hand net and immediately taken alive to the laboratory where they were anaesthetized and measured (body weight and length, snout to vent). To reduce the influence of sex, all of the captured Iberian green frogs were subjected to a previous inspection; females were discarded, and males were kept (until n=5 per site) for further processing. Animals were anesthetized with ethyl ether and sacrificed, and lung, liver, kidney, heart, hind leg muscle, femur (hence forward designated only by bone) and skin were collected and weighed (fresh weight) to the nearest 0.1 mg. In the case of hind leg muscle and kidney, since they were used for both chemical and biochemical analyses, they were previously divided in two pieces and weighed individually to the nearest 0.1 mg. The same procedure was applied to the liver, being nonetheless divided into 3 pieces since a further genetic study is being performed. Tissues for metal quantification were stored at -20 ºC until metal quantification was possible and tissues for biochemical analysis were frozen in liquid nitrogen until preparation for further analysis.

2.2.3. *Water sampling*

Water samples for chemical analyses were collected from the REF site and from the M pond in 0.5 L plastic bottles and 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 WTW330/SET-2 pH meter.

2.2.4. *Chemical analyses*

The total concentrations of Be, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Cd, Ba, Pb and U were quantified in the REF water and in the mine effluent (M), as well as in the liver, kidney, muscle, skin and bone 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. 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, kidney, skin, bone and muscle an initial wet-digestion was required. The tissues were first oven-dried at 105 ºC until a stable weight was achieved. After having been dried, the weight of the tissue was recorded to the nearest 0.1 mg, and they were digested in closed Teflon flasks with 3 ml of nitric acid (suprapur Merck®, 65 %) in a 60 ºC sand-bath. Aliquots of 0.5 ml of suprapur hydrogen peroxide (30 %) MERCK® were then added to free the solution of any organic solid fragments. The final volume of the solution was made up to 5 ml with Milli-Q® water (18.2 Ω). Sample blanks were obtained following the same procedure described for wet-digestion but replacing the tissue volume by Milli-Q® water and replicate measurements in ICP/MS were made for each sample in order to check if a variance coefficient lower than 10% was obtained.

2.2.5. *Biomarkers*

Oxidative stress responses were evaluated in liver, kidney, heart and lung through the enzymatic determinations of CAT, GPx, GRed and GSTs. The extent of lipid peroxidation was assessed through the measurement of thiobarbituric acid reactive substances. Also, LDH was determined in muscle tissue to evaluate alterations in the energy acquisition pathways of organisms.

Liver, kidney, heart and lung, which had been previously frozen individually in liquid nitrogen, were homogenized in ice-cold phosphate buffer (50 mM, pH= 7.0 with 0.1 % TRITON X-100). Homogenates were centrifuged at 10,000 g for 10 min and supernatants were divided into six aliquots, one for each determination (CAT, GPx, GST, GRed and TBARS) and a spare one. Aliquots were stored at -80 ºC until determinations were possible.

GSTs (EC 2.5.1.18) activity was determined by spectrophotometry, according to Habig et al. (1974). GSTs catalyze the conjugation of the substrate 1-

chloro-2,4-dinitrobenzene (CDNB) with glutathione, forming a thioeter (molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$) that can be followed by the increment of absorbance at 340 nm.

GRed (EC 1.8.1.7) activity was determined by spectrophotometry, according the protocol of Carlberg and Mannervik (1985). In this assay the GRed mediated oxidation of NADPH was monitored at 340 nm (molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$).

CAT (EC 1.11.1.6) activity was determined by the spectrophotometric method described by Aebi (1984). This method consists in monitoring the consumption of hydrogen peroxide, at a wavelength of 240 nm (molar extinction coefficient of 0.0394 $mM^{-1}cm^{-1}$) for a period of 30 s.

GPx (EC 1.11.1.9) activity was determined according to Flohé and Günzler (1984), following the oxidation of NADPH at a wavelength of 340 nm (molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$), when GSSG is reduced back to GSH by glutathione reductase. GPx activity was monitored using both hydrogen peroxide (0.255 mM) and cumene hydroperoxide (0.7 mM) as independent substrates, corresponding, respectively, to selenium-dependent glutathione peroxidase and total glutathione peroxidase.

After the muscle was thawed and homogenized in ice-cold Tris/NaCl buffer (pH = 7.2) the LDH (EC 1.1.1.27) activity was measured in the homogenates following the method of Vassault (1983). This method consists in monitoring oxidation of β-NADH by following the decrease in absorbance at a wavelength of 340 nm (molar extinction coefficient of 6.3 $mM^{-1}cm^{-1}$). This oxidation occurs in the presence of LDH and pyruvate substrate.

Enzymatic activities were determined in triplicate and were expressed in µmol (CAT, GRed, LDH) or nmol (GSTs, selenium-dependent GPx and total GPx) of substrate hydrolyzed per min per mg of sample protein (see protein quantification below).

The extent of lipid peroxidation was measured by the quantification of TBARS, according to the protocol described in Buege and Aust (1978). This methodology is based on the reaction of lipid peroxidation by-products, such as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA). The amount of TBARS

was measured spectrophotometrically as a single determination, at a wavelength of 535 nm (molar extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1}$), and results were expressed as nmol of MDA equivalents per mg of sample protein.

Protein concentration of each sample was determined in triplicate, according to the spectrophotometric (wavelength = 595 nm) method of Bradford (1976) adapted to microplate. All biomarkers (CAT, total and selenium-dependent GPx, GRed, GSTs, LDH and TBARS) were expressed as function of the protein content of the corresponding sample. For statistical purposes, and to reduce the variability of biochemical determinations, we averaged the biomarker values of the 5 captured organisms, per sampling site.

2.2.6. *Statistical analysis*

To test for statistical significant differences between animals captured on different sites for metal concentrations, antioxidant enzymes activity and TBARs, data were analyzed through parametric one-way analysis of variance (ANOVA). Before testing, all data were checked for normality and homogeneity to meet statistical demands (Zar, 1996). A level of significance of 0.05 was chosen for rejecting the null hypothesis. Pearson's correlation coefficients (r) were calculated to uncover possible associations between the determined biomarkers and metal concentrations in liver and kidney of organisms from each site.

2.3. Results

2.3.1. *Water samples chemical characterization*

The water quality for both REF and M sites, was assessed by comparing the results obtained from chemical parameters (Table 1) with the maximum recommended values (MRVs) and maximum admissible values (MAVs) for water used for human consumption established by Portuguese legislation (MA 1998). Nonetheless, since these values may be considered overprotective, other benchmark values for metals, available in the literature (Suter and Tsao, 1996) were also taken into consideration and are displayed in Table 1.

Water from the REF presented values of hardness, dissolved O_2, conductivity and pH that were within Portuguese benchmark values (Table 1). On the other hand, M water revealed a 2 times higher value of conductivity relatively to the MRV and a very low pH value, revealing the acidic nature of the effluent. When considering the content of the various metals quantified, M consistently presented higher values than the REF water. For Be, Al, Mn, Fe, Co, Ni and U values in M surpassed every benchmark value presented in Table 1, as well as the MRV and MAV.

Table 1 – Chemical and metal concentration data for REF (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.

	ORNL Tier II Chronic	EC20-SB	EPA R4-SB	MRV	MAV	REF	M	Detection Limit
Hardeness CaCO3 (mg/l)				NLV	500	8.7	358.9	
Dissolved O_2 (mg/l)				NLV	NLV	8.22	4.8	
Conductivity (µS/cm)				400	NLV	42.2	**1093**	
pH				6.5-8.5	NLV	6.7	**3.4**	
Element (µg/l)								
Be	0.66	NLV	0.53	NLV	NLV	BDL	**19.16**	0.10
Mg	NLV	NLV	NLV	30000.00	50000.00	903.54	35871.49	2.00
Al	NLV	75.00	87.00	50.00	200.00	75.84	**3230.59**	5.00
K	NLV	NLV	NLV	10000.00	12000.00	648.51	3694.85	10.00
Ca	NLV	NLV	NLV	100000.00	NLV	1981.84	84599.35	10.00
V	20.00	NLV	NLV	NLV	NLV	BDL	BDL	0.50
Cr	NLV	0.26	11.00	NLV	NLV	BDL	BDL	0.50
Mn	120.00	NLV	NLV	20.00	50.00	12.76	**4771.98**	0.01
Fe	NLV	NLV	1000.00	50.00	200.00	201.40	**2745.93**	10.00
Co	23.00	NLV	NLV	NLV	NLV	0.18	**59.07**	0.01
Ni	NLV	11.00	87.71	NLV	50.00	BDL	**95.09**	0.10
Cu	NLV	0.26	6.54	3000.00	NLV	BDL	21.46	0.50
Zn	NLV	21.00	58.91	500.00	NLV	5.90	356.99	0.50
As	NLV	55.00	190.00	NLV	50.00	1.39	1.64	0.10
Se	NLV	2.60	5.00	NLV	10.00	BDL	BDL	0.50
Sr	1500.00	NLV	NLV	NLV	NLV	14.97	226.96	0.01
Cd	NLV	0.01	0.66	NLV	5.00	BDL	BDL	1.00
Ba	4.00	NLV	NLV	100.00	NLV	4.09	10.94	0.05
Pb	NLV	0.35	1.32	NLV	50.00	BDL	BDL	0.50
U	2.60	NLV	NLV	NLV	NLV	BDL	**1081.00**	0.05

MRV and MAV stands for Maximum recommendable values and Maximum admissible values of waters for human consumption (MA, 1998). ORNL Tier II Chronic – Oak Ridge National Laboratory Tier II Chronic values for allowing the establishment of aquatic benchmarks with fewer data (Suter and Tsao, 1996). EC20 - Sensitive species surface water screening benchmark (Suter and Tsao, 1996). EPA R4 - Chronic Surface water screening benchmark (Suter and Tsao, 1996).
NLV stands for No Legal Values established. The values exceeding the different benchmark values available were written in bold letter. BDL stands for Below Detection Limit.

2.3.2. *Tissues chemical analysis*

The body weight of the captured animals presented a mean value of 27.88 ± 7.86 g and a mean body size of 6.8 ± 0.6 cm. Metal concentrations were recorded in liver, kidney, bones, muscle and skin of animals from both REF and M (Table 2).

Table 2 – Concentration of metals on liver, kidney, bones, muscle and skin of *Pelophylax perezi* (n=5) from REF and M sites.

		Liver	Kidney	Bones	Muscle	Skin
		Average ± SE	Average ± SE	Average ± SE	Average ± SE	Average ± SE
Be	REF	0.38 ± 0.11	BDL	0.07 ± 0.01	BDL	0.24 ± 0.03*
	M	2.32 ± 1.19	BDL	0.16 ± 0.04	BDL	1.53 ± 0.40*
Mg	REF	1070.69 ± 62.41*	ND	3885.40 ± 300.28	1872.12 ± 70.86	1599.72 ± 125.85
	M	810.33 ± 51.54*	ND	4585.53 ± 296.20	1813.60 ± 55.26	1951.15 ± 150.65
Al	REF	35.44 ± 10.12	20.00 ± 3.06	3.68 ± 0.74**	11.06 ± 9.35	33.77 ± 12.44*
	M	790.97 ± 383.82	17.43 ± 2.20	21.47 ± 5.26**	1.83 ± 0.62	113.92 ± 29.97*
K	REF	15736.08 ± 723.38*	ND	5924.41 ± 851.48*	26838.36 ± 1006.79	8731.85 ± 155.62*
	M	12741.09 ± 929.51*	ND	3567.05 ± 307.35*	26368.08 ± 978.10	6849.53 ± 579.12*
Ca	REF	421.48 ± 79.50	ND	217219.31 ± 17102.66*	946.10 ± 158.73	41596.68 ± 4233.70**
	M	378.78 ± 72.48	ND	283980.43 ± 21215.65*	673.85 ± 40.32	74169.71 ± 7498.60**
V	REF	1.98 ± 0.49	ND	0.44 ± 0.05	BDL	BDL
	M	1.17 ± 0.21	ND	0.32 ± 0.04	BDL	BDL
Cr	REF	2.47 ± 0.36	ND	1.48 ± 0.22	1.88 ± 0.19*	2.56 ± 0.26
	M	4.16 ± 1.04	ND	1.41 ± 0.30	1.20 ± 0.10*	2.72 ± 0.36
Mn	REF	20.22 ± 3.06	9.87 ± 1.11*	164.92 ± 50.37	1.80 ± 0.29	104.78 ± 49.03
	M	13.95 ± 1.05	13.70 ± 0.62*	248.09 ± 46.57	1.81 ± 0.20	229.38 ± 65.01
Fe	REF	1265.67 ± 380.19*	213.18 ± 40.16	39.56 ± 5.55	18.08 ± 3.62	37.50 ± 9.71*
	M	6051.50 ± 1391.39*	295.94 ± 23.26	45.88 ± 3.48	26.77 ± 5.85	164.95 ± 45.23*
Co	REF	0.90 ± 0.13**	ND	0.62 ± 0.05**	0.03 ± 0.01*	0.25 ± 0.03*
	M	1.95 ± 0.27**	ND	0.92 ± 0.07**	0.08 ± 0.02*	0.59 ± 0.12*
Ni	REF	1.24 ± 0.38	5.51 ± 2.17	2.66 ± 0.21**	1.22 ± 0.88	2.98 ± 0.53
	M	1.06 ± 0.06	4.37 ± 0.64	3.63 ± 0.19**	0.41 ± 0.10	2.19 ± 0.14
Cu	REF	306.54 ± 62.21*	11.84 ± 1.40	2.06 ± 0.17	3.02 ± 0.38	5.30 ± 0.38
	M	2846.56 ± 900.99*	9.37 ± 0.53	2.37 ± 0.14	3.14 ± 0.39	6.11 ± 0.27
Zn	REF	106.74 ± 8.99*	84.63 ± 16.18	129.20 ± 18.38	51.89 ± 31.45	223.81 ± 32.27*
	M	80.35 ± 4.62*	97.13 ± 9.85	170.95 ± 6.98	21.91 ± 1.63	598.58 ± 129.55*
As	REF	0.73 ± 0.14	0.68 ± 0.16	0.24 ± 0.04*	0.44 ± 0.07**	0.28 ± 0.02*
	M	0.45 ± 0.06	0.44 ± 0.04	0.10 ± 0.02*	0.18 ± 0.02**	0.20 ± 0.02*
Se	REF	2.76 ± 0.31*	ND	0.41 ± 0.04	0.75 ± 0.09*	1.35 ± 0.11
	M	10.31 ± 2.94*	ND	0.51 ± 0.07	1.39 ± 0.24*	1.62 ± 0.23
Sr	REF	1.18 ± 0.34	1.28 ± 0.34	378.05 ± 44.07	0.79 ± 0.21	79.73 ± 13.88
	M	0.86 ± 0.12	0.84 ± 0.17	288.90 ± 41.72	0.38 ± 0.05	72.28 ± 4.94
Cd	REF	3.55 ± 1.58	15.28 ± 3.82	BDL	BDL	BDL
	M	7.75 ± 2.00	10.47 ± 1.40	BDL	BDL	BDL
Ba	REF	12.37 ± 2.94	ND	25.57 ± 3.61*	0.47 ± 0.12	31.92 ± 8.02
	M	14.39 ± 3.59	ND	13.27 ± 3.03*	0.41 ± 0.14	27.95 ± 8.48
Pb	REF	BDL	0.58 ± 0.06	2.03 ± 0.60	BDL	3.65 ± 1.71
	M	BDL	0.42 ± 0.04	1.59 ± 0.41	BDL	2.49 ± 0.51
U	REF	0.18 ± 0.08*	0.21 ± 0.04**	0.26 ± 0.09*	0.10 ± 0.09	0.58 ± 0.25***
	M	243.06 ± 76.75*	9.64 ± 1.85**	106.76 ± 32.27*	0.48 ± 0.17	102.45 ± 12.76***

Concentrations are expressed in µg/g, dry weight.

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

**: represents a statistical significant difference ($p<0.01$) between REF water course and the M pond.

***: represents a statistical significant difference ($p<0.001$) between REF water course and the M pond.

BDL stands for Below Detection Limit.

ND stands for Not Determined.

SE stands for Standard Error.

The quantification of metals in the liver revealed significant higher values in M organisms for Fe ($F = 11.009$; d.f. = 1; $p < 0.011$) Co ($F = 12.819$; d.f. = 1; $p < 0.007$), Cu ($F = 7.910$; d.f. = 1; $p < 0.023$), Se ($F = 6.521$; d.f. = 1; $p < 0.034$) and U ($F = 10.014$; d.f. = 1; $p < 0.013$). From these elements U was the one that presented the most remarkable difference between REF and M frogs, since it was about 1350 times higher in M organisms. In opposition REF animals presented, in this tissue, significant higher values for Mg ($F = 10.347$; d.f. = 1; $p < 0.012$), K ($F = 6.466$; d.f. = 1; $p < 0.035$) and Zn ($F = 6.811$; d.f. = 1; $p < 0.031$), nonetheless for these elements the highest difference obtained between animals from both sites was of 1.3 times for Mg and Zn.

For the kidney significant higher values were only recorded for M organisms for Mn ($F = 10.165$; d.f. = 1; $p < 0.015$) and U ($F = 20.265$; d.f. = 1; $p < 0.003$) being almost 46 times higher in frogs captured in the mine pit.

Bone metal content revealed significant higher values in REF organisms for K ($F = 6.781$; d.f. = 1; $p < 0.031$), Ba ($F = 6.802$; d.f. = 1; $p < 0.031$) and As ($F = 8.451$; d.f. = 1; $p < 0.020$). Arsenic was 2.4 times higher in animals from the REF site. Relatively to the M organisms, the elements which presented significant higher values were Al ($F = 11.212$; d.f. = 1; $p < 0.010$), Ca ($F = 6.002$; d.f. = 1; $p < 0.040$), Co ($F = 13.782$; d.f. = 1; $p < 0.006$), Ni ($F = 13.484$; d.f. = 1; $p < 0.006$) and U ($F = 10.893$; d.f. = 1; $p < 0.011$). The greatest difference was recorded for U which was 410 times higher in M frogs.

Metal values in muscle showed significant higher values of Cr ($F = 10.414$; d.f. = 1; $p < 0.012$) and As ($F = 13.752$; d.f. = 1; $p < 0.006$) in REF animals. Arsenic was, of these two elements, the one that presented the highest difference among sites (2.4 times higher in REF). For M organisms the elements that showed significant higher levels were Se ($F = 6.219$; d.f. = 1; $p < 0.037$) and Co ($F = 5.528$; d.f. = 1; $p < 0.047$). Co was particularly bioaccumulated by these animals (2.6 times more) when compared with those from the REF.

The quantification of metals in the skin revealed that animals in the REF site presented significant higher levels of K (1.2 times higher) ($F = 9.853$; d.f. = 1; $p < 0.014$) and As ($F = 9.451$; d.f. = 1; $p < 0.015$). Organisms from the M site presented significant higher values for Be ($F = 10.248$; d.f. = 1; $p < 0.013$), Al ($F =$

6.103; d.f. = 1; $p < 0.039$), Ca ($F = 14.308$; d.f. = 1; $p < 0.005$), Fe ($F = 7.590$; d.f. = 1; $p < 0.025$) , Co ($F = 7.257$; d.f. = 1; $p < 0.027$), Zn ($F = 7.880$; d.f. = 1; $p < 0.023$), and U ($F = 63.715$; d.f. = 1; $p < 0.001$). Once again uranium was particular bioaccumulated by this organ (177 times more) in frogs captured in the mine.

On overall when considering significant differences between sites, the M organisms were the ones that presented higher differences in metal content, being U the responsible for the highest observed difference (1350 times more).

2.3.3. *Biomarkers*

The determination of GSTs (Fig. 1) revealed only significant different values for the heart ($F = 78.660$; d.f. = 1; $p < 0.001$), with higher levels in *P. perezi* from the M site. As for GRed (Fig. 2) values, lung was the single organ presenting a significant lower average value in organisms from M $F = 7.439$; d.f. = 1; $p < 0.05$). The same pattern was observed for CAT (Fig. 3), presenting also a significantly lower value ($F = 36.664$; d.f. = 1; $p < 0.001$) for M. The selenium dependent GPx (Fig. 4) presented only significant differences for lung ($F = 16.370$; d.f. = 1; $p < 0.01$), revealing higher values in M. Nonetheless the remaining results pointed to a pattern similar to the one observed in total GPx. Regarding values for total GPx (Fig. 4) significantly higher values were observed for M frogs in lung ($F = 7.354$; d.f. = 1; $p < 0.05$), kidney ($F = 5.739$; d.f. = 1; $p < 0.05$) and heart ($F = 12.779$; d.f. = 1; $p < 0.01$). The results for lipid peroxidation (Fig. 5) did not indicate significant differences between organisms from both sites in any organ. The absence of significant differences was also observed for LDH (Fig. 6).

Statistically significant correlations between biomarkers and metals in liver and kidney from organisms from REF and M sites, are shown in Table 3. The obtained correlations in REF were all negative while in M more than a half of the correlations were positive. Results show a positive enzymatic and LPO correlation in the contaminated site, both in liver and kidney, mainly related with non-essential metals such as Be, Al, Sr and U. As for the REF site, the correlations obtained were all negative, regardless of being with essential or non-essential metals. Nonetheless the non-essential metals showing a correlation with biomarkers in

animals from this site (As, Pb and Cd), were not present in the tissue (kidney) in statistically significant higher concentrations than in animals from the M site.

Table 3 – Pearson correlation coefficients for the relationship between biomarkers and metals in kidney and liver of *P. perezi* of REF and M sites.

Site	Tissue	Biomarkers	Correlation coefficient	P value	Metal
REF	Kidney	CAT	-1.000	0.0001	As
		selenium-dependent GPx	-0.962	0.0380	Pb
		GRed	-0.996	0.0045	Cd
	Liver	CAT	-0.905	0.0346	Ca
			-0.915	0.0294	Mn
			-0.924	0.0248	Sr
		selenium-dependent GPx	-0.986	0.0020	K
			-0.927	0.0232	Se
		total GPx	-0.992	0.0008	K
		GRed	-0.922	0.0257	Mg
			-0.955	0.0112	K
		GSTs	-0.930	0.0218	Mg
			-0.985	0.0023	K
		TBARS	-0.962	0.0089	Mg
			-0.931	0.0214	Zn
M	Kidney	total GPx	-0.900	0.0372	Cu
		GSTs	**0.925**	0.0246	**Fe**
		TBARS	**0.904**	0.0352	**Sr**
	Liver	CAT	-0.909	0.0327	K
		selenium-dependent GPx	**0.916**	0.0289	**Be**
			0.929	0.0222	**Al**
			0.935	0.0199	**Se**
		total GPx	-0.948	0.0143	K
			-0.913	0.0307	Ca
			0.933	0.0208	**Ni**
			-0.973	0.0052	Sr
		GRed	-0.928	0.0230	Mn
			0.952	0.0127	**U**
		TBARS	**0.987**	0.0017	**U**

Positive correlation coefficients between biomarkers and metals were written in bold.

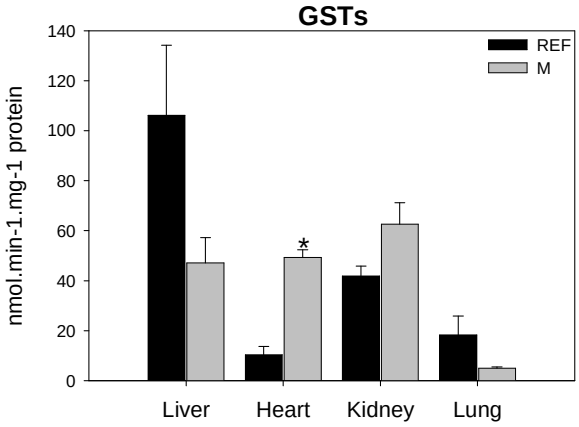

Fig. 1. Mean Glutathione-S-transferases (GSTs) activity in liver, lung, kidney and heart for *P. perezi* from REF and M sites. ⋆: represents a statistical significant difference ($p<0.05$) between REF and M. Error bars represent standard error.

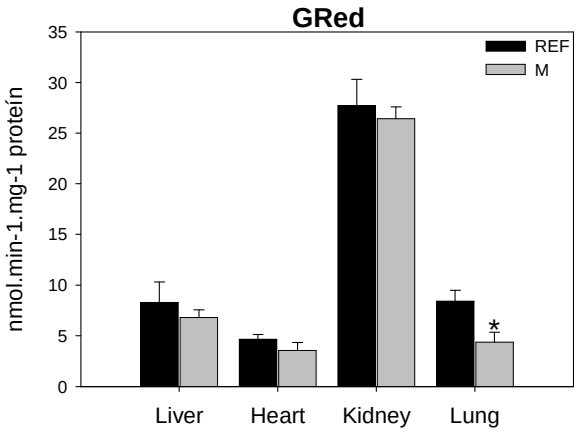

Fig. 2. Mean Glutathione reductase (GRed) activity in liver, lung, kidney and heart for *P. perezi* from REF and M sites. ⋆: represents a statistical significant difference ($p<0.05$) between REF and M. Error bars represent standard error.

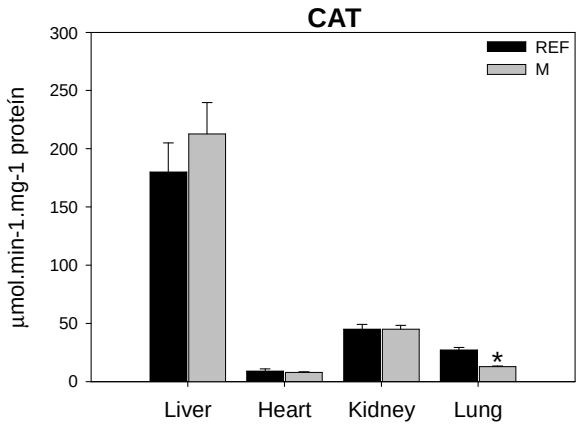

Fig. 3. Mean Catalase (CAT) activity in liver, lung, kidney and heart for *P. perezi* from REF and M sites. ⋆: represents a statistical significant difference ($p<0.05$) between REF and M. Error bars represent standard error.

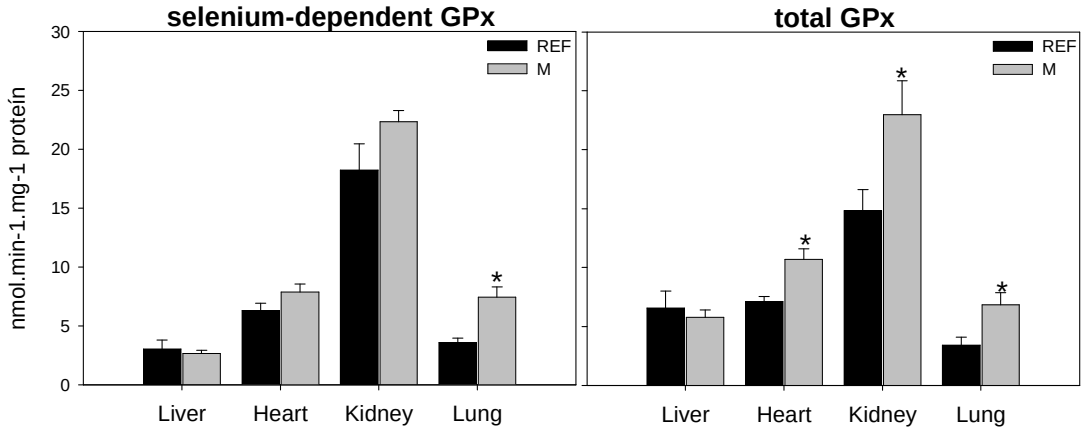

Fig. 4. Mean selenium-dependent Glutathione Peroxidase and mean total Glutathione Peroxidase activities in liver, lung, kidney and heart for *P. perezi* from REF and M sites. *: represents a statistical significant difference ($p<0.05$) between REF and M. Error bars represent standard error.

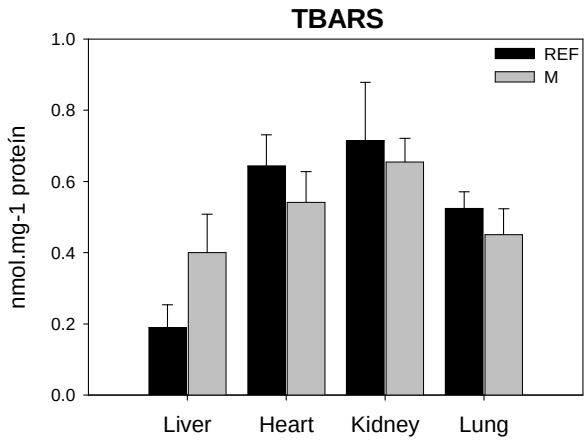

Fig. 5. Mean content of thiobarbituric acid reactive substances (TBARS) in liver, lung, kidney and heart for *P. perezi* from REF and M sites. Error bars represent standard error.

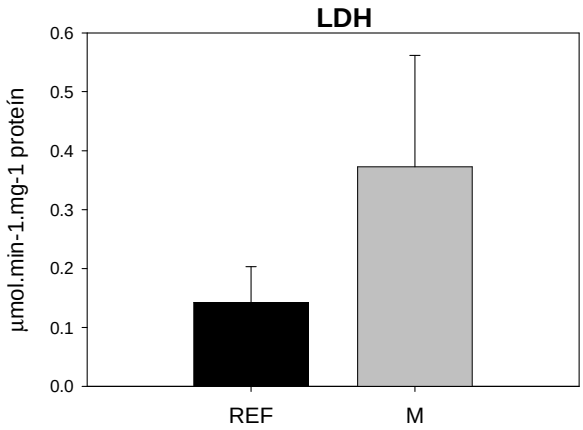

Fig. 6. Mean Lactate Dehydrogenase (LDH) activity in muscle for *P. perezi* from REF and M sites. Error bars represent standard error.

2.4. Discussion

The deactivated uranium mine of Cunha Baixa has been the subject of many studies contemplating terrestrial and aquatic compartments (André et al., 2009; Antunes et al., 2007a, Antunes et al., 2007b, Antunes et al., 2007c; Marques et al., 2008; Pereira et al., 2008; Pereira et al., 2009) and the M site has revealed to be highly contaminated by metals in both. Nonetheless *P. perezi*, a supposedly sensitive species, is found inhabiting this site, using a set of 3 ponds as breeding and living habitat (Marques et al., 2009). The biggest pond (M pond) usually persists through the year and presents the most extreme conditions, due to the direct contact with the mining pit, while the two smaller ones tend to become almost or completely dry during summer. Taking into consideration these facts it is undeniable that Iberian green frogs inhabiting the M site are chronically exposed to contamination by metals. There are several ways in which they may become exposed to metals, such as direct absorption through skin, ingestion of soil or sediment (Linder and Grillitsch, 2000) and ingestion of food (Lefcort et al., 1998). Nonetheless, contaminants bound to sediments are apparently not bioavailable (Antunes et al., 2007c), leaving effluent water as the main source of exposure to metals in the aquatic compartment. Even so, the soil surrounding the ponds might also represent an important input of possibly bioavailable metals since its toxic effects have been demonstrated in edaphic species (Antunes et al., 2008; Lourenço et al., 2011). The close contact with the highly contaminated sludge from the treatment pond (Pereira et al., 2008) resulting from the burrowing behavior described before, might be one of the main causes of the exposure of frogs to metals from the soil. Moreover some edaphic species inhabiting the deactivated mine may also contribute, via food web, to increase amphibian exposure to contamination.

The quantification of metals in the water samples collected both in REF and M sites, revealed that the M pond had the highest values for all the metals determined, when compared to REF (Table 1). Furthermore pH was extremely low, which may influence the solubility and the bioavailability of such metals (ATSDR, 1999; ATSDR, 2004; ATSDR, 2006; Schaller et al., 1994a). The metals

(Be, Al, Mn, Fe, Co, Ni and U) that were present in the M pond in concentrations higher than all the benchmark values considered in Table 1, were also found in at least one of the tissues of M animals (Table 2) at significant higher values than those observed for the same tissue in animals from the REF site. Other authors (Loumbourdis et al., 2007; Stolyar et al., 2008) also observed, for the closely related amphibian *Rana ridibunda*, that in contaminated sites, with higher values of metals, these organisms tend to present higher accumulation of these elements. The absorption of metals in amphibians might result from various factors previously mentioned. But in our particular case and since *P. perezi* is a highly water dependent frog, spending much time submerged, the absorption of contaminants seems to be primarily owed to contact and ingestion of water through skin.

For some metals (Mg, K, Cr, As and Ba), our results revealed that REF animals presented significantly higher levels, nonetheless a further look into the data shows that despite being significantly higher, these values are, in the most extreme case of As in muscle, 2.4 fold higher than those recorded in M organisms. In opposition, frogs from the mine pit presented significantly higher values of U, which reached 1350 times the value recorded in REF animals. The highest differences, in terms of metals accumulation, were obtained for U in all tissues, with exception for muscle. Such result might point to a toxicity mainly owed to this element and also for a preferential accumulation in liver, bones, kidney and skin. The affinity by these organs is coincident with data presented in the toxicological profile for U (ATSDR, 1999). Cooley and Klaverkamp (2000) obtained similar results for *Coregonus clupeaformis*, exposed to a U-rich diet, with significant accumulation in bones, scales, intestine, liver and kidney and no significant accumulation in muscle. Typically kidney and liver tend to present higher accumulation of metals due to their role as detoxifying and excretory organs (Farombi et al., 2007; Fisenne, 1994; Loumbourdis et al., 2007; Malik et al., 2010). As for bones they tend to accumulate Ca analogs, being thus also a target organ for the accumulation of many metals (Arruda-Neto et al., 2004; Drolet-Vives et al., 2009).

The presence of high levels of metals especially in liver and kidney may result in alterations of enzymes involved in oxidative stress response, ultimately leading to LPO (Farombi et al., 2007). Our results showed that in the liver there were no significant differences in any of the enzyme activities measured. Such results were not expected since other authors such as Kelly and Janz (2009) registered alterations, at least in liver GPx activity in *Esox lucius* exposed to uranium. Furthermore Barillet and co-workers (2007) also found alterations in CAT and superoxide dismutase activities in *Danio rerio* exposed to different isotopic compositions of uranium. Nonetheless the biomarker/metal correlation data revealed distinct patterns between livers of animals from both sites, with significantly positive correlations only in the M site, and mainly related with non-essential metals. This fact may indicate that different pathways are involved for essential metals and non-essential metals. As Bozhkov (2010) reviewed the influence of some metals in enzymatic activity, may lead to the formation of a new metabolic pattern which in a complex mixture of metals may help understand some unexpected results. Furthermore other factors must be taken into consideration, such as the possible scavenging action of the metallothioneins, which may vary in a metal concentration and time dependent pattern (Vogiatzis and Loumbourdis, 1997), the existence of melanomacrophagic centers (MMC) (Marques et al., 2009) or simply the basal level of the antioxidant enzymes which could have been high enough to cope with ROS aggression. Metallothioneins are, as Van der Oost et al. (2003) reviewed, a family of proteins rich in cystein which may intercept and bind metal ions and remove them from non-thionein ligands, a mechanism that may be considered a detoxification step. As for MMC, they are cells similar to macrophages, which contain pigments like lipofucin, melanin and hemosiderin (Wolke, 1992) which can act as a free radical scavenger (Różanowska et al., 1999). Even though histopathological alterations have not been analysed in the present work, a previous study by Marques and co-workers (2009) with *P. perezi* captured in the same sites, pointed to an increase in number and size of MMC in organisms from M sampling site. This fact strengthens further the hypothesis of the anti-oxidant action of the MMC, which may have reduced the need for anti-oxidant enzymes intervention, in the liver.

As far as lung was considered, we observed that GRed had a significantly lower activity, as well as CAT, in organisms from the M site, revealing nonetheless a significant increase in both total and selenium dependent GPx. Pandya and co-workers (2010) also obtained similar results in liver of male rats co-exposed to Pb and Cd. Some metals like Al tend to be more concentrated in the lung as age increases (Schaller et al., 1994b) and others, like Be tend to enter via respiratory tract (Rossman, 1994). Furthermore the accumulation of U, one of the main metals present in the M site has been observed in the lungs of rats exposed to this element (Pellmar, 1999), and Periyakaruppan and co-workers (2007) have showed the existence of oxidative stress caused also by U in this tissue. The inhibition of CAT might be related to an excessive presence of hydrogen peroxide (Pigeolet et al., 1990) which may explain the significant increase in the activity of selenium dependent GPx since it is an alternative way of eliminating H_2O_2. Alternatively CAT may also be inhibited by excess of metals (Atli et al., 2006; Singh and Sivalingam, 1982), a response that may be resulting from the direct inhibitory effect of metals (e.g. binding to –SH residues in proteins). Relatively to the lower activity of GRed it might be in part owed to inhibition due to the toxic action of metals (Lalaouni et al., 2007; Tandogan and Ulusu, 2010). This lower activity suggests that fewer glutathione (GSH) is being obtained through GRed cycling of oxidised glutathione (GSSG), since GSH concentration in the cell is associated both to the efficiency of GRed activity and GSH *de novo* synthesis (Meister and Anderson, 1983). Thus, and considering the higher activity of both selenium-dependent GPx and the indispensible availability of GSH for their correct function, *de novo* synthesis of GSH must be occurring in lung cells. Taking into consideration the almost certain contact of lungs with higher levels of metals in the M frogs, the high activity of glutathione peroxidase might be in part responsible for the absence of LPO in this tissue.

Kidney revealed only significant higher values for the activity of total GPx in M organisms. Considering that from the tissues that presented significant accumulation of U, this was the one with the lower difference between sites, lower alterations in antioxidant enzymes were also expected. Also, the biomarker/metal correlation data did not reveal a distinct pattern in this tissue in order to establish a

direct relation between a specific biomarker response and a metal or a group of metals. On the other hand, and taking into consideration the work carried out by Marques et al. (2009) where histopathological alterations were found in kidneys from organisms from the M site, more significant differences in antioxidant defence system or even the occurrence of LPO would not be surprising. Furthermore U is considered a nephrotoxic agent (ATSDR, 1999) and is present in M organisms in values of almost 46 times greater than the ones found in reference organisms. Soluble forms of U in the body tend to complex with bicarbonate and transferrin, being later dissociated in the kidney and recombined in the proximal tubule with surface ligands causing damage (ATSDR, 1999; Fisenne, 1994). Even so our results point to an apparent effective antioxidant defence system where the increase of total GPx gave a contribution to counterbalance any increase in ROS.

As for heart tissue, potentially affected by oxidants due to its high oxygen consumption that may lead to the production of ROS, the significant increase of GSTs and of total GPx may point to the presence of contaminants in this organ and also the existence of an antioxidant response. Some metals like Al tend to accumulate in several organs when intake increases, such is the case of the heart (Schaller, 1994b). The increase of GSTs simultaneously with the increase of total GPx, might point to a possible increase of GSH levels in this tissue. In the presence of metals GSH may be synthesized (Bondy et al., 1998) to help in detoxification. Thus, as Van der Oost (2003) reviewed, since GSTs are necessary to conjugate GSH with electrophiles the increase in GSTs might be a consequence of an increase in GSH resulting from almost certain higher levels of metals in heart. Furthermore to support this idea, the observed significant increase in total GPx activity needs higher levels of GSH, as an electron donor, which are not being further recycled by GRed, since no alterations were observed in its activity.

The activity of LDH was determined in order to assess the respiratory status of the organism, to perceive possible alterations in the mode of energy acquisition. Nonetheless no significant differences were obtained between organisms from REF and M.

In general, the determination of enzymatic activity showed that the tissue presenting most susceptibility to chronic exposure to metals was the lung. Pellmar et al. (1999) and Peryakaruppan et al. (2007) have given examples respectively of the accumulation and induction of oxidative stress in lungs by metals. The higher sensitivity of lungs, when compared to other organs, might result from the fact that both kidney and liver are detoxifying organs, presenting usually higher baselines of antioxidants than other organs (e.g. Behne and Wolters, 1983). In addition, when comparing heart with lungs, the latter are more exposed to metals, since they may be directly in contact with them via respiration. Overall the antioxidant defense system, avoided LPO in all of the four tested organs in M frogs, even though kidney and mainly liver presented significantly higher values of accumulated metals. One of the agents responsible for this protection seems to be glutathione peroxidase since it presents higher activity in all tissues with exception for the liver, nonetheless other factors must not be excluded, such as the glutathione synthesis *de novo* (Meister and Anderson, 1983), the existence of MMC (Marques et al., 2009), or even the action of metallothioneins. In order to further enhance the knowledge of the mechanisms that allow the existence of *P. perezi* in the M site a gene expression study is being made.

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Chapter III

Differential gene expression in Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine

3. Differential gene expression in Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine

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Abstract

Iberian green frogs (*Pelophylax perezi*) were found inhabiting a deactivated uranium mine, especially an effluent pond, seriously contaminated with metals and radionuclides. These animals were previously assessed for oxidative stress parameters and did not revealed significant alterations. In order to better understand which mechanisms may be involved in the ability to withstand permanent contamination gene expression analysis was performed, in the liver through suppression subtractive hybridization (SSH). The SSH outcome in the liver revealed the up-regulation of genes coding for the ribosomal protein L7a and for several proteins typical from blood plasma: fibrinogen, hemoglobin and albumin. Besides their normal function, some of these proteins can play an important role as protective agents against oxidative stress. This work provides new insights on possible basal protection mechanisms that may act in organisms exposed chronically to contamination.

Keywords

SSH; differential gene expression; uranium mine; *Pelophylax perezi*; metals

3.1. Introduction

Metal contaminated sites such as mines are known for exerting deleterious effects in indigenous species (Pereira et al., 2006; Marques et al., 2008, Marques et al., 2009) which may lead to the disappearance of the most sensitive ones (Marqués et al., 2003). In these cases, toxic effects are usually originated from the accumulation of high levels of metals (Cooper and Fortin, 2010; Marques et al., 2009) in body tissues, leading to an alteration of physiological functions such as ageing, energy balance and redox homeostasis (Leonard et al., 2004; Valavanidis et al., 2006). This may result from direct enzyme inhibition by metals (Quig, 1998) or by establishing redox cycles with simultaneous generation of unstable reactive oxygen species (ROS). As reviewed by Galaris and Evangelou (2002), the production of radicals through metal action may result either directly from Fenton-like reactions or through an indirect pathway, forming thiyl radicals resulting from labile bonds between metals and thiol groups. Such complexes, in turn, can react with molecular oxygen and generate ROS. Moreover, and considering that normal aerobic metabolism produces oxygen radicals, the increase in ROS might also result from an inhibition of the antioxidant enzymes due to direct action of metals on them (Stohs and Bagchi, 1995) and not by further increase of ROS through parallel reactions of metals.

A common approach to study the effects of metal contamination on organisms is the use of a biomarker array which normally focuses on the activities of enzymes involved in biotransformation or antioxidant defense system (van der Oost et al., 2003). Nevertheless, the results are not always enlightening. More recently, the need to better understand the molecular mechanisms behind toxicological responses has brought gene expression profiling as a new endpoint for ecotoxicology (Snell et al., 2003). The progress in the molecular description of model organisms has been great and has provided base information for studying non-model organisms which are essential to enhance the knowledge on stress responses (Hansen et al., 2007; Roelofs et al., 2007; Venkatachalam et al., 2009). However, when non-model organisms are studied, usually there is lack of genomic and gene expression data. For these non model organisms, suppression

subtractive hybridization (SSH) can be a valuable strategy to identify genes differentially expressed responding to changes in cellular environment (Diatchenko et al., 1996). The SSH method is based in a selective amplification of target cDNA fragments (differentially expressed) and simultaneously suppression of non-target cDNA amplification. The methodology is supported by a suppression PCR effect, in which long inverted terminal repeats attached to DNA fragments can selectively suppress amplification of undesirable sequences (Diatchenko et al., 1996). SSH also combines normalization and subtraction in a single procedure and presents the advantage of screening the entire genome for genes involved in triggering physiological responses, in particular to stress agents (Snell et al., 2003). Thereafter, genes isolated from subtracted cDNA libraries are identified by homology search in public databases.

Several studies from our team were focused on a population of the amphibian species *Pelophylax perezi*, inhabiting a deactivated uranium mine (Cunha Baixa mine, Centre of Portugal) especially an acidic effluent pond connected directly to the mine pit (Marques et al., 2008, Marques et al., 2009; Marques et al., 2011). Nonetheless, some findings such as the almost undetectable differences in antioxidant responses between organisms from this effluent pond and from a reference site have been quite difficult to understand and explain. Thus, to further enlighten the processes that lie beneath this ability to cope with metal contamination without visible physiological change, a gene expression study based on the SSH technique was carried out. The results were analyzed together with those previously obtained for oxidative stress enzymes: catalase (CAT), glutathione-S-transferases (GSTs), glutathione reductase (GRed), and glutathione peroxidase (GPx) both selenium-dependent and total. In addition, lipid peroxidation (LPO) was also determined in this previous study through thiobarbituric acid reactive substances (TBARS) (Marques et al., 2011). Both gene expression study and enzyme activities were assessed in the liver which is usually a target organ of toxicity due to its role in detoxification and homeostasis (Kerr, 1999).

3.2. Material & Methods

3.2.1. Study site

The present study was conducted on the deactivated uranium mine located near the village of Cunha Baixa (Mangualde, Centre of Portugal) where ore extraction occurred between 1967 and 1993 (Oliveira and Ávila, 1998). After the exploration period, the mine pit was filled with low-grade ore and was flooded with sulphuric acid to extract uranium through an *in situ* leaching process (Santo and Freire, 1983). Presently, there are three small ponds in this area with a variable water level that fluctuates according to the uprising of the underground acidic effluent, which in turn is determined by variations in the level of the aquifer. Due to its origin, this effluent 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. The largest pond (M pond) shows the most extreme conditions due to direct connection with the underground tunnels of the mine pit and, consequently, with the mine effluent. Iberian green frogs (*P. perezi*) are frequently found on the three ponds. However, no egg masses were recorded on the M pond. Furthermore, during their winter lethargic stage, they have been found burrowed (authors' personal observation) in the highly metal contaminated sludge that results from the effluent treatment pond (Pereira et al., 2008). The site chosen for reference (REF) is an unpolluted segment of the Vouga River located to the north of the city of Viseu, a few kilometers from Mangualde, where frogs from the same species were recorded (Marques et al., 2009).

3.2.2. Animal capture and tissue sampling

Adult animals were captured in spring from both REF and M sites using a hand net and immediately taken alive to the laboratory where they were anaesthetized with ethyl ether and where morphometric measures were taken (body weight and snout to vent length). To reduce the influence of sex, all of the captured Iberian green frogs were subjected to an initial inspection. Only males were kept (until n=5 per site) for further processing. Animals were then sacrificed and liver was collected.

Since this organ was used to perform chemical (metal contents), biochemical (oxidative stress enzymes activity) and gene expression analyses it was divided in 3 portions which were weighed individually to the nearest 0.1 mg. The tissue for metal quantification was stored at -20 ºC until processing and tissues for biochemical analysis were frozen in liquid nitrogen until preparation for further analyses (for more details see Marques et al., 2011). For the gene expression analysis the tissue was preserved in RNALater (Qiagen, UK) and stored at -80ºC.

3.2.3. Construction of the cDNA subtraction library

Three liver samples from each site were used in independent RNA extractions. Total RNA from samples stored in RNALater (Qiagen, UK) solution at -80 ºC was extracted after homogenization in liquid nitrogen followed by the addition of Trizol reagent (Invitrogen, USA), according with the manufacturer instructions. The final extract was resuspended in diethylpyrocarbonate (DEPC) treated water. Total RNA integrity was checked by electrophoresis on a 1 % agarose gel and Ethidium Bromide (EtBr) staining. RNA concentration was estimated by spectrophotometry at 260 nm. The isolation of poly $(A)^+$ RNA was carried out using Poly A Spin mRNA Isolation Kit (New England Biolabs, USA) as recommended by the manufacturer. Equivalent amounts of mRNA from the 3 samples from each site were pooled together for suppressive subtractive hybridization (SSH) reactions.

SSH was performed with the PCR Selected Subtractive Hybridization Kit (Clontech, USA) following the manufacturer's instructions, using cDNA from M organisms, containing specific differentially expressed transcripts as tester and cDNA from REF organisms as driver. Briefly, both mRNA pools were converted into cDNA and digested with RSA I, ensuring the maintenance of blunt ended molecules. The tester cDNA was then ligated to adaptors 1 and 2 in independent reactions. Subsequently two sequential hybridizations were performed. The first was prepared by adding an excess of driver to each ligation product, followed by a heat denaturation and subsequent re-annealing. This step allows differentially expressed sequences to be equalized and enriched. The second hybridization was done by combining the two different tester pools, in the presence of fresh heat-denaturated driver, allowing the formation of new hybrids and a preferential

selection of differential expressed sequences in the following steps. After subtraction, two PCR amplifications were performed. The first was done with a primer that has a complementary sequence to the adapters, enabling the exponential amplification of differential transcripts. The non-differentially expressed hybrid sequences are either not amplified or do not have an exponential amplification. The second PCR was a nested PCR with specific primers for each adapter, allowing further enrichment of the differentially expressed sequences and the reduction of background. Subtraction efficiency was evaluated by electrophoresis of PCR products in a 2% agarose gel, as recommended by the kit manufacturer.

PCR products were then purified using Jetquick PCR Product Purification Spin Kit (Genomed GmbH, Germany) and ligated into pCR II T-vector, using TA Cloning Kit (Invitrogen, USA) as described by the manufacturer. After transformation into *Escherichia coli* XL-1 Blue-MRF' competent cells (Stratagene, USA), recombinants were selected in Luria-Bertani (LB) agar plates containing ampicillin (100 µg/ml), IPTG (80 µg/ml) and X-Gal (80 µg/ml). After incubation overnight at 37 ºC, recombinant cells were isolated and scratched onto new plates. The presence of inserts was confirmed by PCR using recombinant cell lysates, prepared in 50 µl of TE with 0.1% of Tween 20 and heating at 100 ºC for 10 minutes. PCR reactions were perform in 50 µl reactions containing 0.5 µM each primer (SP6 and T7) 1x Taq buffer, 0,2 mM each dNTP, 2mM $MgCl_2$, 1U *Taq* polymerase and 3 µl of cell lysate as template DNA. PCR reaction was set as follows: an initial denaturating step, at 94 ºC, for 3 minutes, was followed by 35 cycles of 1 minute denaturation at 94 ºC, 1 minute annealing, at 50 ºC, and 1 minute extension at 72ºC. A final extension step of 3 minutes, at 72ºC, was also included. PCR products were analysed in by electrophoresis in a 1.2% agarose and stained with EtBr. The PCR products revealing the presence of inserts were purified with Jetquick PCR Product Purification Spin Kit (Genomed GmbH, Germany) and sequenced. The corresponding clones were stored at -70 ºC as glycerol stocks.

Sequencing was performed by Macrogen (Seoul, South Korea) using an ABI 3730 XL genetic analyser (Applied Biosystems) and the sequences were

compared with protein databases using BLASTX from National Center for Biotechnology Information (NCBI).

3.3. Results

Overall results obtained by Marques et al. (2011) pointed to a good quality of the water in the REF site. With the exception of iron levels, values from other metals, as well as hardness, dissolved O_2, conductivity and pH were generally in accordance with those established by national legislation (MA, 1998) setting water quality criteria. On the contrary, M water samples presented a very low pH and a high conductivity, being both parameters out of the range considered acceptable. Also several metals (Be, Al, Mn, Fe, Co, Ni and U) surpassed all the established water quality values (Marques et al., 2011; Table 1). In general all metal concentrations were higher in M site than in REF.

From the original metal accumulation data, gathered by Marques et al. (2011), which consisted of five liver samples for each site, the values corresponding to the samples used for SSH were submitted to new statistical analysis in order to have a more related data in the current study. Accordingly, to evaluate statistically significant differences between sites, parametric one-way analysis of variance (ANOVA) were performed. Metal quantification analysis in liver (Table 2) revealed significant higher values in M organisms for six elements: Cr ($F = 11.617$; d.f. = 1; $p < 0.028$), Fe ($F = 300.082$; d.f. = 1; $p < 0.001$), Co ($F = 188.602$; d.f. = 1; $p < 0.001$), Cu ($F = 24.705$; d.f. = 1; $p < 0.009$), Se ($F = 20.950$; d.f. = 1; $p < 0.011$) and U ($F = 74.356$; d.f. = 1; $p < 0.001$). From these, U was the element that presented the most remarkable difference between REF and M frogs, since it was about 2700 times higher in M organisms. In this tissue, for animals from, REF no significant differences were registered.

Results obtained by Marques et al. (2011) for biomarkers (GSTs, GRed, selenium-dependent GPx, total GPx, CAT and TBARS) are also presented (Table 3). Briefly, no statistical significant differences between organisms from M and REF sites were obtained ($p>0.05$). Interestingly, frogs from the M site presented

lower values for all the parameters, with exception for CAT and TBARS (Marques et al., 2011).

Table 1 – Chemical and metal concentration data for REF (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.

	ORNL Tier II Chronic	EPA NRWQC (Chronic)	EPA R4-SB	MRV	MAV	REF	M	Detection Limit
Hardeness CaCO3 (mg/l)				NLV	500	8.7	358.9	
Dissolved O_2 (mg/l)				NLV	NLV	8.22	4.8	
Conductivity (µS/cm)				400	NLV	42.2	**1093**	
pH				6.5-8.5	NLV	6.7	**3.4**	
Element (µg/l)								
Be	0.66	NLV	0.53	NLV	NLV	BDL	**19.16**	0.10
Mg	NLV	NLV	NLV	30000.00	50000.00	903.54	35871.49	2.00
Al	NLV	87.00	87.00	50.00	200.00	75.84	**3230.59**	5.00
K	NLV	NLV	NLV	10000.00	12000.00	648.51	3694.85	10.00
Ca	NLV	NLV	NLV	100000.00	NLV	1981.84	84599.35	10.00
V	20.00	NLV	NLV	NLV	NLV	BDL	BDL	1.00
Cr	NLV	11.00	11.00	NLV	NLV	BDL	BDL	1.00
Mn	120.00	NLV	NLV	20.00	50.00	12.76	**4771.98**	0.01
Fe	NLV	1000.00	1000.00	50.00	200.00	201.40	**2745.93**	10.00
Co	23.00	NLV	NLV	NLV	NLV	0.18	**59.07**	0.10
Ni	NLV	52.00	87.71	NLV	50.00	BDL	**95.09**	0.50
Cu	NLV	NLV	6.54	3000.00	NLV	BDL	21.46	8.00
Zn	NLV	120.00	58.91	500.00	NLV	5.90	356.99	4.00
As	NLV	150.00	190.00	NLV	50.00	1.39	1.64	0.50
Se	NLV	5.00	5.00	NLV	10.00	BDL	BDL	1.00
Sr	1500.00	NLV	NLV	NLV	NLV	14.97	226.96	0.01
Cd	NLV	0.25	0.66	NLV	5.00	BDL	BDL	0.10
Ba	4.00	NLV	NLV	100.00	NLV	4.09	10.94	0.10
Pb	NLV	2.50	1.32	NLV	50.00	BDL	BDL	0.10
U	2.60	NLV	NLV	NLV	NLV	BDL	**1081.00**	0.10

(adapted from: Marques et al., 2011)

MRV and MAV stands for Maximum recommendable values and Maximum admissible values of waters for human consumption (MA, 1998).

ORNL Tier II Chronic – Oak Ridge National Laboratory Tier II Chronic values for allowing the establishment of aquatic benchmarks with fewer data (Suter and Tsao, 1996).

EPA NRWQC - Freshwater chronic values (EPA, 2009)

EPA R4 - Chronic surface water screening benchmark (Suter and Tsao, 1996).

NLV stands for No Legal Values established.

The values exceeding the different benchmark values available were written in bold letter.

BDL stands for Below Detection Limit.

Table 2 – Concentration of metals on liver of *Pelophylax perezi* (n=3) from REF and M sites.

Metal concentration on Liver										
	Be		**Mg**		**Al**		**K**		**Ca**	
	REF	M	REF	M	REF	M	REF	M	REF	M
Average	0.23	3.60	1032.71	773.19	20.13	1211.33	14978.78	12078.63	306.24	279.93
±	±	±	±	±	±	±	±	±	±	±
SE	0.07	1.63	99.13	84.36	6.96	518.56	1004.16	1229.50	46.36	25.45
	V		**Cr**		**Mn**		**Fe**		**Co**	
	REF	M	REF	M	REF	M	REF	M	REF	M
Average	1.32	1.29	**1.96**	**5.57**	17.83	12.67	**729.17**	**8200.31**	**0.71**	**2.34**
±	±	±	±	±	±	±	±	±	±	±
SE	0.22	0.33	**0.14***	**1.05***	3.71	1.01	**190.02****	**387.17****	**0.09****	**0.07****
	Ni		**Cu**		**Zn**		**As**		**Se**	
	REF	M	REF	M	REF	M	REF	M	REF	M
Average	0.69	1.14	**215.33**	**4135.57**	107.74	76.40	0.54	0.46	**2.41**	**14.49**
±	±	±	±	±	±	±	±	±	±	±
SE	0.18	0.02	**19.80****	**788.46****	14.91	2.30	0.01	0.10	**0.27***	**2.63***
	Sr		**Cd**		**Ba**		**Pb**		**U**	
	REF	M	REF	M	REF	M	REF	M	REF	M
Average	0.74	0.75	3.94	10.35	12.10	17.21			**0.13**	**362.52**
±	±	±	±	±	±	±	BDL	BDL	±	±
SE	0.14	0.09	2.82	1.92	4.89	5.47			**0.10****	**42.03****

Concentrations are expressed in µg/g, dry weight.
*: represents a statistical significant difference ($p<0.05$) between REF water course and the M pond.
**: represents a statistical significant difference ($p<0.01$) between REF water course and the M pond.
BDL stands for Below Detection Limit.
SE stands for Standard Error.

Table 3 – Mean activities of GSTs, GRed, selenium-dependent GPx, total GPx, CAT and mean content of TBARS in the liver of P. perezi from REF and M sites.

		Average ± SE
GSTs (nmol.min-1.mg-1 prot)	REF	106.08 ± 28.08
	M	47.12 ± 10.04
GRed (nmol.min-1.mg-1 prot)	REF	8.30 ± 1.99
	M	6.82 ± 0.75
selenium-dependent GPx (nmol.min-1.mg-1 prot)	REF	3.03 ± 0.76
	M	2.65 ± 0.30
total GPx (nmol.min-1.mg-1 prot)	REF	6.57 ± 1.43
	M	5.79 ± 0.62
CAT (µmol.min-1.mg-1 prot)	REF	180.06 ± 25.01
	M	212.68 ± 27.09
TBARS (nmol/mg proteína)	REF	0.19 ± 0.06
	M	0.40 ± 0.11

(adapted from: Marques et al., 2011)

3.3.1. Differential gene expression

SSH was performed to detect differentially expressed genes in *P. perezi* inhabiting M site. Forty nine clones were recovered from the subtracted library. After PCR screening, 20 presented inserts that could represent differential expressed transcripts and were then sequenced (Fig. 1). The molecular weight of these potential transcripts ranged from 541 to 762 base pairs (bp): 11 presented a molecular weight between 500 and 600 bp, 5 between 600 and 700 bp, and 4 between 700 and 800 bp. Homology search for the corresponding sequences was performed using BLASTX in NCBI non-redundant protein database . The sequences were considered homologous when the matching sequence length was at least 25% simultaneously with an e-value lower than e^{-10}. Seven sequences fulfilled these criteria, enabling functional identification (Table 4). Furthermore, best matching scores for these transcripts were found with proteins from other amphibian species, reinforcing their correct identification. The remaining 13 sequences did not show a significant similarity with the sequences in the database. The putative genes obtained were related to fibrinogen α chain (3 sequences), to ribosomal protein L7a (2 sequences), hemoglobin β subunit (1 sequence) and serum albumin precursor (1 sequence). Hemoglobin and serum albumin are transporting proteins, carrying oxygen and metals or hormones, respectively. On the other hand, fibrinogen is a blood coagulation protein and ribosomal protein L7a is involved in the translation process.

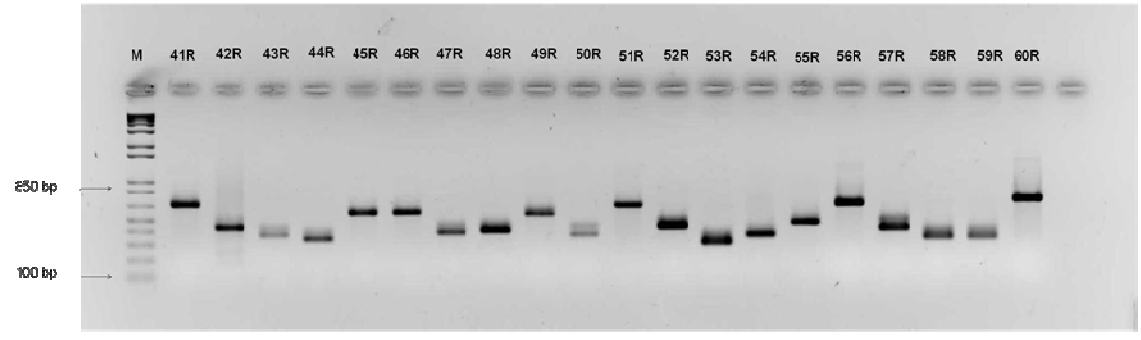

Fig.1. PCR fragments obtained with pCR II vector primers T7 and SP6, representing the potential differential expressed transcripts M - 1Kb plus DNA Ladder (Invitrogen, USA).

Table 4 – Potential up-regulated genes from *P. perezi* inhabiting the M site.

Clone ID	Size (bp)	Accession number	% Identity	% Coverage	E-Value	Putative identity	Matching organim
5 R	627	NP_001080329.1	80%	69%	2.00E-67	fibrinogen alpha chain	Xenopus laevis
18 R	574	NP_001072159.1	96%	29%	3.00E-23	ribosomal protein L7a	Xenopus (Silurana) tropicalis
22 R	567	NP_001072159.1	98%	29%	1.00E-23	ribosomal protein L7a	Xenopus (Silurana) tropicalis
34 R	760	NP_001080329.1	80%	57%	3.00E-67	fibrinogen alpha chain	Xenopus laevis
38 R	711	AAA97879.1	80%	35%	5.00E-23	fibrinogen gamma subunit	Rana sylvatica
43 R	653	P02134.1	78%	39%	6.00E-30	Hemoglobin subunit beta	Rana esculenta
49 R	730	AAD09358.1	91%	72%	7.00E-82	serum albumin precursor	Rana shqiperica

3.4. Discussion

The studies carried out by our team with the amphibian species *P. perezi* have revealed much information about toxicological effects in both adult organisms inhabiting the mining site and larval stages exposed to the mine effluent (Marques et al., 2008, Marques et al., 2009, Marques et al., 2011). Nonetheless there is the need to enhance the knowledge and understanding of the mechanisms that allow the survival of a population of this species in such a contaminated environment. This particular population may become exposed to metals by several ways. First, this species is highly dependent of water, spending much time submerged in the effluent, which increases exposure through contact with skin. Moreover, its burrowing behavior in sediment as an evasive tactics and in sludge contaminated soil in order to have shelter during their lethargic state in winter (authors' personal observation), allows an additional exposure (Antunes et al., 2007c; Lourenço et al., 2011; Pereira et al., 2008).

The results of metal quantification in the water samples from both REF and M sites, revealed that the M pond had the highest values for all the metals determined (Marques et al., 2011; Table 1). In addition, the extremely low pH observed may influence the solubility and the bioavailability of metals (ATSDR, 1999; ATSDR, 2004; ATSDR, 2006; Schaller et al., 1994). Iron, Co and U were present in the M pond in concentrations higher than all the benchmark values (Table 1) and were also found in liver at significant higher levels than those observed for the animals from the REF site (Table 2). Several authors

(Loumbourdis et al., 2007; Stolyar et al., 2008) observed metal accumulation in a similar pattern for the closely related amphibian *Rana ridibunda*. Nonetheless, the metal accumulation data revealed that frogs from both M and REF sites have bioaccumulated significant higher levels of some metals. However, most of these metals were corresponding to essential metals (Cr, Fe, Co, Cu and Se). The only exception was U, a toxic metal only found in organisms from the contaminated site. This fact, as well as the magnitude of the difference in U content among sites points that this metal is one of the main responsible elements for toxicological effects in the liver. Due to its role as a detoxifying organ, liver tends to present higher metal accumulation than other organs (Fisenne, 1994; Loumbourdis et al., 2007; Malik et al., 2010). This fact, together with the high environmental levels of uranium and its known affinity for liver (ATSDR, 1999; Cooley and Klaverkamp, 2000; Marques et al., 2011), provides an explanation for the observed accumulation. Despite such fact, the enzymatic activities and the LPO values did not reveal any significant difference between animals from M and REF sites. This was an unexpected result since other authors (e.g. Kelly and Janz, 2009) reported differences in GPx activity of *Esox lucius* when exposed to lower levels of metals in a uranium mill effluent. However some factors may explain the absence of these responses like the previously reported increased number of melanomacrophagic centers (MMC) (Marques et al., 2009), which are cells similar to macrophages containing pigments that can act as a free radical scavengers (Różanowska et al., 1999).

The results obtained for differential gene expression analysis revealed the possible up-regulation of genes coding for 4 different proteins: hemoglobin β subunit, ribosomal protein L7a, fibrinogen α chain and serum albumin precursor.

Hemoglobin is known to represent the chemical basis for O_2 transportation in all vertebrates. However, other functions have been recognized for this protein (Giardina et al., 1995). Hemoglobin presents an enzymatic activity similar to monooxygenases and Ferraiolo et al. (1984) confirmed that this activity is primarily related to the β subunit. Monooxygenases such as cytochrome P450 are responsible for catalyzing many compounds in phase I of biotransformation which may involve processes such as oxidation, reduction or hydrolysis (van der Oost et

al., 2003). Some authors (e.g. Izzotti et al., 2002) have obtained an enhanced gene expression of various monoxygenases coding genes with metals exposures. Considering these hemoglobin features, the up-regulation of hemoglobin β subunit might be a consequence of the chronic contamination that M organisms are exposed to. Furthermore some metals (e.g. Pb) may disrupt heme synthesis (Goering, 1993), leading to an up-regulation of genes coding for heme-binding proteins (e.g. hemoglobin) as a compensatory response to reduced heme levels (Garcia-Reyero et al., 2009). The present results support those obtained by Marques et al. (2009), which observed a lower frequency of immature erythrocyte in adult green frogs captured in this mine, suggesting a slower erythropoiesis as a consequence of the undergoing environmental exposure. Considering that higher erythrocytic abnormalities were observed and that abnormal cells tend to be removed faster from blood circulation than normal cells (Das and Nanda, 1986), a probable result would be a decrease in the number of erythrocytes. Thus an up regulation of hemoglobin coding gene would be required to compensate the hemoglobin loss by the abnormal erythrocytes removal. Nevertheless, the possibility of a higher hemoglobin demand in order to cope with higher oxygen requirements by M organisms must not be excluded. Usually animals in exposed to contamination have higher energetic demands due to detoxification processes (Smolders et al., 2003).

Ribosomal proteins as rpL7a are known to be involved in the translation process. Nonetheless, they can participate in extra ribosomal activities (Noara and Naora, 1999; Wool, 1996) and their over expression has been reported in carcinomas (Barnard et al., 1992; Chester et al., 1989; Wang et al., 2000; Zhu et al., 2001). In particular, the ribosomal protein L7a has revealed to be DNA-damage inducible (Ben-Ishai et al., 1990) and some authors have established a relationship between higher expression levels of its coding gene in the presence of contaminants and tumorogenesis (Ben-Ishai et al., 1990; Zhu et al., 2001). In fact, some metals like Co and U have been reported as genotoxic or even carcinogenic (ATSDR, 2004; Barillet et al., 2005; Bucher et al., 1999) and were present in significant higher values in M frogs' liver. Thus, the presence of high

concentrations of these genotoxic metals in this organ and their consequent damage in cell's DNA could explain the up-regulation of the rpL7a coding gene.

Albumin is a transport protein with many functions and having a broad range of ligands such as hormones or metals (Bal et al., 1998; Sadler and Viles, 1996). In addition, some research works have reported other possible roles such as the antioxidant activity (Bourdon et al., 1999; Cha and Kim, 1996). Indeed, the results of Cha and Kim (1996) pointed the human serum albumin as a possible extracellular major antioxidant, acting like a glutathione-linked thiol peroxidase, which removes reactive oxygen species from blood plasma. Furthermore, this protein may act as a detoxifying agent due to its ability to bind metals, including those that are recognized as non-essential elements (Sadler and Viles, 1996; Bal et al., 1998). Up-regulation albumin coding gene may indicate either an early response to metal exposure or a feedback regulation resulting from albumin depletion since it may act as a "sacrificial" antioxidant (Halliwell, 1995; Roche et al., 2008), as this activity can imply structural changes and impairment of the biological activity of albumin (Bourdon et al., 1999). The absence of significant activity differences for enzymes involved in oxidative stress responses in M organisms' liver may result from the protective role of albumin.

Fibrinogen is biosynthesized in the liver and is involved in blood coagulation, with a main role in platelet aggregation. However, there are growing evidences that this protein has also an important antioxidant role (Kaplan et al., 2001; Olinescu and Kummerow, 2001). The study of Kaplan and coworkers (2001) showed that fibrinogen acted as an antioxidant by preventing oxidation of β-lipoproteins in various conditions. This antioxidant activity should be attributed to the inhibition of the oxidation caused by the oxidizing agent and not by its chelation. This protein, like plasma proteins that possess globular portions, may protect other molecules because it can inhibit free radical mediated oxidation. In this process, the globular portions become slowly oxidized and degraded. Furthermore, fibrinogen contains fibrinopeptides which may be especially rich in electrons (reducing power), further enhancing its antioxidant potential (Hantgan et al., 1994). Thus, due to their amino acid constitution, this protein along with other abundant plasma proteins (e.g. albumin) may act as "sacrificial" antioxidants

(Olinescu and Kummerow, 2001). Accordingly, the results obtained for the oxidative stress biomarkers and for the LPO (Marques et al., 2011), where no significant differences were observed between M and REF animals, can be explained by the concerted activity of albumin and fibrinogen. The up-regulation of the corresponding genes may result from a regulatory feedback mechanism since both fibrinogen and albumin lose their primary function when acting as antioxidants.

In general, the absence of enzymatic activity and LPO differences between organisms from the reference and contaminated sites, together with the results of our previous studies (Marques et al., 2008, Marques et al., 2009, Marques et al., 2011) suggest that other mechanisms besides those concerning the enzymes analyzed are involved in order to cope with metal exposure. The present work revealed an up-regulation of various genes coding for proteins that, besides their usual role may present activities typical of proteins that are involved in the response to external aggressions, such as the ones reviewed by van der Oost et al. (2003). Furthermore, even though they are synthesized in the liver, these proteins are characteristic of blood plasma. If the up-regulation of their coding genes is indicative of a higher synthesis of these proteins, then a prompt response to the effects of the contaminants in the organisms is occurring in blood plasma. This hypothesis would explain the absence of structural damage in liver (Marques et al., 2009) as well as the nonexistence of differences in the biomarkers studied in the liver (Marques et al., 2011). Furthermore, this could explain why the main enzymatic changes observed by Marques et al. (2011) were detected in lungs. Lungs can be exposed directly to contaminated particles inhaled by frogs during normal respiration, having a reduced access to the protection provided by blood proteins since these act mainly internally.

The overall information available on basal protection mechanisms in organisms inhabiting contaminated sites is scarce, especially on amphibians. Beyond acute effects like mortality, research works are usually focused on the impact of contamination on enzymatic biomarkers (Falfushinska et al., 2008) and do not clarify many aspects about responses to contamination. The results presented in this study contribute to a better understanding of the mechanisms

that allow the presence and survival of the *P. perezi* population in this metal contaminated site and brings new insights on possible basal protection that may be present in organisms chronically exposed to contamination.

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Chapter IV

Evaluation of growth, biochemical and bioaccumulation parameters in *Pelophylax perezi* tadpoles, following an *in situ* exposure to three different effluent ponds from a uranium mine

4. Evaluation of growth, biochemical and bioaccumulation parameters in *Pelophylax perezi* tadpoles, following an *in situ* acute exposure to three different effluent ponds from a uranium mine

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Abstract

Mining activities produce invariably metal contaminated effluents. Depending on factors such as pH and metal concentration the toxicity of the effluent may vary. To assess the effects of three characteristically different effluent ponds from a deactivated uranium mine, with toxicological relevant data, an *in situ* exposure with *Pelophylax perezi* tadpoles*,* was conducted. Tadpoles were exposed to the three effluent ponds, sorted by increasing order of metals concentration levels (REF, M1, M2). Survival, growth, metal accumulation, oxidative stress response enzymes' activity (catalase, glutathione peroxidase and glutathione reductase) and lipid peroxidation (LPO) were determined in tadpoles, as well as, physical and chemical parameters of the effluents were measured. Death percentage was highest in the M2 pond, where the highest acidity level (pH ≈ 3.77) was registered. From the three effluents M2 tadpoles had the lowest growth. However metal accumulation was highest in tadpoles exposed to M1, while metal contents in M2 tadpoles were quite similar to those recorded in REF tadpoles, indicating low uptake of metals. Antioxidant enzymatic activity was only affected in the case glutathione peroxidase (GPx) with significantly higher activity in M1, being in accordance with the highest accumulation of metals. LPO, usually associated with metal accumulation, had the following pattern M1 > REF > M2. Overall, effluent toxicity in tadpoles exposed to M2 effluent seems to be primarily an effect of pH while in M1 toxicity is mainly owed to high metal concentrations. The effluent acidity seems to reduce metal accumulation probably due to damage in the integument, affecting ion uptake. The results obtained bring a better understanding of the toxicological processes that local *P. perezi* population is subjected to, mainly in the early life stages.

Keywords: oxidative stress; tadpoles; metals; pH; *in situ* testing

4.1. Introduction

The production of metal contaminated effluents as a by-product of mining activities is well known. One of the main factors that influences the hazardousness of these effluents, is the mineralogical composition of the site under exploration (Williams, 2001) since it has a remarkable role on its chemical content and acidity and thereafter in the kind and magnitude of hazard posed to fauna. Furthermore, depending on pH, metal content and bioavailability an effluent can present different levels of toxicity (Lopes et al., 1999). The toxicity mechanism of acidity and metal is quite different. Low pH values tend to affect osmotic regulation in animals. An example is the study carried out by Freda and Dunson (1984) which has shown that the exposure of amphibian larvae to low pHs (2.5-4.0) leads to an increased efflux of sodium and an inhibition of its influx, resulting in death when 50% of the net body sodium was lost. Metals, on the other hand, once the uptake has occurred, act, very frequently, by establishing redox cycles originating subsequently highly unstable reactive oxygen species (ROS) such as the superoxide and hydroxyl anions (Galaris and Evangelou, 2002). ROS can then cause damage in lipids, proteins and DNA (Stohs and Bagchi, 1995) and ultimately cause cell death. However as reviewed by van der Oost et al. (2003) there are various enzymes that can act as an antioxidant response system preventing, in many cases, greater damage to cells. For this role we can account enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which act by neutralizing ROS. Also other proteins may have antioxidant roles like Cha and Kim (Cha and Kim, 1996) suggest for albumin.

Physiological alterations occurring in field animals exposed either to pH or metal contamination have already been the subject of various studies (Farombi et al., 2007; Frisbie and Wyman, 1992) nonetheless due to the complexity of data interpretation or even the difficulty in developing field work the majority of the existing studies are based in laboratory exposures. Thus, there is still the need to betake *in situ* testing, gathering as much information as possible, to generate more realistic and toxicological relevant data.

Amphibians are one of the groups were most work is necessary, given that, until recently was largely overlooked from an ecotoxicological point of view , when compared to other vertebrates (Sparling et al., 2000), regardless of their potential as bioindicators and as sources of contamination transference between aquatic and terrestrial compartments. Their early-life stages are especially sensitive to contaminants, and even though there are some species able to avoid potentially toxic breeding sites, as reviewed by Rowe and Freda (2000), there is always the possibility of unexpected contaminant inputs in uncontaminated sites through several processes like runoff or leaching. Thus, water dependent early-life stages become frequently in contact with contamination (Weir et al., 2010). In addition, the role of amphibians in trophic chains is extremely important since, depending on their habitat, they may occupy both the role of prey and top predators (Murphy et al., 2000b) being a key element in the accumulation and transfer of toxic substances.

Some studies have shown that amphibian tolerance to contaminants, metals included, are species and contaminant dependent (Birge et al., 2000; Bridges et al., 2002). An example of tolerance is been followed by our research group. Previous works (Marques et al., 2009; Marques et al., 2008) reported the presence of *Pelophylax perezi* (Iberian green frog) in a deactivated uranium mine were a highly toxic effluent is produced (Antunes et al., 2007a; Antunes et al., 2007b). Adults from the population inhabiting the mining complex have already been studied for histopathological and biochemical alterations as well as for metal accumulation (Marques et al., 2011; Marques et al., 2009). Furthermore, there are also gene expression studies which were made with the same species, under the same exposure conditions (Marques et al., *in prep* a; Marques et al., *in prep* b). So far the results showed metal accumulation in various tissues of *P. perezi* as well as histological alterations in liver kidney and lungs. As for oxidative stress biomarkers, GPx seemed to be the most responsive enzyme in the organisms inhabiting the contaminated site, which may contribute to mild oxidative stress (Livingstone, 2001). Also gene expression in *P. perezi* inhabiting the mining complex indicated the possibility of up-regulation of genes coding for proteins that may be included in an antioxidant basal protection mechanism (e.g. albumin).

Despite these data, information on the response of the more sensitive early life-stages to the effluent is scarce. The exception made is for acute laboratory assays, where the effect of dilutions of the effluent with pH correction for 8.0, was assessed on *P. perezi* egg masses and subsequent development (Marques et al., 2008). Thus, the present work was undertaken with the objective of complementing the information available on the toxicological effect of the uranium mine effluent and subsequent treatment in the early-life stages of *P. perezi* (Marques et al., 2008), with more realistic and toxicological relevant data. To meet this overall objective the activity of various enzymes, CAT, GPx and glutathione reductase (GRed), involved in oxidative stress response was assessed, as well as lipid peroxidation (LPO) in tadpoles exposed *in situ* to the effluent, before and after a chemical treatment has been performed to neutralize pH and precipitate radionuclides. Survival and growth of exposed tadpoles were also evaluated. Furthermore to establish a cause-effect relation, effluent physical and chemical parameters were determined, as well as, metal body burden and effluent metal content were quantified.

4.2 Materials & Methods

4.2.1. Study site

Cunha Baixa (Mangualde, Viseu) is a small village in the centre of Portugal, where a deactivated uranium mine is located close to the population. The ore extraction on this mine occurred between 1967 and 1993 (Oliveira and Ávila, 1998) and in the later period of exploration the main mine pit was filled with low-grade ore and extraction was carried through an *in situ* leaching process with sulphuric acid (Santo and Freire, 1983). From the exploration process various ponds with different grades of contamination were formed and are still present (Antunes et al., 2007a; Antunes et al., 2007b) being used as refuges and as breeding sites for Iberian green frogs inhabiting the mining complex (Marques et al., 2009; Marques et al., 2008). Three ponds with different chemical and physical properties still reflect various processes that occurred during the exploration period. The first one (REF) (Fig. 1, REF) resulted from an open pit where extraction was stopped after

a short period due to the low quality of the ore. According to previous studies, the accumulated effluent (Antunes et al., 2007b) meets the Portuguese legislation requirements (MA, 1998) to be used as crop irrigation water. The second pond (M1) (Fig. 1, M1) is a decantation basin, constructed for receiving the acidic mine effluent, generated in the third pond, after treatment with lime and barium chloride (Oliveira and Ávila, 2001). M1 effluent has a complex mixture of metals and a practically neutral pH (≈ 8). The third pond (M2) (Fig. 1, M2) is in direct contact with the mine pit were the *in situ* leaching process occurred, presenting a highly acidic effluent (pH≈3.5) and also a complex mixture of metals (Antunes et al., 2007b). This pond has 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.

Fig. 1 – *In situ* exposure sites, adapted from: Google earth (http://earth.google.com)

4.2.2. Test organisms

Pelophylax perezi eggs from the same clutch and in the first stage of development (Gosner, 1960) were collected in an uncontaminated segment of Vouga River located in the north part of the city of Viseu, a few kilometers from Mangualde. The

egg mass was immediately transported to the laboratory in a clean plastic container, where it was maintained in FETAX medium (Dawson and Bantle, 1987) at a constant temperature (20ºC±1) and photoperiod (14hL:10hD) until hatching occurred and tadpoles reached stage 21 (Gosner, 1960).

4.2.3. In situ exposure chambers

For the exposure of *P. perezi* tadpoles in the field, specific test chambers were designed. Each test chamber consisted of a 24 well polystyrene culture plate with lid. All of the plate's wells were perforated evenly, until a total of 16 holes of 1 mm diameter, to allow effluent/medium circulation. Also, the top of the plate's lid was replaced by a 300 µm nylon mesh (Fig. 2a), which was sealed to the lid's sides with thermal white glue already tested for toxicity (Pereira et al., 1999). In order to minimize external physical interactions with the test chambers during the exposure time, the plates were placed inside containers. Since, in the experimental design, *in situ* controls were used, two different kinds of containers had to be designed, one for controls and another one for direct effluent exposure. Thus for the chambers intended to be in direct contact with the effluent a polyethylene terephtalate (PET) container with two openings of 15 cm height and 10 cm width, in opposite sides, was used per chamber. In these containers the chambers were placed inside and maintained stable in the middle of the container through nylon strings (Fig. 2b). After placing the chambers inside the containers the openings were sealed with a 300 µm nylon mesh and kept in place with nylon cable ties. For the control test chambers the containers consisted of a polypropylene box covered on top with a 300 µm nylon mesh sewed to the box with nylon string. In order to keep the effluent from entering the control container and simultaneous keep almost the entire container submerged, four closed cylindrical PET containers filled with polyurethane were attached, one on top of each side, outside the container (Fig. 2c) allowing thus the flotation of the container.

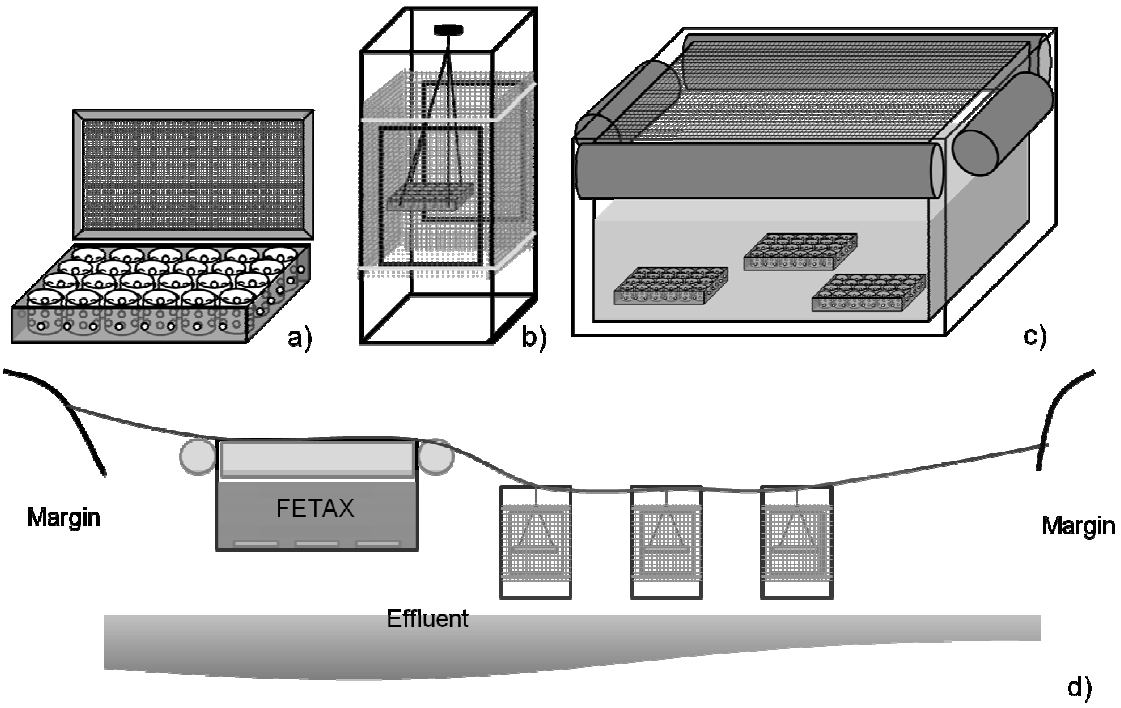

Fig. 2 – a) *In situ* 24 well test chamber. b) Container with test chamber for direct effluent exposure. c) Container with test chambers for *in situ* control with FETAX medium. d) Schematic representation of *in situ* exposure.

4.2.4. In situ acute assay

The *in situ* exposure was made for 96h, in REF, M1 and M2 ponds. For each pond 3 containers, with one test chamber each, were submersed to assess direct exposure to the effluent. Also in each pond a control container was placed (Ctrl REF, Ctrl M1, Ctrl M2) containing 3 exposure chambers each. Every control container was filled with 10 L of FETAX medium and then partially submersed in the effluent to maintain similar temperature. In all chambers 22 tadpoles, in stage 21, were placed individually in the wells making a total 132 (3x22 effluent; 3x22 control) tadpoles *per* pond. In addition to field controls a laboratory control (Ctr LAB) was also established with 3 replicas of 22 tadpoles exposed to FETAX medium at a constant temperature (20ºC±1) and photoperiod (14hL:10hD). During the assay (both in laboratory and *in situ*) the animals were not fed and mortality was recorded daily as well as pH (WTW 330/SET-2 pH meter), dissolved oxygen (WTW 315i/SET Oxi meter) and conductivity (LF 330/SET conductivity meter). The dead tadpoles found, during the daily observation were removed. At the end of the

assay the total body length of 20 randomly chosen tadpoles among the 3 replicas for each condition (Ctrl REF, REF, Ctrl M1, M1, Ctrl M2, M2 and Ctrl LAB) was measured using an Olympus SZX9 stereoscope. Also 3 pools of 10 tadpoles from each condition were frozen in liquid nitrogen and stored at -80ºC to evaluate oxidative stress responses and 5 randomly chosen tadpoles from among the 3 replicas of each condition were removed both after 48 and 96h of exposure, allowed to swim in FETAX medium for one minute to remove metals in excess from the body surface, and stored at -20^{o}C until metal quantification was possible.

4.2.5. Effluent sampling

Water samples for chemical analyses were collected from the REF, M1 and M2 ponds and in 0.5 L plastic bottles and acidified with pro analysis nitric acid MERCK® (65 %), to a pH below 2, to prevent metal adsorption. The same procedure was followed for a FETAX medium sample. The bottles used to store the samples were previously filled with nitric acid (50 %, v/v) left overnight and after this period thoroughly rinsed with distilled water.

4.2.6. Chemical analyses

The total concentrations of Be, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Cd, Ba, Pb and U were quantified in REF, M1 and M2 effluent and in the FETAX medium, as well as in tadpoles from all testing conditions, by inductively coupled plasma mass spectrometry (ICP/MS) (APHA, 1995). 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 tadpoles an initial wet-digestion was required. The tadpoles were first oven-dried at 105 ºC until a stable weight was achieved. After having been dried, the weight of the tissue was recorded to the nearest 0.1 mg, and they were digested in closed Teflon flasks with 3 ml of nitric acid (suprapur Merck®, 65 %) in a 60 ºC sand-bath. Aliquots of 0.5 ml of suprapur

hydrogen peroxide (30 %) MERCK® were then added to free the solution of any organic solid fragments. The final volume of the solution was made up to 5 ml with Milli-Q® water (18.2 Ω). Sample blanks were obtained following the same procedure described for wet-digestion but replacing the tissue volume by Milli-Q® water and replicate measurements in ICP/MS were made for each sample in order to check if a variance coefficient lower than 10% was obtained.2

4.2.7. Oxidative stress biomarkers

Oxidative stress responses were evaluated in tadpoles through the enzymatic determinations of CAT, GPx, and GRed. The extent of lipid peroxidation was assessed through the measurement of thiobarbituric acid reactive substances (TBARS).

Tadpoles which had been previously frozen individually in liquid nitrogen, were homogenized in ice-cold phosphate buffer (50 mM, pH= 7.0 with 0.1 % TRITON X-100). Homogenates were centrifuged at 10,000 g for 10 min and supernatants were divided into four aliquots, one for each determination (CAT, GPx, GRed and TBARS) and a spare one. Aliquots were stored at -80 ºC until determinations were possible.

GRed (EC 1.8.1.7) activity was determined by spectrophotometry, according the protocol of Carlberg and Mannervik (1985). In this assay the GRed mediated oxidation of NADPH was monitored at 340 nm (molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$).

CAT (EC 1.11.1.6) activity was determined by the spectrophotometric method described by Aebi (1984). This method consists in monitoring the consumption of hydrogen peroxide, at a wavelength of 240 nm (molar extinction coefficient of 0.0394 $mM^{-1}cm^{-1}$) for a period of 30 s.

Total GPx (EC 1.11.1.9) activity was determined according to Flohé and Günzler (1984), following the oxidation of NADPH, at a wavelength of 340 nm (molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$), when GSSG is reduced back to GSH by glutathione reductase. GPx activity was monitored using cumene hydroperoxide (0.7 mM) as substrate.

Enzymatic activities were determined in triplicate and were expressed in

µmol (CAT) or nmol (GRed and total GPx) of substrate hydrolyzed per min and per mg of sample protein (see protein quantification below).

The extent of lipid peroxidation was measured by the quantification of TBARS, according to the protocol described by Buege and Aust (1978). This methodology is based on the reaction of lipid peroxidation by-products, such as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA). The amount of TBARS was measured spectrophotometrically as a single determination, at a wavelength of 535 nm (molar extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1}$), and results were expressed as nmol of MDA equivalents per mg of sample protein.

Enzymatic activities were determined in triplicate and were expressed in nmol of substrate hydrolyzed per min and per mg of sample protein (see protein quantification below).

Protein concentration of each sample was determined in triplicate, according to the spectrophotometric (wavelength = 595 nm) method of Bradford (1976). For statistical purposes, each pool was considered a replica.

4.2.8. Statistical analysis

Statistically significant differences in body size ($p<0.05$) were tested initially between *in situ* FETAX controls (Ctrl REF, Ctrl M1, Ctrl M2) and laboratory FETAX control (Ctrl LAB) through a one-way analysis of variance (ANOVA) followed by a Dunnet test (Zar, 1996). Interactions, for biochemical parameters and growth, among the different sites (REF, M1, and M2 ponds) and the exposure media (FETAX and effluent from the respective ponds) were tested through a two-way ANOVA. When interaction was significant ($p<0.05$), differences between groups were tested with simple main effects ANOVAs followed by a multi-comparison Tukey test, otherwise one-way ANOVAs followed by Tukey test were used.

4.3. Results

The chemical characterization of all effluents and FETAX medium is present in Table 1 in parallel with benchmark values established by Portuguese legislation for

Table 1 – Metal concentration and hardness data for FETAX medium and REF, M1 and M2 effluents.

Element (µg/l)	Detection Limit	ORNL Tier II Chronic	EC20-SB	EPA R4-SB	MRV	MAV	FETAX	REF	M1	M2
Be	0.10	0.66	NLV	0.53	NLV	NLV	BDL	0.4	0.3	**16.0**
Mg	2.00	NLV	NLV	NLV	30000.0	50000.0	14613.1	1497.8	33319.6	48508.4
Al	5.00	NLV	75.00	87.00	50.0	200.0	5.5	74.7	25.3	**1519.3**
K	10.00	NLV	NLV	NLV	10000.0	12000.0	**15325.2**	1047.4	5142.9	5035.2
Ca	10.00	NLV	NLV	NLV	100000.0	NLV	20618.0	3058.3	**179761.6**	**120332.0**
V	0.50	20.00	NLV	NLV	NLV	NLV	1.3	BDL	BDL	BDL
Cr	0.50	NLV	0.26	11.00	NLV	NLV	BDL	BDL	BDL	BDL
Mn	0.01	120.00	NLV	NLV	20.0	50.0	1.8	24.2	**304.9**	**5831.7**
Fe	10.00	NLV	NLV	1000.00	50.0	200.0	BDL	977.2	29.4	**1299.0**
Co	0.01	23.00	NLV	NLV	NLV	NLV	BDL	0.2	0.9	**46.7**
Ni	0.10	NLV	11.00	87.71	NLV	50.0	BDL	BDL	4.1	66.5
Cu	0.50	NLV	0.26	6.54	3000.0	NLV	BDL	BDL	BDL	8.8
Zn	0.50	NLV	21.00	58.91	500.0	NLV	BDL	BDL	BDL	223.5
As	0.10	NLV	55.00	190.00	NLV	50.0	BDL	39.9	1.7	1.2
Se	0.50	NLV	2.60	5.00	NLV	10.0	BDL	BDL	BDL	BDL
Sr	0.01	1500.00	NLV	NLV	NLV	NLV	6.8	12.4	207.2	321.4
Cd	1.00	NLV	0.01	0.66	NLV	5.0	BDL	BDL	BDL	0.8
Ba	0.05	4.00	NLV	NLV	100.0	NLV	BDL	0.8	58.6	17.5
Pb	0.50	NLV	0.35	1.32	NLV	50.0	0.2	0.7	BDL	6.0
U	0.05	2.60	NLV	NLV	NLV	NLV	0.2	**19.4**	**254.9**	**571.9**
Hardness $CaCO_3$ (mg/l)							111.66	13.80	586.07	500.23

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.

MRV and MAV stands for Maximum recommendable values and Maximum admissible values of waters for human consumption (MA, 1998).

ORNL Tier II Chronic – Oak Ridge National Laboratory Tier II Chronic values for allowing the establishment of aquatic benchmarks with fewer data (Suter II and Tsao, 1996).

EC20 - Sensitive species surface water screening benchmark (Suter II and Tsao, 1996).

EPA R4 - Chronic Surface water screening benchmark (Suter II and Tsao, 1996).

NLV stands for No Legal Values established.

The values exceeding the different benchmark values available were written in bold letter.

BDL stands for Below Detection Limit.

human consumption water (MA, 1998). However, since these values may be considered overprotective other benchmark values, which have taken in consideration ecotoxicological parameters (Suter II and Tsao, 1996) are reported in the same table. Our results, revealed that various elements (Be, Al, Ca, Mn, Fe, Co and U) present in the effluent, mainly in M2, exceeded all the benchmark values used for comparison. Parameters registered in all conditions during *in situ* exposure (dissolved O_2, conductivity pH and temperature) revealed that M1 and M2 ponds presented the highest values of conductivity (Table 2) while, in opposition, REF presented the lowest values even when comparing to the FETAX controls. Also pH values were all close to neutral, with the exception of M2 which presented a markedly acid pH. Temperatures registered in the field were fairly similar among them presenting, however, an average temperature about 7ºC higher than the Ctrl LAB. This fact as well as the significant lower body size of the Ctrl LAB in comparison to the other controls led to its removal it from other statistical analyses. Nonetheless, the purpose of this control was to guarantee the good physiological conditions of the hatched tadpoles and this was achieved since no mortality was observed. As expected, data for metal accumulation in tadpoles revealed that accumulation increased with the exposure duration (Table 3). Furthermore, the majority of the elements quantified (Be, Mg, Al, Ca, Mn, Co, Ni, Ba and U) had higher concentrations in tadpoles exposed to M1 effluent.

Table 2 - Abiotic parameters and respective values during *in situ* exposure for each tested condition.

	Ctrl LAB	Ctrl REF	REF	Ctrl M1	M1	Ctrl M2	M2
O_2 (mg/l)	6.73 ± 0.45	6.12 ± 0.27	7.50 ± 0.09	6.38 ± 0.36	8.21 ± 0.16	7.89 ± 0.36	8.22 ± 0.06
Conductivity (µS/cm)	545.7 ± 22.5	510.7 ± 14.6	80.0 ± 1.7	509.5 ± 16.3	1015.3 ± 9.4	509.0 ± 17.0	1007.2 ± 12.5
pH	7.81 ± 0.09	7.89 ± 0.06	7.35 ± 0.25	7.87 ± 0.02	7.89 ± 0.18	7.91 ± 0.08	3.77 ± 0.01
Temperature (ºC)	20 ± 0	28.0 ± 0.1	28.0 ± 0.1	27.6 ± 0.8	27.6 ± 0.8	26.2 ± 0.9	26.2 ± 0.9

Table 3 - Metal concentration in *P. perezi* tadpoles (pool, n=5) at 48 and 96h exposure for all *in situ* exposure conditions

Element (µg/g)	Ctrl REF (48h)	Ctrl REF (96h)	REF (48h)	REF (96h)	Ctrl M1 (48h)	Ctrl M1 (96h)	M1 (48h)	M1 (96h)	Ctrl M2 (48h)	Ctrl M2 (96)	M2 (48h)	M2 (96h)
Be	BDL	BDL	BDL	4.4	BDL	BDL	BDL	**5.5**	BDL	BDL	BDL	BDL
Mg	822.7	1865.9	1012.7	1804.3	721.9	1575.0	1429.6	**3029.9**	768.9	958.1	511.8	1995.8
Al	BDL	1939.6	102.8	2202.6	BDL	1194.9	136.3	**5981.1**	BDL	74.2	51.8	2213.0
K	BDL	**8995.0**	681.3	BDL	BDL	3501.4	BDL	BDL	BDL	2885.2	3710.0	7388.9
Ca	7153.3	17561.3	10094.0	13343.8	7070.0	20505.3	12508.5	**22536.3**	7108.9	17838.3	5291.4	9819.4
V	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Cr	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Mn	BDL	102.9	BDL	250.9	BDL	114.4	104.1	**1046.3**	BDL	BDL	17.1	134.0
Fe	BDL	3381.9	514.7	5035.6	BDL	2549.9	654.8	5868.9	BDL	1028.1	BDL	**10150.0**
Co	BDL	BDL	BDL	BDL	BDL	BDL	BDL	**8.1**	BDL	BDL	BDL	BDL
Ni	BDL	BDL	BDL	BDL	BDL	BDL	BDL	**21.2**	BDL	BDL	BDL	BDL
Cu	16.9	31.2	BDL	33.9	18.4	**36.3**	BDL	31.9	17.7	BDL	11.5	33.1
Zn	347.0	**565.3**	403.2	326.6	345.6	487.7	427.5	287.6	346.3	350.5	189.2	364.4
As	BDL	BDL	BDL	**49.2**	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Se	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Sr	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Cd	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Ba	BDL	BDL	BDL	BDL	BDL	71.3	163.0	**5371.6**	BDL	BDL	BDL	BDL
Pb	BDL	18.5	BDL	12.5	BDL	**18.9**	BDL	13.7	BDL	13.2	BDL	10.2
U	BDL	14.0	6.6	132.4	BDL	5.7	20.2	**278.4**	BDL	BDL	5.1	124.1

Concentrations are expressed in µg/g, dry weight
BDL stands for Below Detection Limit
The highest concentration for each element is written in bold

At the end of the assay, mortality had only occurred in tadpoles exposed to the effluents, with average mortalities of 3.17, 9.84 and 42.86% for REF, M1 and M2 respectively. All surviving tadpoles (Ctrl LAB included) were in stage 25 (Gosner, 1960) and the statistical analysis of body length did not revealed interactions among different exposure sites (ponds) and exposure media (FETAX *versus* pond effluent). However, statistical differences between organisms exposed in different ponds were found between REF and M2 ($F = 6.770$; $df = 2$; $p < 0.01$) (Fig. 3) with organisms in M2 presenting a lower body size. The statistical analysis of the biochemical data showed no interaction between factors (ponds *versus* exposure media) and no statistical differences between levels of each factor for CAT and GRed (Fig. 4a and 4b respectively). Also no significant interaction between ponds and exposure medium was obtained for GPx, however, within M1 pond, differences in the activity of this enzyme ($F = 38.946$; $df = 1$; $p < 0.01$) were found between organisms exposed to the FETAX medium and those exposed directly to the effluent, presenting a higher activity in M1. Furthermore differences in GPx activity were also found between the M1 and M2 effluents ($F = 5.880$; $df = 2$; $p < 0.05$) (Fig. 4c) with M2 presenting a lower activity. Analysis of LPO via TBARS determination revealed a significant interaction between the ponds and the exposure media ($F = 14.654$; $df=2$; $p < 0.001$) and further analyses through simple main effects ANOVAs and Tukey multi-comparison showed significant differences between all effluent ponds ($F=27.46$; $df=2$; $p < 0.01$) (Fig. 4d) with the following increasing order of LPO M2<REF<M1. Also within the M1 pond differences were found ($F = 57.94$; $df = 1$; $p<0.01$) between Ctrl M1 and M1 with the latter presenting higher lipid peroxidation.

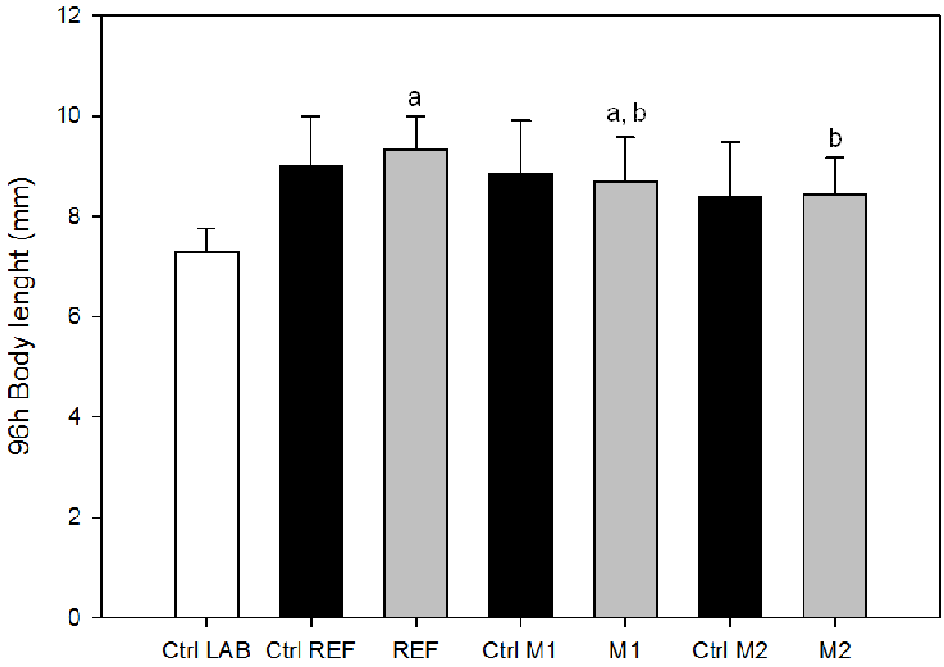

Fig. 3 – Larvae body length after the 96h *in situ* exposure assay. Ctrl LAB was not included in statistical analyses since exposure temperature was in average 7ºC lower. Statistical analysis was performed within exposure sites, between different controls and between different effluents. Effluents were not compared with controls from other sites. a and b: represent statistically significant different groups ($p < 0.05$).

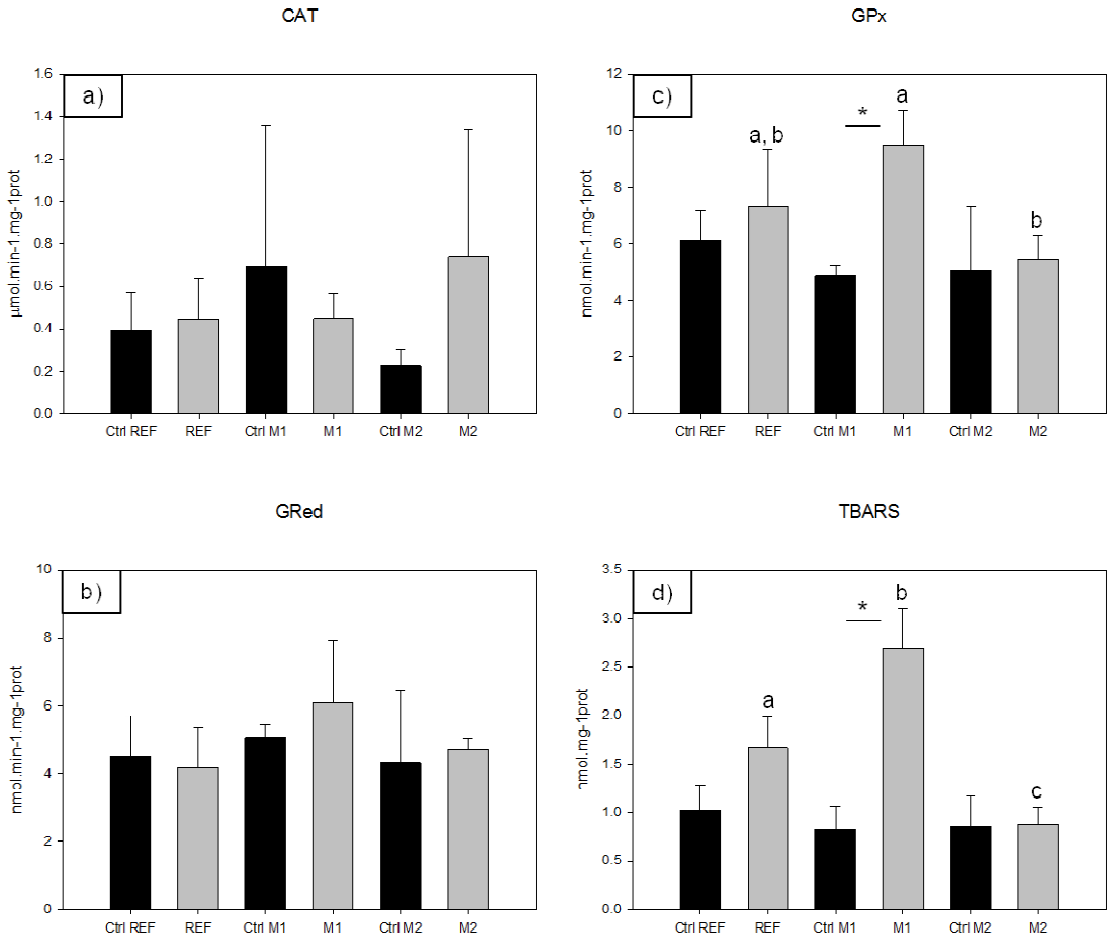

Fig. 4 – a) Mean CAT activity in *P. perezi* tadpoles after 96h exposure to REF, M1 and M2 effluents and respective controls Ctrl REF, Ctrl M1 and Ctrl M2. b) Mean GRed activity in *P. perezi* tadpoles after 96h exposure to REF, M1 and M2 effluents and respective controls Ctrl REF, Ctrl M1 and Ctrl M2. c) Mean GPx activity in *P. perezi* tadpoles after 96h exposure to REF, M1 and M2 effluents and respective controls Ctrl REF, Ctrl M1 and Ctrl M2. d) Mean TBARS content in *P. perezi* tadpoles after 96h exposure to REF, M1 and M2 effluents and respective controls Ctrl REF, Ctrl M1 and Ctrl M2. Error bars represent standard deviation. Statistical analysis was performed within exposure sites, between different controls and between different effluents. Effluents were not compared with controls from other sites. a, b and c: represent statistically significant different groups ($p < 0.05$). *: represents statistical significant differences ($p < 0.05$) within control and effluent of a given pond.

4.4. Discussion

Various studies have already been carried out in the deactivated uranium mine of Cunha Baixa. These have focused both terrestrial and aquatic compartments and covered chemical and ecotoxicological characterization (Antunes et al., 2007a;

Antunes et al., 2007c; Antunes et al., 2008; Marques et al., 2009; Marques et al., 2008; Pereira et al., 2008). This characterization revealed that the M2 pond effluent is very toxic to aquatic species and possesses the most extreme physical conditions of all the local studied ponds. Nonetheless, Iberian green frogs are known to inhabit the mining area and to use the M2 pond as a refuge (Marques et al., 2009). Also, located near this pond, there are two smaller ponds with very different physical and chemical characteristics that *P. perezi* used as a breeding site (authors' personal observation). These ponds, depending on the amount of rainfall, can become in contact with M2 allowing local biota to become exposed to this extremely metal rich acid effluent. Tadpoles are especially sensitive to chemical alterations in water (Freda and Dunson, 1985; Horne and Dunson, 1995a) since they possess a highly permeable skin. Furthermore in the earlier stages of development most have external gills which may render them more susceptible to contaminants (Ortiz-Santaliestra et al., 2006).

Mortality in our *in situ* exposure increased in the following order REF<M1<M2, which followed also the increase in the effluent metal content. Nonetheless, taking into consideration metal body burden, the M1 tadpoles accumulated higher levels of metals than M2, which may point that the higher toxicity of the M2 effluent is owed mainly to low pH. This is corroborated by Marques et al. (2008) who carried out a laboratorial acute assay with *P. perezi tadpoles* with the M2 effluent adjusted to a pH of 8 and obtained only 28% mortality. It must be pointed out that when comparing the values of the elements quantified by Marques et al. (2008) (Be, Mn, Fe, Ni, Zn, Sr, Pb and U) in M2 and our data, our values were all lower with exception for Sr. Thus, if mortality was to be owed mainly by metal content in M2, the mortality in the present study should have been lower than 28%. Furthermore, many authors testing the effect of acidity in larval amphibian have obtained lethal effects (Cummins, 1986; Freda and Dunson, 1985; Horne and Dunson, 1995a). As Freda (1986) reviewed the main physiological effect of low pH on larvae is the inhibition, at the gills, of the active uptake of Na and Cl and simultaneously an increase of their efflux. The decrease in the uptake of sodium is a consequence of the disruption of the Na^+/H^+ exchange mechanism and when its body net loss reaches 50% death occurs (Freda and

Dunson, 1984). Besides lethal effects, growth was also another parameter affected, when effluent exposures were compared. The fact that M2 tadpoles presented a smaller body size than REF tadpoles might, be also owed to the effect of low pH (Cummins, 1986). However, metals must also contribute for the smaller body size, since Marques et al. (2008) had similar results for tadpoles exposed to M2 effluent but with a pH of 8.

Metal body burden in tadpoles generally increased in all exposures from 48h to 96h indicating a continuous uptake of contaminants. Despite of being expected a higher accumulation of metals in M2 tadpoles, due to the high metal content and acidic nature of the effluent, which is one of the factors that increases metal's bioavailability, the M1 tadpoles were the ones with the highest accumulation. Two possible reasons, or even both combined, may be pointed as responsible for this lower bioaccumulation. The first is that at low pH, such as the one registered in M2, H^+ ions compete with metal ions for binding sites, reducing thus metal interaction with cells (Blackwell et al., 1995; Tobin et al., 1984). The second is that cutaneous and gill ion uptake might be impaired by damage to the integument of tadpoles caused also by low pH (Meyer et al., 2010). This last hypothesis would indicate that other factors, such as pH, influencing physiologically M2 tadpoles, would have a greater effect in bioaccumulation rather than bioavailability of metals. Nonetheless, in both hypothesis, and based on previous studies with low pH (Freda, 1986; Freda and Dunson, 1984; Rowe et al., 1992), the most probable outcome for tadpoles exposed continuously to M2 effluent is death.

The determination of the activity of enzymes involved in the response to oxidative stress along with the quantification of LPO allowed us to evaluate, at least in part, the physiological conditions of the exposed tadpoles. CAT activity did not reveal any significant alteration even in M1 tadpoles where metal accumulation was the highest for most of the analyzed metals. A previous study (Marques et al., 2011) on antioxidant responses of various tissues of adult *P. perezi*, inhabiting the present study site, also revealed a lack of significant differences in the activity of this enzyme comparing with animals from a reference site. Indeed no positive correlation could be established between the activity of this enzyme and the

amount of accumulated metals. However, in the same study, both activities of selenium dependent GPx and mainly of total GPx were significantly higher in animals from the contaminated site. A similar pattern was observed in this study in M1 tadpoles for GPx activity, presenting significant higher activities than those from the respective control and M2 tadpoles. This response can be related with the higher amount of metals accumulated in M1 tadpoles. The difference in responses of CAT and GPx can be explained by the fact that CAT acts specifically on hydrogen peroxide, while GPx has a broader action spectrum, acting on various kinds of peroxides (van der Oost et al., 2003) that might be generated in the presence of metals. Thus, we can point out that the higher activity observed in GPx may be mainly owed to peroxides other than hydrogen peroxide.

Accordingly with this response of GPx, the activity of GRed, even with no statistically significant differences, is slightly higher in M1. The activity of GRed is essential to regenerate GSSG generated during GPx activity to the previous form GSH to allow the continuous activity of GPx (Winston and Di Giulio, 1991).

The quantification of LPO showed that M2 tadpoles presented similar values to all the controls, being from all the effluents the one with lowest values. Comparing M2 with REF this last one presented significant higher damage, which may be owed mainly, to the presence of As in these organisms since the majority of the other elements were accumulated in similar concentrations. Along with the production of reactive oxygen species, As has been related with the resulting LPO. As reviewed by Lyn Patrick (2003), this element was listed in 2003 by the Agency for Toxic Substances and Disease Registry (ATSDR), in the top seven of the 275 most hazardous substances in the environment, based on the toxicity of the substance and the potential for exposure. Thus, this element should have special attention when present in the environment and accumulated by organisms. Furthermore, and despite the detected concentration of As in REF being under the maximum admissible value (50 µg/L), in Portuguese legislation for waters for human consumption, Birge et al. (1979) established an LC_{50} for the amphibian *Gastrophryne carolinensis* of 40 µg/L. Even though LPO in REF was higher than M2 it was still significantly lower than the levels recorded in tadpoles exposed to M1. In these tadpoles it is difficult to point a main responsible for the LPO value

since several metals are present in higher values (e.g. Be, Al, Fe, Co, Ni), however it is possible to conclude that, even with high activity, the antioxidant GPx is unable to avoid oxidative damage of membranes certainly resultant from ROS production due to high metal content (Ercal et al., 2001; Stohs and Bagchi, 1995).

Overall, based on the biological and chemical data gathered in our study, it is possible to assume that effluent acidity may assume a primary role over metal content in its toxic effect. The results of metal accumulation clearly reveal that even at low pH, where most metals have increased bioavailability, accumulation may be low. Such may be a result of damage in the integument (Meyer et al., 2010) or of H^+ competition with metal ions binding sites (Blackwell et al., 1995; Tobin et al., 1984). Furthermore our study strengthens the idea that *in situ* exposures need always to be complemented not only by environmental chemical analyses and physical parameters but also by chemical and biochemical analyses on organisms, to support the establishment of a cause effect relationship. Indeed, these results brought a better knowledge of the toxicological processes that local *P.perezi* population might be subjected, mainly in the early life stages. Also, on a global perspective, we can conclude that even neutralizing the mine effluents, with lime and barium chloride (as M1), these effluents are still hazardous to local fauna, which may be exposed in receiving freshwater systems. Hence, further treatment should be considered before its release.

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Chapter V

Antioxidant response and metal accumulation in tissues of Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine

5. Effects of a chronic exposure of *Pelophylax perezi* tadpoles to various pH ranges of a uranium mine effluent: from physiological parameters to gene expression

Sérgio Marques, Sandra Chaves, Fernando Gonçalves, Ruth Pereira

Abstract

In this study we aimed to assess the chronic (90 days) effects of an acidic effluent from a deactivated uranium mine and to perceive the influence of different pH ranges ([8.0-7.5], [7.0-6.5], [6.0-5.5], [5.0-4.5], [4.0-3.5]) in the toxicity of this effluent to the amphibian *Pelophylax perezi*. In order to do so *P. perezi* eggs were exposed to the mine effluent and FETAX medium (control) adjusted for different pH ranges. Mortality and growth were registered until day 90. At the end of the exposure metal accumulation and oxidative stress biomarkers (CAT, GRed, GSTs, selenium dependent-GPx, total-GPx) were measured and differential gene expression in tadpoles exposed to both mediums at [5.0-4.5] pH was performed. Mortality at [5.0-4.5] and [4.0-3.5] pH ranges was 50% and 100% respectively, in both FETAX and mine effluent. Despite higher metal accumulation, growth was significantly higher ($p<0.05$) in the effluent relatively to FETAX for the two higher pHs - [8.0-7.5] and [7.0-6.5]. Within each medium the highest growth was obtained in the lower pH. Biomarkers did not respond coherently, except selenium dependent-GPx and total-GPx that were inhibited tadpoles that have been exposed to the effluent. However differential gene expression pointed for the possibility of two main mechanisms contributing for the endurance of tadpoles in the acidic effluent - [5.0-4.5] pH. The suggested mechanisms are increased osmotic regulation trough up regulation of stomatin and peptidyl-prolyl cis/trans isomerase FKBP2 (FKBP) and in energy investment acquisition related to the up-regulation of voltage dependent anion channel and trypsin.

Keywords

amphibians; oxidative stress biomarkers; metals; SSH; pH; *Pelophylax perezi*

5.1. Introduction

Amphibians are found inhabiting the most varied habitats, being subject to diverse sources of contamination. One of the sources of contamination of amphibian habitats are mining explorations (Lee and Stuebing, 1990). The effluents produced on these areas are characterized by a high metal load and by a generally acidic pH. Several studies have addressed the effects of low levels of pH on both early-life stages and adult amphibians (e.g. Frisbie and Wyman, 1992; McDonald et al., 1984; Rowe et al., 1992). In a general way, parameters commonly assessed by these studies are hatching success, mortality, sodium balance, growth and time to metamorphosis (Frisbie and Wyman, 1992; McDonald et al., 1984; Muths et al., 2003; Rowe et al., 1992). Overall results, as reviewed by Freda (1986), indicate that the toxicity of low pH results mainly from the disruption of Na and Cl balance, usually characterized by an increased efflux and a decreased influx of Na^+. Meyer et al. (2010) has pointed gill damage in tadpoles as a probable cause for such unbalance. Complementary to these studies a more complex approach was made by other authors (Cummins, 1986; Horne and Dunson, 1995a; Horne and Dunson, 1995b) in order to evaluate the joint effect of both low pH and metals such as Al, Cu, Fe, Pb and Zn. Diversified effects as decreased and increased toxicity of pH were observed, depending on the tested metal and on the water chemistry (Horne and Dunson, 1995a).

Research on metal toxicity in amphibians has increased recently, evaluating effects such as mortality, abnormalities, growth, behavioral changes and metal accumulation (Berzins and Bundy, 2002; Calevro et al., 1998; Lefcort et al., 1998; Selvi et al., 2003). The majority of these studies consist in the exposure of organisms to a given concentration of one or a mixture of two or three metals, being scarce in comparison, assays focusing the toxicity of complex metal effluents (e.g. Marques et al., 2008), such as the ones from mining activities. Despite the difficulty in the interpretation of data generated by these assays, the use of the whole effluent toxicity (WET) tests provides more relevant ecotoxicological data, as they represent common environmental exposures. Nevertheless, the complexity in the interpretation of the toxicological data provided

by such exposures relates to the fact that different metals may contribute more or less for altering different parameters (Herkovits and Alejandra Helguero, 1998; Herkovits et al., 2000). Generally, the toxic effect of metals is attributed to their potential to originate reactive oxygen species (ROS) causing subsequently lipid peroxidation (LPO) (Stohs and Bagchi, 1995), even though, as reviewed by Leonard et al. (2004), some metals can exert toxic effects by binding directly to DNA or proteins.

From an ecotoxicological point of view, it is relevant to understand which molecular mechanisms are involved in the responses of amphibians to complex acidic and metal-rich effluents. Such may be obtained through the suppression subtractive hybridization (SSH) technique (Diatchenko et al., 1996), which allows the identification of genes differentially expressed responding to changes in cell's environment. This methodology has the advantage of screening the differentially expressed genes, even without prior genome information. Despite the great potential of gene expression profiling, in ecotoxicology, there are still few examples of its application with complex mixtures of stressors (e.g. Hansen et al., 2007; Poynton et al., 2008).

Our research group has carried out various studies with the amphibian species *Pelophylax perezi*, both with tadpoles and adult life-stages, exposed to the acidic effluent of a deactivated uranium mine (Cunha Baixa mine, Centre of Portugal) (Marques et al., 2011; Marques et al., 2009; Marques et al., 2008). The main goal has been to understand how these amphibians withstand this kind of contamination, since this species is found inhabiting the above mentioned mining complex Overall, the results obtained, both in adults and tadpoles showed that effectively metal accumulation occurs, namely uranium (Marques et al., 2009; Marques et al., 2008). In adults, genotoxic effects have been observed in erythrocytes, and some histopathological alterations, such as dilatation of the renal tubules lumen associated with tubular necrosis were reported (Marques et al., 2009). Additionally, oxidative stress parameters, mainly glutathione peroxidase (GPx) presented an increased activity in organisms from the contaminated site. Similar results were observed *in situ* exposed tadpoles (Marques et al., *in prep*. b). Despite this wide variety of toxicological effects assessed the differential gene

expression study in the liver, Marques et al. (*in prep.* a) unveiled the potential role of basal protection mechanisms (e.g. up-regulation of genes coding for the antioxidant albumin and fibrinogen) in the tolerance and survival of organisms exposed chronically to contamination. Nonetheless and despite the amount of work done with this species and in this contaminated site there are still many questions to be answered.

In order to increase the knowledge on the toxicological effects and the possible genes involved in triggering physiological responses to chronic exposures, to mine effluent, in early life stages of *P. perezi,* the present study was undertaken. Furthermore, the study of the effect of different pH ranges, on the effluent toxicity was also an objective. To achieve these goals *P. perezi* eggs from a reference site were exposed to the effluent and to FETAX medium at five different pH ranges: ([8.0-7.5]; [7.0-6.5]; [6.0-5.5]; [5.0-4.5]; [4.0-3.5]) in laboratorial conditions, for 90 days. Survival, growth, metal accumulation, oxidative stress biomarkers (catalase, total-glutathione peroxidase, selenium dependent-GPx glutathione reductase, glutathione-S-transferases) were assessed. Furthermore a differential gene expression study, based on the SSH technique, was carried out on tadpoles after 90 days of exposure in the [5.0-4.5] pH, that corresponded to the most extreme condition in terms of pH at the end of the assay.

5.2. Materials & Methods

5.2.1. Study site

The present study was conducted with effluent from the deactivated uranium mine located near the village of Cunha Baixa (Mangualde, Centre of Portugal) where ore extraction occurred between 1967 and 1993 (Oliveira and Ávila, 1998). After the exploration period, the mine pit was filled with low-grade ore and was flooded with sulphuric acid to extract uranium through an *in situ* leaching process (Santo and Freire, 1983). Presently, there are three small ponds in this area, with a variable water level, that fluctuates according to the uprising of the underground acidic effluent, which in turn is determined by variations in the piezometric level of the aquifer. Due to its origin, this effluent 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. The largest pond (M pond) shows the most extreme conditions due to direct connection with the underground tunnels of the mine and, consequently, with the mine effluent. Iberian green frogs (*P. perezi*) are frequently found on the three ponds. However, no egg masses were recorded on the M pond. Furthermore, during their winter lethargic stage, they have been found burrowed (authors' personal observation) in the highly metal contaminated sludge coming from the effluent treatment pond (Pereira et al., 2008) which is placed nearby these lagoons. The site chosen as a reference for egg masses collection is an unpolluted segment of the Vouga River (VR) located to the north of the city of Viseu, a few kilometers from Mangualde, where frogs from the same species were recorded (Marques et al., 2009).

5.2.2. Effluent sampling

The effluent for the assay was collected in the M pond in 5 L plastic containers. These containers 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 collected in VR and M pond, 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. FETAX medium (Dawson and Bantle, 1987), despite being prepared in laboratory, was sent for analysis as well. These samples were then stored at 4ºC until chemical analyses was possible. Two samples of the mine effluent were analysed; one at the beginning of tadpoles exposure and the other at the end, to perceive if the effluent suffered a great variation in any of its elements, during this 90-days period.

5.2.3. Test organisms

Pelophylax perezi eggs from the same clutch and in the first stage of development

(Gosner, 1960) were collected in VR. The egg mass was immediately transported to the laboratory to begin the chronic assay.

5.2.4. Chronic assay

The 90 days bioassay was adapted, with a few changes, from ASTM recommendations for conducting acute toxicity assays with effluents (ASTM, 1997), since it is the only available for aqueous ambient samples. The experimental setup consisted in 3 replicas for each pH range ([8.0-7.5], [7.0-6.5], [6.0-5.5], [5.0-4.5], [4.0-3.5]) and medium: the M effluent and FETAX medium (Dawson and Bantle, 1987) making a total of 10 different conditions being tested. From these conditions FETAX [8.0-7.5] can be considered the control since it encompasses the pH normally established for FETAX medium (Dawson and Bantle, 1987). Previously to pH adjustments, the effluent was filtered by cellulose nitrate ALBET® filters with a 47 mm diameter and a 0.20 µm pore to eliminate possible bacteria and parasite contamination. To obtain the desired pH range the acidity was adjusted with 5M NaOH and 5M HCl for the effluent and FETAX respectively. At the beginning of the assay 20 eggs, from the same egg mass, in the second stage of development (Gosner, 1960), were placed in each one of the 3 replicas of each testing condition. For the first 96h, exposure was carried in plastic Petri dishes with 20 ml of the respective media. Additionally 5 more tadpoles randomly selected across the 3 replicas (2+2+1) for every condition, were removed, pooled together and stored at -20 ºC for metal quantification. Afterwards the remaining larvae (13+13+14), were transferred to plastic vessels, keeping the previous number of replicas, containing 1000 ml of the same medium and pH condition, to which they have been exposed since the beginning of the assay. The exposure conditions were the following, constant temperature (20±1ºC), photoperiod (14hL:10hD), renewal of the media every other day and smooth aeration after the first 96h. The pH was checked after every renewal of the media.

The evaluated parameters were mortality, growth, metal accumulation, oxidative stress biomarkers and differential gene expression, the latter specifically for the [5.0-4.5] pH range, since it corresponded to the most extreme condition in terms of pH at the end of the assay (mortality was total in [4,0-3.5] pH).

Mortality was registered every 24h during the test and dead animals were removed. Growth was assessed through measuring body length (head to tail) of 20 tadpoles randomly selected equitably across the 3 replicas at days 4, 6, 11, 19, 24, 30, 35, 40, 45, 50, 57, 63, 71, 76, 81, 85 and 90, using an Olympus SZX9 stereoscope. For metal accumulation, sampling occurred at two distinct times, day 4 previously mentioned and day 90. A pool of three tadpoles randomly selected, at day 90, was obtained across the 3 replicas (1 tadpole *per* replica) for each condition and stored at -20 ºC until metal quantification was possible. Prior to freezing, tadpoles obtained at different exposure times, from all conditions, were allowed to swim for one minute in freshly made FETAX medium to remove metal rich-effluent from the body surface. For the oxidative stress biomarkers 3 pools of 5 tadpoles (1 pool per replica) randomly obtained from each condition were frozen in liquid nitrogen and stored at -80ºC. At the end of the assay three tadpoles (1 per replica) from the pH [5.0-4.5] were stored at -80 ºC in RNALater (Qiagen, UK) for gene expression analysis. During the assay tadpoles were feed daily *ad libitum* with a suspension of grossly ground Tetramin® (Tetrawerke®, Germany).

5.2.5. Chemical analyses

The total concentrations of Be, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Cd, Ba, Pb and U were quantified in VR water, M effluent and FETAX medium, as well as in tadpoles from all testing conditions, by inductively coupled plasma mass spectrometry (ICP/MS) (APHA, 1995). 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 tadpoles a previous wet-digestion was required. The tadpoles were first oven-dried at 105 ºC until a stable weight was achieved. After having been dried, the weight of the tissue was recorded to the nearest 0.1 mg, and they were digested in closed Teflon flasks with 3 ml of nitric acid (suprapur Merck®, 65 %) in a 60 ºC sand-bath. Aliquots of 0.5 ml of suprapur hydrogen peroxide (30 %) MERCK® were then added to free the solution of any

organic solid fragments. The final volume of the solution was made up to 5 ml with Milli-Q® water (18.2 Ω). Sample blanks were obtained following the same procedure described for wet-digestion but replacing the tissue volume by Milli-Q® water and replicate measurements in ICP/MS were made for each sample in order to check if a variance coefficient lower than 10% was obtained.

5.2.6. Oxidative stress biomarkers

Oxidative stress responses were evaluated in tadpoles measuring the activity of catalase (CAT), selenium dependent-glutathione peroxidase (selenium dependen-GPx), total GPx (total-GPx) glutathione reductase (GRed), and glutathione-S-transferases (GSTs) enzymes.

Pools of 5 tadpoles previously frozen in liquid nitrogen, were homogenized in ice-cold phosphate buffer (50 mM, pH 7.0 with 0.1 % TRITON X-100). Homogenates were centrifuged at 10 000 g for 10 min and supernatants were divided into five aliquots, one for each determination (CAT, selenium dependent-GPx, total-GPx, GST, GRed). Aliquots were stored at -80 ºC until determinations were possible.

GSTs (EC 2.5.1.18) activity was determined by spectrophotometry, according to Habig et al. (1974). GSTs catalyze the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, forming a thioeter (molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$) that can be followed by the increment of absorbance at 340 nm.

GRed (EC 1.8.1.7) activity was determined by spectrophotometry, according the protocol of Carlberg and Mannervik (1985). In this assay the GRed mediated oxidation of NADPH was monitored at 340 nm (molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$).

CAT (EC 1.11.1.6) activity was determined by the spectrophotometric method described by Aebi (1984). This method consists in monitoring the consumption of hydrogen peroxide, at a wavelength of 240 nm (molar extinction coefficient of 0.0394 $mM^{-1}cm^{-1}$) for a period of 30 s.

GPx (EC 1.11.1.9) activity was determined according to Flohé and Günzler (1984), following the oxidation of NADPH at a wavelength of 340 nm (molar

extinction coefficient of 6.22 $mM^{-1}cm^{-1}$), when GSSG is reduced back to GSH by glutathione reductase. GPx activity was monitored using both hydrogen peroxide (0.255 mM) and cumene hydroperoxide (0.7 mM) as independent substrates, corresponding, respectively, to selenium dependent-GPx and total-GPx.

Enzymatic activities were determined in triplicate and were expressed in nmol of substrate hydrolyzed per min and per mg of sample protein (see protein quantification below).

Protein concentration of each sample was determined in triplicate, according to the spectrophotometric (wavelength = 595 nm) method of Bradford (1976). For statistical purposes, each pool was considered a replica.

5.2.7. Differential gene expression

Three tadpoles from FETAX [5.0-4.5] and from M [5.0-4.5] were used in independent RNA extractions. Total RNA from samples stored in RNALater (Qiagen, UK) solution at -80 ºC was extracted after homogenization in liquid nitrogen followed by the addition of Trizol reagent (Invitrogen, USA), according with the manufacturer instructions. The final extract was resuspended in diethylpyrocarbonate (DEPC) treated water. Total RNA integrity was checked by electrophoresis on a 1 % agarose gel and Ethidium Bromide (EtBr) staining. RNA concentration was estimated by spectrophotometry at 260 nm. The isolation of poly $(A)^+$ RNA was carried out using Poly A Spin mRNA Isolation Kit (New England Biolabs, USA) as recommended by the manufacturer. Equivalent amounts of mRNA from the 3 samples from each condition were pooled together for suppressive subtractive hybridization (SSH) reactions.

SSH was performed with the PCR Selected Subtractive Hybridization Kit (Clontech, USA) following the manufacturer's instructions, using cDNA from M organisms, containing specific differentially expressed transcripts as tester and cDNA from FETAX organisms as driver. Briefly, both mRNA pools were converted into cDNA and digested with RSA I, ensuring the maintenance of blunt ended molecules. The tester cDNA was then ligated to adaptors 1 and 2 in independent reactions. Subsequently two sequential hybridizations were performed. The first was prepared by adding an excess of driver to each ligation product, followed by a

heat denaturation and subsequent re-annealing. This step allows differentially expressed sequences to be equalized and enriched. The second hybridization was done by combining the two different tester pools, in the presence of fresh heat-denaturated driver, allowing the formation of new hybrids and a preferential selection of differential expressed sequences in the following steps. After subtraction, two PCR amplifications were performed. The first was done with a primer that has a complementary sequence to the adapters, enabling the exponential amplification of differential transcripts. The non-differentially expressed hybrid sequences are either not amplified or do not have an exponential amplification. The second PCR was a nested PCR with specific primers for each adapter, allowing further enrichment of the differentially expressed sequences and the reduction of background. Subtraction efficiency was evaluated by electrophoresis of PCR products in a 2% agarose gel, as recommended by the kit manufacturer.

PCR products were then purified using Jetquick PCR Product Purification Spin Kit (Genomed GmbH, Germany) and ligated into pCR II T-vector, using TA Cloning Kit (Invitrogen, USA) as described by the manufacturer. After transformation into *Escherichia coli* XL-1 Blue-MRF' competent cells (Stratagene, USA), recombinants were selected in Luria-Bertani (LB) agar plates containing ampicillin (100 µg/ml), IPTG (80 µg/ml) and X-Gal (80 µg/ml). After incubation overnight at 37 ºC, recombinant cells were isolated and scratched onto new plates. The presence of inserts was confirmed by PCR using recombinant cell lysates, prepared in 50 µl of TE with 0.1% of Tween 20 and heating at 100 ºC for 10 minutes. PCR reactions were perform in 50 µl reactions containing 0.5 µM each primer (SP6 and T7) 1x Taq buffer, 0,2 mM each dNTP, 2mM $MgCl_2$, 1U *Taq* polymerase and 3 µl of cell lysate as template DNA. PCR reaction was set as follows: an initial denaturating step, at 94 ºC, for 3 minutes, was followed by 35 cycles of 1 minute denaturation at 94 ºC, 1 minute annealing, at 50 ºC, and 1 minute extension at 72ºC. A final extension step of 3 minutes, at 72ºC, was also included. PCR products were analysed in by electrophoresis in a 1.2% agarose and stained with EtBr. The PCR products revealing the presence of inserts were purified with Jetquick PCR Product Purification Spin Kit (Genomed GmbH,

Germany) and sequenced. The corresponding clones were stored at -70 ºC as glycerol stocks.

Sequencing was performed by Macrogen (Seoul, South Korea) using an ABI 3730 XL genetic analyser (Applied Biosystems) and the sequences were compared with protein databases using BLASTX from National Center for Biotechnology Information (NCBI).

5.2.8. Statistical analysis

To analyse the effect of exposure medium (FETAX *vs* effluent), time (from day 4 to day 90) and pH ranges ([8.0-7.5], [7.0-6.5], [6.0-5.5], [5.0-4.5]) on the growth of tadpoles, as well as to check for a significant interaction between the three factors a three-way analysis of variance (ANOVA) was performed. Since significant interactions between the three factors were recorded, the mean square error of the ANOVA was used to compute the standard error of the difference between groups, in the post-hoc Tukey multiple comparison test, to correct for simple main effects (Quinn and Keough, 2002; Shoshan-Barmatz and Israelson, 2005; Zar, 1996).

The comparison of growth patterns of tadpoles exposed to the mine effluent *versus* the FETAX medium, at different pH ranges was performed first obtaining regression lines for each medium, considering time and growth as independent and as depend variables, respectively. The significance of each regression line was tested using a Student's t statistic (Zar, 1996). Thereafter, for each pH range the slopes of the two regression lines obtained for the two mediums, were compared through a Student's-t test (Zar, 1996). The same procedure was followed to compare growth patterns of tadpoles exposed to different pH ranges of both the mine effluent and the FETAX medium. The slopes of regression lines obtained for different pH ranges were compared by an analysis of covariance as described by (Zar, 1996), followed by a Tukey multiple comparison assay when the analysis of covariance concluded about significant differences among slopes (Zar, 1996).

For biochemical parameters, a significant effect of pHs and medium on enzyme activities, was analyzed by two-way ANOVAs. When a significant interaction between both factors was recorded ($p<0.05$), differences between

groups were tested by simple main effects ANOVAs followed by a multi-comparison Tukey test, otherwise one-way ANOVAs followed by Tukey test were used.

5.3. Results

The chemical characterization of VR water, FETAX medium and M effluent is presented in Table 1 as well as the benchmark values established by Portuguese legislation for evaluating the quality of water for human consumption (MA, 1998). Even being overprotective, these benchmark values were chosen since for other water uses (e.g. irrigation, recreation) benchmark values available account for fewer elements. To fill this gap other benchmark values established by EPA (2009) and Suter II and Tsao (1996), based in chronic ecotoxicological parameters, are also reported (Table 1). Our results revealed that Be, Mg, Al, Ca, Mn, Co, and U in the M effluent surpassed all the benchmark values available for comparison. On the other hand the values reported for VR water and FETAX medium were, with exception of K in FETAX, lower than the benchmark values presented in Table 1. These results assure, for the determined parameters, the reference condition of VR where egg masses were collected as well as the quality of the FETAX medium. The mortality results for the 90 days-chronic exposure allowed to validate the assay since for the three FETAX replicas at the pH range [8.0-7.5] (considered as the control pH, since it is the normal pH established to conduct assays with this medium (Dawson and Bantle, 1987) only a 2.5% average mortality occurred. Overall at the end of the assay, mortality in FETAX was of 2.5% for pH [8.0-7.5], [7.0, 6.5] and [6.0-5.5] treatments; 50% for pH [5.0-4.5] and 100% for pH [4.0-3.5]. As for the effluent, at pH ranges of [8.0-7.5], [7.0-6.5], [6.0-5.5], [5.0-4.5] and [4.0-3.5] average percentages of mortality of 12.5, 17.5, 32.5, 50 and 100%, respectively, were recorded. We must highlight that by day 6 all tadpoles exposed to pH [4.0-3.5] in both FETAX and M effluent were dead and many did not even hatch.

Table 1 – Chemical and metal concentration data for FETAX, M effluent and VR egg mass collection site. 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.

Element (µg/L)	Detection Limit	Freshwater CCC	ORNL Tier II Chronic	EPA R4-SB	MRV	MAV	VR	FETAX	EFFLUENT (Mean ± SD)
Be	0.10	NLV	0.66	0.53	NLV	NLV	BDL	BDL	**15.4** ± 1.1
Mg	2.00	NLV	NLV	NLV	30000.0	50000.0	14432.2	14613.1	**49763.4** ± 3633.1
Al	5.00	87.00	NLV	87.00	50.0	200.0	6.1	5.5	**1507.0** ± 238.1
K	10.00	NLV	NLV	NLV	10000.0	12000.0	1729.9	**15325.2**	5314.8 ± 384.9
Ca	10.00	NLV	NLV	NLV	100000.0	NLV	22571.0	20618.0	**123939.0** ± 5780.4
V	1.00	NLV	20.00	NLV	NLV	NLV	1.2	1.3	BDL
Cr	1.00	11.00	NLV	11.00	NLV	NLV	BDL	BDL	BDL
Mn	0.01	NLV	120.00	NLV	20.0	50.0	2.0	1.8	**6076.3** ± 502.5
Fe	10.00	NLV	NLV	1000.00	50.0	200.0	BDL	BDL	605.0 ± 252.8
Co	0.10	NLV	23.00	NLV	NLV	NLV	BDL	BDL	**46.2** ± 5.0
Ni	0.50	52.00	NLV	87.71	NLV	50.0	BDL	BDL	65.0 ± 10.7
Cu	8.00	NLV	NLV	6.54	3000.0	NLV	BDL	BDL	10.8 ± 0.0
Zn	4.00	120.00	NLV	58.91	500.0	NLV	5.0	BDL	213.9 ± 59.6
As	0.50	150.00	NLV	190.00	NLV	50.0	BDL	BDL	0.6 ± 0.0
Se	1.00	5.00	NLV	5.00	NLV	10.0	BDL	BDL	BDL
Sr	0.01	NLV	1500.00	NLV	NLV	NLV	7.6	6.8	331.6 ± 27.6
Cd	0.10	0.25	NLV	0.66	NLV	5.0	BDL	BDL	0.8 ± 0.2
Ba	0.10	NLV	4.00	NLV	100.0	NLV	1.4	BDL	19.6 ± 4.4
Pb	0.10	2.50	NLV	1.32	NLV	50.0	1.1	0.2	1.4 ± 1.6
U	0.10	NLV	2.60	NLV	NLV	NLV	0.1	0.2	**526.7** ± 95.3
Hardness (mg/L)							115.79	111.66	579.89 ± 348.72

MRV and MAV stands for Maximum recommendable values and Maximum admissible values of waters for human consumption (MA, 1998).

ORNL Tier II Chronic – Oak Ridge National Laboratory Tier II Chronic values for allowing the establishment of aquatic benchmarks with fewer data (Suter and Tsao, 1996).

Freshwater CCC (Criterion Continue Concentration) – National recommended water quality criteria (EPA, 2009)

EPA R4 - Chronic surface water screening benchmark (Suter and Tsao, 1996).

NLV stands for No Legal Values established.

The values exceeding the different benchmark values (after summing SD) available were written in bold letter.

BDL stands for Below Detection Limit.

Metal quantification in tadpoles exposed to both FETAX and M effluent, for almost every quantified element (exception made for Cu, Zn and Pb), revealed an increased accumulation from day 4 to day 90 (Table 2). Moreover, the higher values reported were mainly for effluent exposed tadpoles with particularly higher concentrations for Al and U. Within the FETAX tested pHs, the accumulation of metals by tadpoles was higher at the pH range of [6.0-5.5]. On the other hand, in the M effluent tested pHs, depending on the element, the higher concentrations were recorded both in [8.0-7.5] and [5.0-4.5] exposed tadpoles. Specifically within the effluent pHs, the tadpoles from the [8.0-7.5] pH presented the highest values of Be, Ca, Mn, Zn, Sr and Ba while in the [5.0-4.5] pH were the Mg, K, Fe, Cu and U that were accumulated in higher concentrations.

Table 2 - Metal concentration in *P. perezi* tadpoles at day 4 (pool, n=5) and day 90 (pool, n=3) exposures for all pH ranges in both FETAX and M effluent.

	FETAX								EFFLUENT							
pH	[8.0-7.5]		[7.0-6.5]		[6.0-5.5]		[5.0-4.5]		[8.0-7.5]		[7.0-6.5]		[6.0-5.5]		[5.0-4.5]	
Element (µg/g)	day 4	day 90	day 4	day 90	day 4	day 90	day 4	day 90	day 4	day 90	day 4	day 90	day 4	day 90	day 4	day 90
Be	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	14.1	BDL	13.7	BDL	8.9	BDL	6.3
Mg	594.7	1006.1	547.4	1022.5	674.9	1396.2	516.4	917.4	676.7	978.6	809.8	1037.3	1011.5	1031.0	580.3	1079.9
Al	BDL	BDL	BDL	0.3	BDL	28.3	BDL	BDL	BDL	2334.4	BDL	2362.2	BDL	1945.4	BDL	1897.0
K	2403.4	9569.2	1605.1	10460.3	2397.8	10142.4	1875.7	8676.1	2013.7	8391.5	1332.2	9215.2	2551.0	9199.4	925.5	10062.5
Ca	BDL	9919.6	BDL	8812.6	BDL	21133.9	BDL	8310.3	147.8	15606.6	240.6	9376.5	1200.0	7639.5	BDL	11568.5
V	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Cr	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Mn	BDL	5.3	BDL	4.5	BDL	4.2	BDL	2.5	48.6	170.6	68.0	123.6	77.0	110.6	48.2	86.1
Fe	BDL	182.6	BDL	122.0	BDL	221.3	BDL	146.2	BDL	518.7	BDL	507.1	BDL	473.3	BDL	985.1
Co	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	2.3	BDL	1.4	BDL	1.1	BDL	1.2
Ni	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	3.1	BDL	3.1	BDL	2.4	BDL	BDL
Cu	41.8	24.7	41.3	27.7	42.4	54.3	30.1	26.5	29.3	26.3	38.3	47.4	44.1	40.7	34.2	57.4
Zn	423.7	329.8	374.2	368.8	412.4	502.3	350.0	304.3	307.8	799.7	417.0	461.8	515.5	327.8	331.6	189.7
As	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Se	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Sr	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	11.3	BDL	6.0	BDL	BDL	BDL	4.4
Cd	BDL	0.4	BDL	0.4	BDL	BDL	BDL	0.3	BDL	1.7	BDL	1.2	BDL	0.9	BDL	0.4
Ba	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	9.2	BDL	5.8	BDL	3.1	BDL	1.9
Pb	BDL	1.0	BDL	0.8	BDL	1.6	3.9	1.1	BDL	0.7	BDL	0.8	BDL	0.9	3.8	1.1
U	BDL	BDL	BDL	BDL	BDL	2.9	BDL	0.3	5.3	335.5	3.9	369.8	4.4	369.9	3.1	506.0

Concentrations are expressed in µg/g, dry weight

BDL stands for Below Detection Limit

The highest concentration for each element is written in bold

For the body length data, the three factors: exposure time, medium and pH ranges had a significant effect on this parameter ($F = 1773.1$, d.f. = 16, $p = 0.000$; $F = 41.7$, d.f. = 1, $p = 0.000$; $F = 94.4$, d.f. = 3, $p = 0.000$; respectively). Interactions between factors were tested as well by the three-way ANOVA performed. Specifically we tested exposure media (FETAX and effluent) vs time vs pH, obtaining a significant interaction among them ($F = 3.4$, d.f. = 48, $p = 0.000$). Also, there were significant interactions in media vs time ($F = 10.9$, d.f. = 16, $p = 0.000$),

media vs pH ($F = 6.8$, d.f. = 3, $p = 0.000$) and time vs pH ($F = 7.8$, d.f. = 48, $p = 0.000$). After the three-way ANOVA a post-hoc Tukey multiple comparison test, corrected for simple main effects was performed, to check for significant differences among tadpoles exposed to different media and pH ranges. Our results revealed that at low pHs ([6.0-5.5] and [5.0-4.5]) there were no significant differences in tadpoles' body length between FETAX and M effluent media. However for the highest pHs ([8.0-7.5] and [7.0-6.5]) there were differences between the body length of tadpoles, exposed to different media, at the end of the assay. When complementing this analysis with the comparison of slopes of the regression lines expressing the growth of animals throughout time (Fig. 1) we confirmed that the increment in growth was higher in tadpoles exposed to the M effluent both at [8.0-7.5] and [7.0-6.5] pHs (correspondingly Fig. 1a and 1b) in comparison with tadpoles exposed to the FETAX medium at the same pH ranges. Such observation is drawn from the significant differences among the slopes of linear regressions obtained for each effluent at [8.0-7.5] and [7.0-6.5] pHs ranges ($t = 55.3$, d.f. = 676, $p < 0.05$ and $t = 60.5$; d.f. = 676, $p < 0.05$ respectively). This higher increment in growth has originated a significantly higher body length in tadpoles, at the end of the assay, has it was demonstrated by the previous analysis. On the other hand way around, despite having no significant difference in body length, at the end of the assay, the comparison of regression lines obtained for both effluents showed significant differences in slopes obtained for the lowest pH ranges ($t = 60.0$, d.f. = 676, $p < 0.05$ for [6.0-5.5] and $t = 64.4$, d.f. = 676, $p < 0.05$ for [5.0-4.5]) (Fig. 1c and 1d, respectively). The increment in growth throughout time was always higher in tadpoles exposed to the effluent, despite its richness in toxic metals. To assess the effect of pH in growth, differences among the slopes of the regression lines obtained for different pH ranges, within FETAX medium (Fig. 2a) and within effluent medium (Fig. 2b) were evaluated. Initial analysis revealed significant differences among FETAX pH ranges slopes ($F_{(3;1352)} = 21.301$, $p<0.05$). Further slope multi-comparison analysis in FETAX revealed significantly differences among slopes obtained for [5.0-4.5] pH compared to [8.0-7.5], [7.0-6.5] and [6.0-5.5] ($q = 11.276$, d.f. = 1352, $p < 0.05$; $q = 5.719$, d.f. = 1352; $p < 0.05$ and $q = 5.479$, d.f. = 1352, $p < 0.05$, respectively). The increment in

growth throughout the assay in tadpoles exposed to the FETAX medium was higher at the lowest pH. In opposition additionally significant differences among slopes were recorded as well between [8.0-7.5] pH further compared to [7.0-6.5] and [6.0-5.5] ($q = 5.977$, $d.f. = 1352$, $p < 0.05$ and $q = 5.499$, $d.f. = 1352$, $p<0.05$ respectively). This result demonstrates that the increment in growth was always lower at the highest pH. As far as effluent exposures are considered the initial analysis revealed significant differences among the pH slopes of linear regressions obtained for tadpoles growth ($F_{(3;1352)} = 26.147$, $p<0.05$). The subsequent slope multi-comparison analysis revealed a similar pattern to the one obtained for FETAX. Specifically the slope obtained at [5.0-4.5] pH was significantly different from those computed by regression analysis at [8.0-7.5], [7.0-6.5] and [6.0-5.5] pHs ($q = 12.460$, $d.f. = 1352$, $p < 0.05$; $q = 6.721$, $d.f. = 1352$; $p < 0.05$ and $q = 6.462$, $d.f. = 1352$, $p < 0.05$ respectively). Also, as verified for FETAX when the slope at the pH range of [8.0-7.5] was compared to [7.0-6.5] and [6.0-5.5] slopes ($q = 8.117$, $d.f. = 1352$, $p<0.05$ and $q = 6.530$, $d.f. = 1352$, $p < 0.05$ respectively) the increment in growth was always lower at the highest pH range. For the intermediate pH ranges, no differences were obtained, between the slopes of linear regressions computed for both media. Globally, and after applying a post-hoc Tukey multiple comparison test after the three-way ANOVA, to compare differences in tadpoles body size at the end of the assay, promoted by medium and pH ranges, we observed that higher body lengths were obtained in lower pHs [5.0-4.5] in both mediums ($p<0.05$).

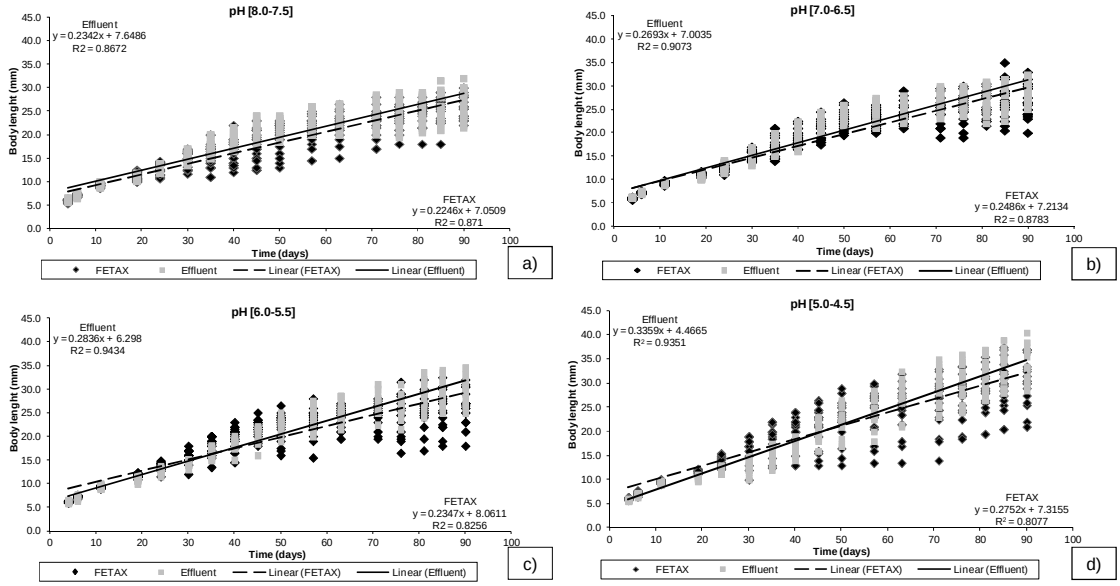

Fig. 1 – Growth regression analyses comparing different media within each pH range. Statistical significant differences ($p<0.05$) were obtained in all situations. a) Growth regression analysis between FETAX and M effluent for [8.0-7.5] pH. b) Growth regression analysis between FETAX and M effluent for [7.0-6.5] pH. c) Growth regression analysis between FETAX and M effluent for [6.0-5.5] pH. d) Growth regression analysis between FETAX and M effluent for [5.0-4.5] pH.

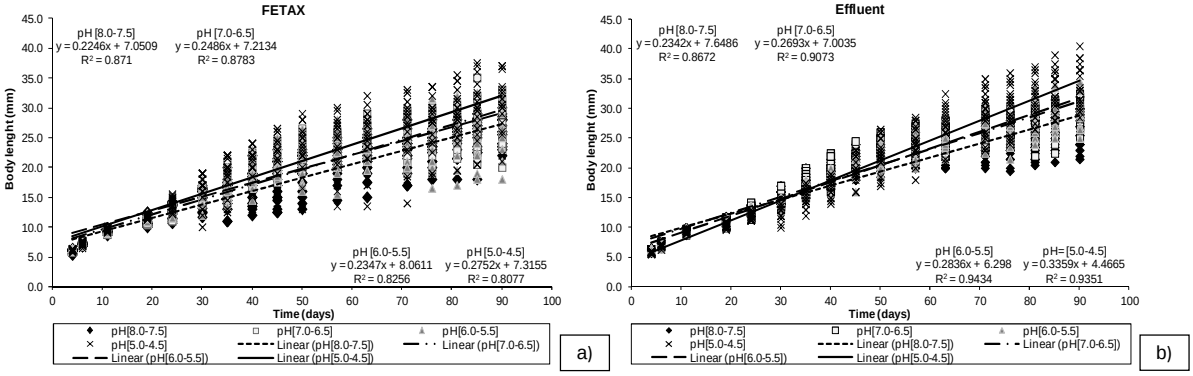

Fig. 2 – Growth regression analyses comparing different pHs within each medium. Statistical significant differences ($p<0.05$) were obtained in all situations except between [6.5-6.0] and [5.5-5.0] in both media. a) Growth regression analysis between [8.0-7.5], [7.0-6.5], [6.0-5.5] and [5.0-4.5] for FETAX. b) Growth regression analysis between [8.0-7.5], [7.0-6.5], [6.0-5.5] and [5.0-4.5] for M effluent.

The effect of medium and pH ranges on oxidative stress biomarkers activity (CAT, GRed, selenium dependent-GPx, total-GPx and GSTs) as well as interactions between factors were tested by two-way analyses of variance. A significant interaction between both factors was recorded for CAT, selenium dependent-GPx and GSTs activities ($F = 5.430$, d.f. = 3, $p = 0.004$; $F = 4.769$, d.f.=3, $p = 0.007$ and $F = 5.64$, d.f. = 3, $p = 0.002$ respectively). Further statistical analyses, after testing the interaction, were performed for each enzyme, to test for activity differences within pH ranges of FETAX or M effluent and also to test for differences between exposure media within each pH. For this purpose individual one-way analysis of variance, followed by a multiple comparison test, both corrected for simple main effects, were performed. For CAT (Fig. 3a) activity significant differences within media, for different pH ranges, were only observed in effluent tadpoles ($F = 9.530$, d.f. = 3, $p < 0.000$). Despite the great variation recorded for this parameter among different pH ranges, a decreasing tendency for lower pHs was observed. Only at [7.0-6.5] pH a significant difference between media was recorded, with tadpoles from the effluent showing a significant inhibition in the activity of these biomarkers, when compared with tadpoles exposed to the FETAX medium. As for the activity of GRed (Fig. 3b) the only worth mentioning fact was the significantly higher activity ($F = 8.704$, d.f. = 1; $p = 0.018$) recorded in effluent tadpoles at pH [5.0-4.5] when compared to the activity reported for the FETAX tadpoles. Relatively to the selenium dependent-GPx activity (Fig. 3c) the comparison within medium for different pHs ranges, revealed significant differences only among tadpoles exposed to the FETAX medium ($F = 4.008$, d.f. = 3, $p = 0.026$). At lower pH ranges, the activity of this enzyme tended to stabilize between a medium and maximum value recorded for [8.0-7.5] and [7.0-6.5] pH, respectively. When differences among mediums are considered, at each pH range, the activity of this enzyme was always significantly inhibited in tadpoles exposed to the effluent, in comparison with those from the FETAX medium at [7.0-6.5], [6.0-5.5] and [5.0-4.5] pHs ($F = 11.670$, d.f = 1, $p = 0.009$; $F = 47,591$, d.f. = 1, $p < 0.001$ and $F = 7.394$, d.f. = 1, $p = 0.026$ respectively). As far as total-GPx activity is considered (Fig. 3d) no significant differences were recorded between tadpoles exposed to different pH ranges of the M effluent. Within FETAX

exposures, significant differences among tadpoles exposed to different pH ranges were recorded only between [8.0-7.5] and [6.0-5.5] pHs (F = 4.752; d.f. = 3; p = 0.015) with lower activity in the latter range. When comparison of total-GPx activity between effluents was made, for specific pH ranges, a generalized inhibition in its activity was obtained in M effluent exposed tadpoles, with exception for [6.0-5.5].

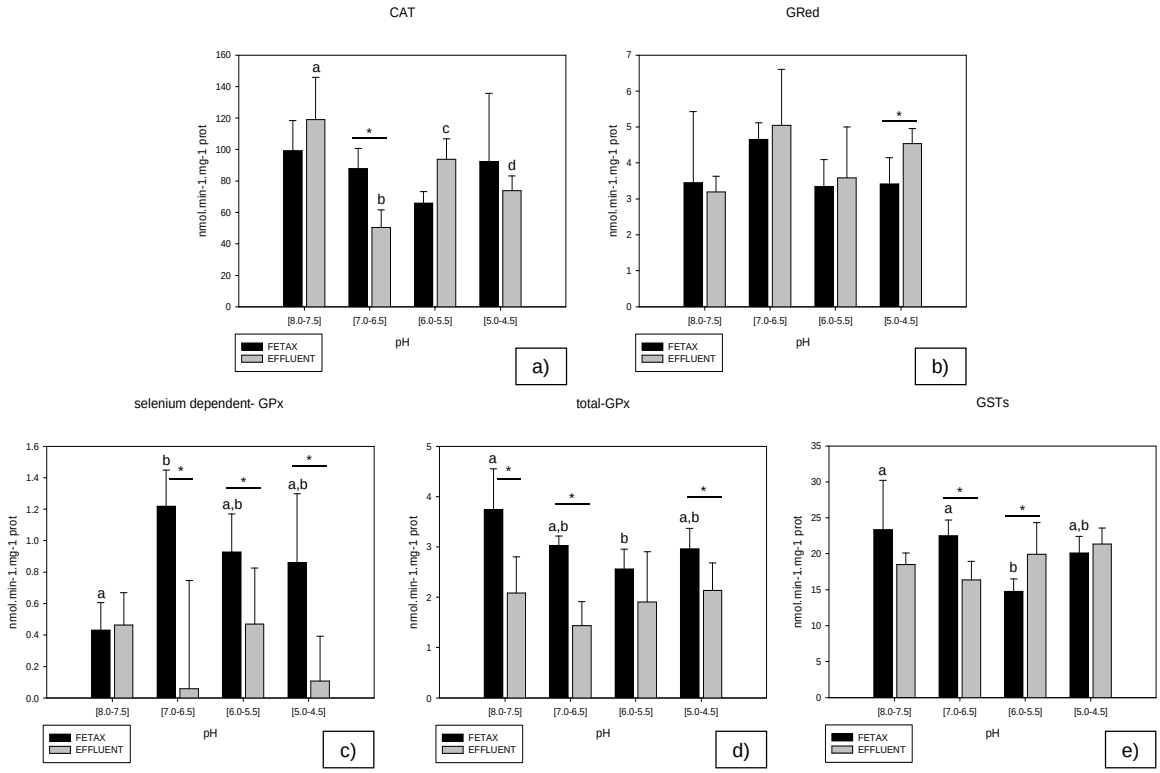

Fig. 3 – a) Mean CAT activity in *P. perezi* tadpoles after 90 days exposure to FETAX and M effluent at the pH ranges of [8.0-7.5], [7.0-6.5], [6.0-5.5] and [5.0-4.5]. b) Mean GRed activity in *P. perezi* tadpoles after 90 days exposure to FETAX and M effluent at the pH ranges of [8.0-7.5], [7.0-6.5], [6.0-5.5] and [5.0-4.5]. c) Mean selenium dependent-GPx activity in *P. perezi* tadpoles after 90 days exposure to FETAX and M effluent at the pH ranges of [8.0-7.5], [7.0-6.5], [6.0-5.5] and [5.0-4.5]. d) Mean total-GPx activity in *P. perezi* tadpoles after 90 days exposure to FETAX and M effluent at the pH ranges of [8.0-7.5], [7.0-6.5], [6.0-5.5] and [5.0-4.5]. e) Mean GSTs activity in *P. perezi* tadpoles after 90 days exposure to FETAX and M effluent at the pH ranges of [8.0-7.5], [7.0-6.5], [6.0-5.5] and [5.0-4.5]. Error bars represent standard deviation.

Statistical analysis was performed within pH ranges of FETAX and M effluent separately, and between FETAX and M effluent for each pH range.

a, b, c and d: represent statistically significant different groups ($p < 0.05$).

*: represents statistical significant differences ($p < 0.05$) between FETAX and M effluent of a pH range.

When comparison between effluents was made for specific pH ranges, in a general way the activity of total-GPx was inhibited in tadpoles exposed to the M effluent, this inhibition was significant for all the pH ranges, except for [6.0-7.5]. Finally for the GSTs activity (Fig. 3e) there were significant differences only among the pH ranges of FETAX ($F = 6.376$, d.f. = 3, $p = 0.005$), Despite the tendency for reduction with decreasing pH, the lowest activity was recorded at [6.0-5.5]. When differences among tadpoles exposed to different media were analyzed, significant differences were recorded only for intermediate ranges of pH namely [7.0-6.5] and [6.0-5.5] ($F = 8.005$, d.f.= 1, $p = 0.022$ and $F = 5.670$, d.f. = 1, $p = 0.044$), however no clear pattern of variation was found.

Table 3 – Potential up-regulated genes from *P. perezi* tadpoles after 90 days exposure to M effluent at a [5.0-4.5] pH range.

Clone ID	Size (bp)	Accession number	% Identity	% Coverage	E-Value	Putative identity	Matching organim
2G	427	XP_002938991.1	89%	53%	6.00E-28	apolipoprotein A-I-binding protein-like	Xenopus (Silurana) tropicalis
5G	537	CAC69536.1	70%	68%	4.00E-59	thrombin	Crocodylus niloticus
10 G	427	XP_002938991.1	89%	53%	7.00E-35	apolipoprotein A-I-binding protein-like	Xenopus (Silurana) tropicalis
17 G	585	NP_001080162.1	94%	53%	4.00E-41	stomatin	Xenopus laevis
19G	753	NP_001080162.1	95%	46%	2.00E-44	stomatin	Xenopus laevis
20 G	419	XP_003225465.1	100%	41%	2.00E-32	40S ribosomal protein S3-like	Anolis carolinensis
23 G	651	NP_001089607.1	97%	56%	5.00E-57	ribosomal protein L35	Xenopus laevis
30 G	670	NP_001016193.1	92%	64%	2.00E-89	voltage-dependent anion channel 2	Xenopus (Silurana) tropicalis
33 G	554	NP_988916.1	62%	54%	3.00E-23	ribosomal protein L35	Xenopus (Silurana) tropicalis
40 G	465	NP_001158699.1	96%	38%	1.00E-33	peptidyl-prolyl cis-trans isomerase FKBP2	Oncorhynchus mykiss
42 G	749	XP_002944675.1	88%	51%	6.00E-74	lysozyme C-like, partial	Xenopus (Silurana) tropicalis
64 G	525	NP_001079770.1	83%	32%	4.00E-28	trypsin precursor	Xenopus laevis

SSH was performed to detect differentially expressed genes in *P. perezi* tadpoles after 90 days exposure to M effluent at a pH of [5.0-4.5]. Sixty six clones were recovered from the subtracted library. After PCR screening, 40 presented inserts that could represent differential expressed transcripts and were then sequenced (Fig. 4). The molecular weight of these potential transcripts ranged from 415 to 753 base pairs (bp): 14 presented a molecular weight between 400 and 500 bp, 13 between 500 and 600 bp, 11 between 600 and 700 bp and 2 between 700 and 800 bp. Homology search for the corresponding sequences was performed by BLASTX with NCBI non-redundant protein database. The sequences were considered homologous when the matching sequence length was at least 25% simultaneously with an e-value lower than e^{-10}. Twelve sequences fulfilled these criteria, enabling functional identification (Table 3). Furthermore, best matching scores for these transcripts were found mainly with proteins from

other amphibian species, reinforcing their correct identification. The remaining 28 sequences did not show a significant similarity with the sequences in the database. The putative genes obtained were related to apolipoprotein (2 sequences), ribosomal proteins (40S, L35) (3 sequences), thrombin (1 sequence), stomatin (2 sequences), voltage-dependent anion channel 2 (1 sequence), peptidyl-prolyl cis-trans Isomerase (1 sequence), lysozyme C-like (1 sequence) and trypsin (1 sequence).

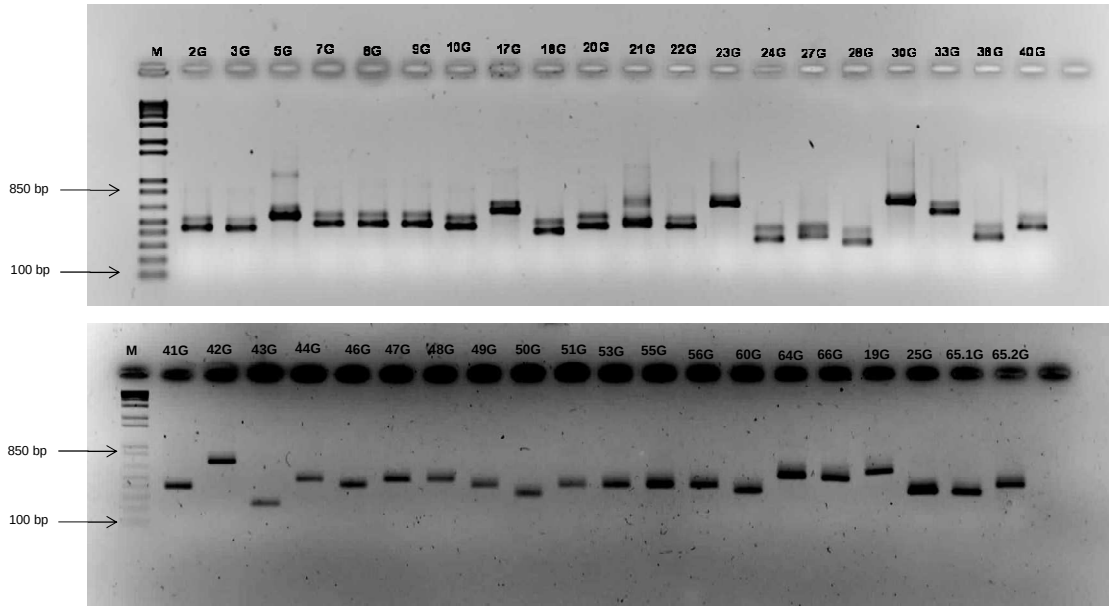

Fig.4. PCR fragments obtained with pCR II vector primers T7 and SP6, representing the potential differential expressed transcripts M - 1Kb plus DNA Ladder (Invitrogen, USA).

5.4. Discussion

Previous laboratorial and also *in situ* studies carried out with *P. perezi* (Marques et al., 2011; Marques et al., 2009; Marques et al., 2008); Marques et al., *in prep* a; Marques et al., *in prep* b) have provided a fairly good insight on toxicological effects on amphibians, resulting from the exposure to acid mine effluents. The toxicological effects have been assessed in various organizational levels such as individual (Marques et al., 2008), tissues (Marques et al., 2009), enzymes activity (Marques et al., 2011) and DNA molecules (Marques et al., 2009). Nonetheless the chronic effects of the M effluent on *P. perezi* larvae have never been assessed

as well as the influence of pH in its toxicity. Previous acute *in situ* exposure of *P. perezi* tadpoles to the M effluent with a pH of 3.77 resulted in a mortality of almost 50%, after 96h, however it was suggested that a longer exposure could lead to 100 % mortality (Marques et al., *in prep* b). In fact that occurred in the present study, under laboratorial conditions after 6 days (144h) of exposure to the M effluent at a [4.0-3.5] pH. However that was also verified for FETAX at the same pH. Similar results were also reported for the pH range of [5.0-4.5] where mortality reached 50% in both FETAX and M effluent medium. These related results obtained for the two lower tested pHs, in FETAX and M effluent, indicate that toxicity owed to pH supplants toxicity from the M effluent metal content, at least until a pH of 5.0. Other studies where amphibians were submitted to pH (pH ≈ 3.5 – 6.5) variation revealed that at low pH amphibian mortality frequently occurs (Cummins, 1986; Meyer et al., 2010). This fact is mainly owed to the body net loss of sodium due to a disruption of the Na^+/H^+ exchange mechanism (Freda and Dunson, 1984) which leads to an inhibition of sodium influx and an increase in efflux (Meyer et al., 2010). Also in the [4.0-3.5] pH many eggs did not hatch in either FETAX or M effluent, which as Freda (1986) reviewed is common in extremely low pH. It is suggested that mortality within the eggs results from vitelline membrane dysfunction due to the inactivation of the enzyme responsible for hatching (Dunson and Connell, 1982). This enzyme catalyzes changes in the vitelline membrane that allow it to respond to the osmotic influx of water (Carroll and Hedrick, 1974). For the highest ranges of pH tested ([8.0-7.5], [7.0-6.5], [6.0-5.5]) the mortality in the M effluent decreased with the increase of the pH while simultaneously in the FETAX medium it remained at 2.5%, for the different pH ranges. These results suggest that a possible interaction between toxic metals and slightly acidic pHs may result in an increased acute toxicity, when concomitant exposures occur. Horne and Dunson (1995b) also observed an interaction among pH and metal toxicity with metals presenting higher toxicity at lower pHs (pH = 4.5). At this point it is important to mention that accumulation of metals in M effluent exposed tadpoles has shown a tendency to decrease with pH decrease (Be, Al, Mn, Zn, Sr, Cd, Ba) for the same pH ranges ([8.0-7.5], [7.0-6.5], [6.0-5.5]). The elements K, Mg and U were the exception with a slight increase with

decreasing pHs. The general decrease of bioaccumulation with a decrease of pH has probably resulted from the competition between H^+ ions and metal cations for binding sites, reducing thus metal interaction with cells (Blackwell et al., 1995; Tobin et al., 1984). This may have occurred despite the great bioavailability of metals expected for the M effluent, at acidic pHs. Relatively to body length of tadpoles, for both FETAX and M effluent medium the increase in this parameter with the decrease of pH was intriguing. Indeed studies reporting similar results are very scarce (e.g. Fioramonti et al., 1997) in contrast there are more studies reporting the inhibition of growth due to exposures to low pH conditions (Barth and Wilson, 2010; Horne and Dunson, 1995b; Jung and Jagoe, 1995). Nonetheless the species that Fioramonti et al. (1997) used in their study, for which a higher growth rate was observed at lower pH, was *R. lessonae*, a frog that is closely related with *P. perezi* forming even hybrids (Pagano et al., 2001). Hence, it might be possible the same feature with *P. perezi*, since there seems to be a common genetic background. An intriguing result obtained in our study is the fact that for the higher pHs, when comparing tadpoles exposed to FETAX and to the M effluent, is that the body length attained was significantly higher in the M effluent. This might result from a stimulatory effect from the metals present in the M effluent leading to a higher body size. Indeed even without significant differences in body length, after 90 days the increment in growth throughout the exposure was significantly higher for tadpoles exposed to the M effluent at lower pHs, in comparison with those from the FETAX medium, leading into believing that this effect is also present in lower pHs. A previous work, in which *P. perezi* tadpoles were exposed to a series of dilutions of the M effluent also revealed a stimulatory effect but in this case for the dilutions of 12.5 and 25 % (Marques et al., 2008). However in the same study other assay reported that tadpoles recently hatched in the effluent (100 %) at a pH of 8, presented higher body length than all the other exposure conditions (FETAX control included).

Since metals are known for originating ROS (Stohs and Bagchi, 1995) we determined the activity of various enzymes known to be involved in cellular antioxidant defense to test their performance. However the biomarkers analyzed did not allow perceiving a clear response pattern associated to pH or metal

toxicity. In fact the only coherent response was verified for the GPx, both selenium dependent and total. These enzymes were, significantly inhibited in tadpoles exposed to the M effluent, in practically all the pH ranges, which may be related to high metal content or to low pH as verified by other studies (Chatziargyriou and Dailianis, 2010; Cossu et al., 2000; Grose et al., 1987; Reddy et al., 1981; Ying et al., 1999). In fact, inhibition absence in selenium dependent-GPx activity, has only occurred for the M effluent at pH [8.0-7.5]. Previously obtained results by Marques et al. (*in prep*. b) where an increase in GPx acivity was observed in tadpoles exposed to the treated effluent could lead us to expect also an increase of GPx activity at the end of our assay. However it is possible that after a long exposure to high metal content enzymatic inhibition occurs (e.g. (Congiu et al., 2000; Sidhu et al., 1993). Relatively to the activity of CAT in tadpoles exposed to the mine effluent it is possible to admit that there was a tendency for a decreasing activity with decreasing pH. However the absence of significant differences between tadpoles exposed to FETAX and to the M effluent, with exception for exposures occurring at [7.0-6.5], does not allowed us to suggest further conclusions. Overall in metal complex mixtures there are many factors confounding the results, for instance some metals (e.g. Zn), may have ameliorative effects (Horne and Dunson, 1995a) or interact with others and display higher toxicity (Lefcort et al., 1998).

The differential gene expression analysis performed in our study revealed the possible up-regulation of genes coding for 9 different proteins: apolipoprotein A-I, thrombin, stomatin, ribosomal proteins (S3, L35), voltage-dependent anion channel 2, peptidyl-prolyl cis-trans isomerase FKBP2 , lysozyme C-like and trypsin precursor.

From these possible differentially expressed genes some have caught our attention, specially stomatin, peptidyl-prolyl cis/trans isomerase FKBP2 (FKBP) and voltage dependent anion channel (VDAC) since all are in some way related with regulation of homeostasis of cell by being ionic channels or by helping controlling channel gating (Göthel and Marahiel, 1999; Zaid et al., 2005; Zhang et al., 2001). Due to the high metal content this would be expected since osmotic balance may become affected. However the function of these proteins, for which the up regulated genes are coding for, is different. For example stomatin acts on

acid-sensing ion channels by inhibiting them (Price et al., 2004). A study has even reported that the absence of stomatin in red blood cells was associated with massive leak of Na^+ (Mannsfeldt et al., 1999). On the other hand VDAC is a major mitochondrial outer membrane transporter with an important role in energy production and Ca^{2+} homeostasis (Shoshan-Barmatz and Gincel, 2003; Shoshan-Barmatz and Israelson, 2005). As for FKBP it can act as part of receptor complexes such as Ca^{2+} release channels as reviewed by (Schiene-Fischer and Yu, 2001). Despite their different role proteins may play a conjunct action. For example VDAC can provide higher energy to compensate energy spent in osmoregulation, while stomatin may be inhibiting acid-sensing ion channels to impede the loss of the essential Na^+ and FKBP may be modulating Ca^{2+} release according to cell and mitochondria needs, since Ca^{2+} is vital for energy production (Brookes et al., 2004) and signaling in the cell. Relatively to the ribosomal proteins they are usually involved in the translation process, however there are some extra ribosomal activities reported (Wool, 1996). Specifically for ribosome protein S3 it has been hypothesized to have a role in DNA repair caused by ROS (Choi et al., 2006; Yacoub et al., 1996). Being this the case, it is not unexpected since many of the accumulated metals are known to produce ROS (e.g. Fe) (Stohs and Bagchi, 1995) and in our study the activity of some enzymes involved in antioxidant responses (namely total and selenium dependent GPx) have proved to be inhibited in tadpoles exposed to the M effluent at this lower pH range. Another protein which has been hypothesized to be affected by ROS is trypsin (Tian et al., 2004), which is an extremely important digestive enzyme. Once again taking into consideration the accumulated metals (much higher for Al, Fe and U) it would be expected to have high levels of ROS which can affect various proteins (Stadtman and Oliver, 1991) such as trypsin. Furthermore metals, depending on their concentration have also been shown to inactivate trypsin (Griffin and Steven, 1982; Steven et al., 1979). Being so, the possible up regulation of its coding gene could have resulted from the depletion of trypsin necessary to normal digestive process. However another hypothesis exists and is related to the need of higher levels of trypsin, not due to its depletion but by the necessity of a more efficient digestion due to higher energetic requirements to maintain osmoregulation (Pérez-

Pinzón and Lutz, 1991). Relatively to apolipoprotein A-I it is a major constituent of high-density lipoprotein (HDL) particle and has a unique ability: to capture and solubilise free cholesterol. This ability enables HDL to remove excess peripheral cholesterol. As a curios note recently, Vidaud et al. (Vidaud et al., 2005) showed that, apolipoprotein A-I as well as other serum proteins, is able to bind U, which was one of the metals most highly accumulated in the tadpoles exposed to the mine effluent.

For the last two possible up-regulated genes, these concern coagulation process and possibly immunological process (thrombin and lysozyme respectively). Nonetheless, to the authors' knowledge there are no remarkable features, besides their main attributed function, that could provide a more linear relationship with metal induced stress, and justify their up-regulation.

Overall this study has shown that acidity effects have surpassed those of toxic metals at low pHs ([5.0-4.5], [4.0-3.5]) in tadpoles exposed to these metal rich effluent. Nonetheless as pH increases the joint toxicity of metal and pH becomes visible, for instance at pH [6.0-5.5] FETAX exposed tadpoles had a 2.5% average mortality while for the same pH range in M effluent mortality was of 32.5%. The similar effect of decreasing mortality with increasing pHs was further observed for the other M effluent higher pH ranges ([7.0-6.5], [8.0-7.5]). Also a stimulatory effect on growth was visible for M effluent exposed tadpoles, in comparison to FETAX tadpoles. As for the biomarkers evaluated in this assay, they did not allow to draw any major conclusions, nonetheless, as in previous studies GPx (Marques et al., 2011); Marques et al., *in prep* b) was the enzyme that had the most consistent response. Finally the SSH assay provided some clues on the mechanisms that allow the enduring of tadpoles in the effluent. These mechanisms seem mainly related with osmotic regulation (stomatin, FKBP) and energy production/acquisition (VDAC and possibly trypsin).

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Chapter VI

Concluding Remarks

6. Concluding Remarks

This final chapter aims at providing a holistic overview of the previous four chapters (II-V), integrating the results accordingly to the main objective of this thesis. All of the work was carried out using the effluent (M) produced in the deactivated uranium mine of Cunha Baixa as a source of metal contamination. The central aim of our work was to understand how *P. perezi* frogs, inhabiting the Cunha Baixa deactivated uranium mine, in particular the M pond area, were able to survive in such a contaminated environment. With this aim and considering that there are two major life stages (larval and adult) in these organisms, one totally dependent of water and another terrestrial, we had to adopt two different approaches, one for adult frogs and the other for tadpoles. Nonetheless, and considering that contaminants were metals, we assessed, in both cases, the activity of enzymes involved in oxidative stress responses (CAT, GRed, GSTs, GPx).

The approach for adult frogs consisted in capturing animals from the contaminated site (M) and from a reference site (VR), for comparison of biomarkers and also to perform differential gene expression analysis. Since adults may become exposed to metals through various ways, we determined antioxidant enzymatic activity and quantified LPO in different tissues (liver, kidney, heart and lung). For the differential gene expression analysis, liver was selected as the target organ due to its major role in synthesizing a wide variety of proteins involved in many functions, ranging from detoxification to digestion. As for tadpoles we choose controlled exposures, assessing various effluents as well as the effect of pH variation on the toxicity of the M effluent. The first exposure was carried out *in situ* during 96h, evaluating the ecotoxicological effect of three different effluents with different degrees of contamination, by assessing mortality, growth, biochemical parameters and metal bioaccumulation. The second exposure was carried in laboratory with a duration of 90 days, exposing *P. perezi* eggs to five different ranges of pH ([8.0-7.5], [7.0-6.5], [6.0-5.5], [5.0-4.5], [4.0-3.5]) to evaluate the influence of pH variation in the toxicity of the effluent. Besides mortality, growth and oxidative stress biomarkers, we have chosen tadpoles exposed to the effluent

at the lowest tested pH, [5.0-4.5], to perform a differential gene expression analysis. The chemical analysis of the M effluent revealed high content of various elements (e.g. Be, Al, Mn, Fe, U). Among the various chemical quantifications, made during the elaboration of this thesis, Be, Al, Mn, Co and U remained always above all benchmark values used for comparison, revealing that these are likely the most concerning elements of this hazardous effluent. Curiously, since the beginning of the study, in 2008, with the elaboration of the work in Chapter II, and through the following chronologically organized chapters (III, IV, V) we can notice a lowering of the M effluent chemical content of the elements Be, Al, Fe, Co, Ni, Zn, As and U. Furthermore, in 2006, when the first study with *P. perezi* in M effluent was made (Marques et al., 2008), the chemical values presented strengthen this evidence, with higher values for 8 of the 9 elements quantified then. Such fact is not a surprise, since the acid that was spread in this area, and which has contributed for the production of the acidic effluent, with the seasonal variances in the level of the aquifer, is likely washing the geological material and depleting it from the dominant and more available elements. The analysis of metal residues in tissues of adult frogs, in comparison with frogs from the VR site, revealed that M frogs have higher metal body burden. However, unlike the expected, few oxidative stress biomarkers have shown meaningful alterations. In fact, total-GPx was the biomarker that presented a more consistent pattern across the various tissues analysed. Generally, lung was the tissue displaying more alterations in the evaluated biomarkers. The correlation data between biomarker/metal revealed that many enzyme activities in the liver of M frogs were positively related with the accumulation of non-essential metals. After differential gene expression analysis, there was a suggestion that resistance to metal contamination could be resulting from a basal protection mechanism provided by blood plasma proteins such as albumin or fibrinogen (Marques et al., unpublished results) which can act both as antioxidants. Furthermore the higher presence of melanomacrophagic centres in the liver of mine inhabiting *P. perezi*, has also been pointed as an antioxidant protection (Marques et al., 2009).

Considering the studies carried out in this thesis with early life stages, (Chapter IV and V), we can state that the M effluent (in Chapter IV designated as

M2) is lethal for these stages. Furthermore, metal uptake is not proportional to the effluent metal content, but it is variable, depending on the pH and also from the analysed element. In addition, our data suggests that biological factors such as tadpole's integument damage, impeding metal uptake (Meyer et al., 2010), are possibly influencing metal accumulation. That was particularly remarkable in the *in situ* assay, were tadpoles exposed to M2 effluent had similar levels of accumulated metals when compared to REF tadpoles. It is important to mention that among the two sites there were great differences among metal content (higher in M2) and that pH levels would point for higher bioavailability in M2. Nonetheless, metal accumulation did occur in a time dependent way. Considering the bioaccumulation and the mortality data in both *in situ* acute assay and chronic laboratorial assay we can point that when pH levels are too low, acidity assumes the main toxicological role. From our data, we can presume that above a pH of [5.0-4.5] the toxicity of acidity is reduced on *P. perezi*, acting nonetheless as a stressor. In both chronic (first exposure days) and *in situ* assays, tadpoles exposed to M effluent presented lower body length when compared to respective controls. However, the results of the chronic assay indicate that tadpoles inverted that tendency during the exposure, which resulted in a higher growth. Apparently, through their development, they must activate a mechanism that allows the survival in both low pHs and metal contamination. The selected oxidative stress biomarkers used in both assays revealed that mainly GPx has a coherent behaviour within exposures, revealing opposite responses between assays. Nonetheless, it is expectable that during long term metal contamination exposures the depletion of antioxidant enzymes occurs (Chatziargyriou and Dailianis, 2010; Reddy et al., 1981). The differential gene expression analysis in tadpoles points to the possibility of increased osmoregulation as well as an increase investment in energy acquisition mechanisms, being this fact most probably to compensate higher energy loss in osmoregulation. These mechanisms provide a new insight on how *P. perezi* tadpoles may withstand metal contamination and apparently even present better fitness, considering body growth. Nonetheless, we must remember that even with these probable "positive" features, half of the exposed tadpoles at a [5.0-4.5] pH died.

Summing up, the work carried out in the present thesis allowed to perceive that both adult frogs and tadpoles are able to withstand highly contaminated areas such as the one in Cunha Baixa's deactivated uranium mine, possibly trough activating basal protection mechanisms. Since exposure to contamination differs among tadpoles and adult frogs, the resulting protection mechanism will possible differ. Apparently, in tadpoles it will consist of higher osmoregulation investment and energy acquisition, and in adults it is based in the increase of basal antioxidant defences. Despite this fact, within the pH range of [4.0-3.5] mortality was total, affecting inclusively egg hatching. At higher effluent pHs, metal accumulation occurred as well as mortality, which tended to decrease with the increase of pH. Furthermore, oxidative stress biomarkers do not seem to respond accordingly to metal contamination, with exception of GPx. Nonetheless, its response was dependent on exposure conditions (acute *vs* chronic).

6.1. Future perspectives

Taking into consideration the results obtained and discussed in the present thesis *P. perezi* has proved to be a species able to endure metal contaminated sites apparently at the cost of some altered basal mechanisms. In addition, the selected oxidative stress biomarkers proved to be non-responsive in this species, or at least unable to provide a coherent response in accordance to metal exposure or bioaccumulated metal. In the future it would be interesting to confirm the gene expression results and further increase the present knowledge of mechanisms impaired through metal contamination. This improvement could eventually lead to the development of new biomarkers with higher response power. Thus, it would be important to continue this line of research in the future to allow the design of species and contaminant specific biomarkers. Also it would be important to clarify the mechanisms behind the higher growth of *P. perezi* at low pHs. This could be accomplished with an approach similar to the one undertaken in Chapter V applying also the SSH technique to the pH extremes exposed tadpoles.

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