Universidade de Aveiro Departamento de Biologia
Ano 2019

DAVID MANUEL BRANCO BROTAS

Toxicity of silver, lead an nanoplastics to early life stages of amphibians.

Toxicidade de prata, chumbo e nanoplásticos em diferentes estádios de desenvolvimento de anfíbios.

DECLARAÇÃO

Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

Universidade de Aveiro Ano 2019 Departamento de Biologia

DAVID MANUEL BRANCO BROTAS

Toxicidade de prata, chumbo e nanoplásticos em diferentes estádios de desenvolvimento de anfíbios.

Toxicity of silver, lead an nanoplastics to early life stages of amphibians.

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica da Doutora Isabel Maria Cunha Antunes Lopes e do Doutor Marcelino Miguel Guedes de Jesus Oliveira, ambos Investigadores Auxiliares do CESAM – Centro de Estudos do Ambiente e Mar, do Departamento de Biologia da Universidade de Aveiro.

o júri

Presidente	Professora Doutora Maria Helena Abreu Silva Professora Auxiliar, Universidade de Aveiro
Arguente	Doutora Cátia Alexandra Ribeiro Venâncio Investigadora Contratada, Universidade de Coimbra
Orientadora	Doutora Isabel Maria Cunha Antunes Lopes Equiparada a Investigadora Auxiliar, Universidade de Aveiro

Agradecimentosw

Agradeço à Doutora Isabel Lopes por ter aceite o desafio de me orientar neste trabalho. Pelo incansável acompanhamento em todas as etapas deste trabalho e por toda a confiança, motivação, simpatia e disponibilidade que sempre demonstrou.

Agradeço ao Doutor Marcelino Miguel Oliveira por me ter recebido com toda a simpatia, honestidade e disponibilidade. E por me guiar e ensinar os bons modos de como trabalhar em laboratório.

Agradeço à Sara, ao Bruno, Cátia e ao Emanuele que me receberam e me ensinaram como trabalhar no grupo dos anfíbios. Por toda a ajuda e bons momentos no laboratório.

À minha família e amigos, por todo vosso apoio e presença tanto nas vitórias como nos tempos mais difíceis. Independentemente da distância estão sempre ao meu lado e disponíveis

E não podia deixar de agradecer também aos vários organismos que participaram (ainda que involuntariamente) nos meus ensaios, sem eles é que o trabalho não podia mesmo ter sido realizado.

palavras-chave

Metais, efeitos subletais, nanoplásticos de poliestireno, *Xenopus laevis,* linhas celulares de rim.

Resumo

Nos últimos 40 anos as populações de anfíbios têm sofrido um declínio a nível mundial, tendo sido identificadas espécies de anfíbios a desaparecer por completo dos locais onde naturalmente ocorrem. Este declínio deve-se principalmente a atividades antropogénicas e aos efeitos negativos que elas podem provocar neste grupo de vertebrados. Os anfíbios são considerados organismos muito sensíveis a alterações ambientais, principalmente porque possuem uma pele altamente permeável. Até recentemente, a avaliação de riscos de produtos químicos para anfíbios era baseada em dados ecotoxicológicos gerados para peixes (para estádios de vida aquáticos) e para aves e mamíferos (para estádios de vida terrestres). Contudo, o uso destes dados pode levar a que seja subestimado o risco de certos produtos para os anfíbios. Deste modo, torna-se fundamental obter dados de toxicidade especificamente gerados para diferentes estádios de desenvolvimento de anfíbios, com o intuito de promover a sua proteção e conservação, de forma precisa. Este estudo teve como objetivo principal avaliar a toxicidade de dois metais e um tipo de nanoplástico para os estádios de vida iniciais aquáticos de uma espécie de anfíbio, *Xenopus laevis*. Para além disso, pretendeu-se avaliar a adequabilidade de ensaios *in vitro*, com linhas celulares de anfíbios, como substitutos de ensaios *in vivo* para avaliar a toxicidade de produtos químicos e nanopartículas para este grupo de organismos. Embriões e girinos de *X. laevis* foram expostos a uma gama de concentrações de prata, chumbo e nanoplásticos de poliestireno, tendo sido avaliados os seguintes parâmetros: em embriões – mortalidade, malformações, taxa de eclosão, comprimento corporal; e em girinos – mortalidade, taxa de crescimento (ganho de peso e aumento do comprimento do corpo), estádio de desenvolvimento e batimento cardíaco. Concentrações de chumbo iguais ou superiores a 0.22 mg/L afetaram significativamente a taxa de eclosão e comprimento rostro-cloaca das larvas expostas desde a fase de embrião. No caso dos girinos, foram identificadas alterações significativas na taxa de ganho de peso e de crescimento corporal em concentrações de chumbo iguais ou superiores a 0.49 mg/L. Os ensaios *in vitro* demonstraram uma redução da viabilidade celular em concentrações de chumbo iguais ou superiores a 0,078 mg/L. Em relação à prata, concentrações tão baixas como 0.013 mg/L induziram o aparecimento de malformações, eclosão precoce e diminuição do comprimento rostro-cloaca em larvas. Esta mesma concentração causou um aumento da taxa de crescimento da cauda, e corporal. Os ensaios *in vitro* apresentaram maior sensibilidade a prata, tendo havido efeitos significativos na viabilidade celular a partir da concentração de 0.0004 mg/L. A exposição a nanopartículas de poliestireno não induziu efeitos significativos nem em embriões nem em girinos para concentrações entre 3,95 e 30 mg/l de poliestireno. Em suma, os embriões e girinos de *X. laevis* demostraram uma sensibilidade semelhante aos produtos químicos testados. Nos ensaios *in vitro* com células A6, os dados obtidos, no caso dos ensaios com chumbo, demonstram que as células são ligeiramente mais sensíveis a este contaminante que os girinos e os embriões. Nos dados obtidos para a prata, houve efeitos significativos nas células, em concentrações mais baixas que as utilizadas nos ensaios com embriões e girinos. Estes dados sugerem que, pelo menos para esta linha celular, a prata é muito mais tóxica do que o que seria expectável dos dados dos restantes ensaios. Os resultados obtidos neste estudo sugerem que em estádios iniciais de avaliação de risco, ensaios *in vitro* podem ser usados como uma ferramenta

sensível para a avaliação de toxicidade, tendo o valor acrescentado de permitir evitar a experimentação em animais.

Keywords Metals, Sublethal effects, Polystyrene nanoplastics, *Xenopus laevis,* kidney cell line.

Abstract

Over the last 40 years amphibian populations are reported to be increasingly declining each year with amphibian species disappearing completely from their natural place of occurrence. This decline is mostly due to anthropogenic disturbances and the negative effects they pose to this group of vertebrates. Amphibians are considered very sensitive to environmental changes, namely because they are characterized by a highly permeable skin. Until recently, the risk assessment of chemicals for amphibians was based on the data generated for fish (for aquatic life stages) and for birds and mammals (for terrestrial life stages). However, it has been reported that the use of data from these three groups of vertebrates may underestimate the risk of some chemicals to amphibians. It is, therefore needed to generate toxicity data specifically for amphibians in order to promote its accurate protection and conservation. The present study aimed at assessing the toxicity of two metals and a nanoplastic to aquatic early life stages of an amphibian species. It also intended to assess the adequacy of using *in vitro* assays, with amphibian cell lines, as surrogates of the *in vivo* assays to assess the toxicity of the selected chemicals to this group of organisms. To attain these objectives the anuran *Xenopus laevis* was used as test model species. Embryos and tadpoles of *X. laevis* were exposed to a set of concentrations of silver, lead or nanoplastics of polystyrene and the following endpoints were monitored: for embryos – mortality, malformations, hatching rate and body length; and for tadpoles – mortality, growth rate (as weight gain and body length increase), developmental stage, and heart beat rate. Lead concentrations equal or above 0.22 mg/L induced significant adverse effects in the hatching rate and snout-to-vent length of larvae exposed to lead since the embryonic stage. Tadpoles exposed to concentrations of lead equal or above 0.49 mg/L showed significant changes in the weight gain and total body growth rate. The *in vitro* assays with lead revealed a significant reduction of cells viability at concentrations equal or higher than 0.078 mg/L. Regarding silver, concentrations as low as 0.013mg/L induced the appearance of malformations, an early hatching and increased snout-to-vent length of larvae. This same concentration induced an increase in the tail and total body growth rates. The *in vitro* assay revealed to be very sensitive to silver, concentrations as low as 0.0004 mg/L of silver affected negatively the viability of cells. Nanoparticles of polystyrene induced no significant effects on both embryos and tadpoles. Overall, the embryos and tadpoles of *X. laevis* exhibited a similar sensitivity to the tested chemicals. The *in vitro* assays performed with lead, revealed that A6 cell lines are slightly more sensitive to lead than embryos or tadpoles of *X. laevis*. In the case of silver, toxic effects in the cell lines were observed at concentrations much lower than those inducing effects in embryos and tadpoles. The obtained results suggest that for early stages of risk assessment frameworks, *in vitro*

assays may be used for a first toxicity screening in order to avoid running animal experimentation.

Index

List of Figures

Chapter I

Introduction

1. Introduction

1.1 Amphibian populations and their ecological importance

Amphibians have a central role in the freshwater ecosystems, being both prey and predators at the center of the food web (Whiles et al., 2006). They are relevant species and bioindicators of both aquatic and terrestrial niches, since a high percentage of the species from this Class of vertebrates exhibit a life cycle that presents aquatic larvae and terrestrial adults (Benvindo-Souza et al., 2020; Gonçalves et al., 2015). Because of this last characteristic, these organisms may be affected by various anthropogenic factors, like pollutants, that are present in the aquatic and terrestrial environment. The high sensitivity of amphibians to pollutants has been associated with their highly permeable skin, as well as their varied lives, which maximize their exposure: they dwell on land and water, and eat both plants and animals at various stages of their life cycle (Burlibaşa & Gavrilacaron;, 2011).

Amphibians are important model animal species in biology used in studies on early embryonic development and cell biology. Amphibians have played a key role in the elucidation of the mechanisms of early development over the last century. Amphibian embryos remained the choice for experimental embryologists for many decades (Burlibaşa & Gavrilacaron, 2011) and much of our knowledge about the mechanisms of vertebrate early development comes from studies using *X. laevis*.

1.2 Worldwide decline of amphibian populations

The amphibian populations have suffered significant declines, in the past 30 years, in many areas of the planet. Distinct causes are thought to be responsible for such declines and include habitat destruction (e.g. associated with agricultural expansion; (Gonçalves et al., 2015)) fragmentation, and alteration; introduction of exotic species; resource over-exploitation; increased UV-B radiation; chemical contaminants and emerging of infectious diseases (Stuart et al., 2003).

The worldwide decline in amphibian populations suggests that these organisms are particularly sensitive to water quality, implying that amphibians may be useful as a species indicating water degradation (Haywood et al., 2004).

1.3 Metal and nanoplastics contamination

Environmental pollution is a global problem associated with contamination of soils, water and air, environmental compartments with which all organisms interact. The adverse consequences on the ecosystems have been widely reported, as a factor influencing habitats' degradation and species' decline. For example, the worldwide decline in amphibian populations in recent decades (Berzins & Bundy, 2002).

The presence of high concentrations of metals in the environment is commonly associated with anthropogenic activities. The levels of these contaminants in aquatic ecosystems are a problem in different areas of the globe with potential to affect environmental and human health. Present in all environments at low baseline concentrations, these substances may be very dangerous reaching high concentrations (above the normal tolerated physiological levels) due to their non-biodegradable and bioaccumulation characteristics (Shafiuddin Ahmed et al., 2019; Srivastava, 2008). Metals may enter aquatic ecosystems, as a result of industrial activities, mining, textile processes, pesticides application, among others (Yologlu & Ozmen, 2015). Although in small amounts certain metals are essential for life, in large quantities they can be extremely toxic due to their accumulation in the body of organisms (as a result of ingestion or directly absorption). High levels of metals may inhibit enzymatic activities, physiological processes, and cause histological damage, and ultimately affect the survival of individuals (Haywood et al., 2004). Metals can also cause oxidative stress when reactive oxygen species (ROS) are formed, including superoxide, peroxide and hydroxide radicals. These radicals are highly reactive and toxic to cellular macromolecules and can cause oxidative damage to proteins, lipids and even nucleic acids (Yologlu & Ozmen, 2015).

Metal contamination can interact with other anthropogenic impacting factor: water acidification; which may affect the speciation of metals, increasing their bioavailability and potentially their toxicity. Biologically available metals are generally present in the environment as free metal ions or as colloids with a diameter of less than 45 μm and therefore readily available for uptake by living organisms (Haywood et al., 2004). Given that all freshwater life forms need to actively absorb ions in order to maintain ion homeostasis in the osmoregulatory process, they are particularly susceptible to certain ionic forms of metals (Schnizler et al., 2007).

The presence of plastic particles in the environment is an emerging concern. As a result of improper management of plastic residues have been found in all kind habitats: oceans, soils, sediments and surface waters. Plastics may be degraded into increasingly smaller particles, by mechanical abrasion, photo-oxidation and biological degradation and generate micro (sizes between 5 mm and 100 nm) and nanoplastic (sizes below 100 nm) (Oliveira, et al., 2019). The concern associated with the presence of plastics in the environment are related to direct toxicity to biota, toxicity caused by the additives used in the production of plastics and their role as vectors for other environmental contaminants and invasive organisms/pathogens (Ferreira et al., 2019). These particles are likely to be an additional stressor to the amphibian community and thus, the knowledge of their potential effects becomes highly relevant.

2. Objectives

The present study aimed at assessing the toxicity of two metals (lead and silver) and one type of nanoparticles (50 nm polystyrene) on early aquatic life stages of *Xenopus laevis*. This study also aimed to compare the sensitivity of *in vitro* assays (with amphibian cell lines) and *in vivo* assays (with embryos and tadpoles), in order to identify the adequacy of the early methodology as a surrogate for animal experimentation regarding amphibian's risk assessment.

The metals used in this study, silver and lead, were selected based on their widespread presence in several aquatic environments, resulting from industrial processes and diverse applications. Furthermore, since plastic has become an emerging problem for all aquatic species, the assessment of polystyrene nanoparticles toxicity was also included into this study.

Chapter II

Evaluation of the toxicity of lead to aquatic life stages and to a kidney cell line of *X. laevis*

2. Evaluation of the toxicity of lead to aquatic life stages and to a kidney cell line of *X. laevis*

2.1 Introduction

According to the International Union for Conservation of Nature (IUCN), among the groups of endangered animals', amphibians are the most imperiled, with 41% of the known species facing the threat of extinction (Monastersky, 2014). Amphibian decline can be caused by a panoply of factors and by their interactions: UV radiation, habitat destruction, global warming, human exploitations, pollution, among others (Hayes, T.B. et al. 2010; Blaustein, et al., 2011). Concerning pollution, amphibian hold specific characteristics that confers them a high sensitivity to this type of environmental perturbations (Slaby et al., 2019). Namely, these organisms present external fertilization and embryonic development, the eggs lack a shell while embryos are not involved by the amniotic membrane, and most species exhibit a life cycle that requires a metamorphosis phase (some associated with the changing of aquatic life stages to terrestrial ones) (Slaby et al., 2019; Sparling, et al., 2010). Furthermore, they exhibit a naked skin that is highly permeable, and is involved in physiological homeostasis (namely in gas, water and electrolyte exchange with the surrounding environment; Helmer et al., 2005; Quaranta et al., 2009). The reproduction through amniotic eggs and the highly permeable skin turns amphibians especially dependent on aquatic environments. Considering that the aquatic ecosystems have been pointed as major receptors of chemical contamination [through direct, release of domestic/industrial effluents) or indirect inputs (runoff or leachates from surrounding contaminated soils)], amphibian populations worldwide are and will continue to be exposed to a large diversity of chemicals (Wagner & Lötters, 2013). This requires generating knowledge on the ecotoxicity of chemicals to aquatic life stages of amphibians, in order to adequately protect them. Among the many chemicals that enter the aquatic ecosystems, metals are of special concern since most of them are not easily metabolized into less toxic forms, are persistent in the environment and some have been shown to be highly toxic to the biota (Venne et al., 2006). Lead is a very stable and persistent metal (with a half-time of over 20 years) that has been historically used in a large variety of industrial activities, including the production of batteries, gasoline, inks, pigments and many other products (Rowe et al., 2001; Berzins &

Bundy, 2002). As a result of such industrial applications, its release into the environment greatly increased over the past decades and, at present, is considered a worldwide environmental problem with air, soil, sediments and water being affected by lead contamination (Stansley, et al., 1997; Sparling, et al, 2006). Adding to the industrial applications, activities associated with hunting and coal combustions have also been pointed as important sources of environmental lead contamination, mainly due to the bullets discharge into the soil and release of lead-rich ashes into the atmosphere (Stansley et al., 1997; Rowe et al., 2001). In surface water bodies, levels of lead ranging from 0.18 to 179 µg/L have been measured across the world (Mutia et al., 2012).

Several studies have been published on the ecotoxicity of lead to freshwater biota, including microalgae (*e.g.*, Saçan et al., 2007), cladocerans (*e.g.*, Cooper et al., 2009), gastropoda (*e.g.*, Grosell et al., 2006), mussels (*e.g.*, Keller and Zam, 1991), fish (e.g., Paul et al., 2014), amphibians (e.g., Herkovits and Pérez-Coll, 1991; Berzins & Bundy, 2002), among others. Specifically, for amphibians, 1 mg/L of lead was shown to impair the survival of larvae of *X. laevis* and induce genotoxicity in the erythrocytes of those animals (Mouchet et al., 2006). Yeung (1978) showed that exposure of pre-metamorphic stages of *Rana utricularia* to this same concentration of lead (1mg/L) delayed the completion of metamorphosis and induced histopathologies in the thyroid, which presented follicles poorly differentiated and without the presence of colloidal material. Sparling et al. (2006), studied the effects of lead-contaminated sediments in tadpoles of *Rana sphenocephala* and reported that, within 5 days, all tadpoles exposed to lead concentrations equal or higher than 3940 mg/Kg (equivalent to 13,579mg/L in pore water) were death. Most of the available studies on the effects of lead to amphibians are focused on larvae and adults, only a few focusing the effects on embryos (e.g. Berzins & Bundy, 2002). Furthermore, none aimed at comparing the sensitivity of embryos and larvae amphibians to this metal. However, within a perspective of risk assessment of chemicals, the comparative sensitivity of these life stages is important, aiming to determine if embryos may be used to predict risks for larval stages, and thus contribute to decrease the use of animals during the process of risk assessment. Embryos and larvae experience distinct exposure routes and exhibit different biochemical, physiological and morphological parameters that may determine differential sensitivities (Venturino et al., 2003).

According to the above, the present work intended to assess the sublethal toxicity of lead to aquatic life stages of the amphibian model species *X. laevis*. Furthermore, following the rational for reducing animal experimentation in risk assessment frameworks, is was also aimed to determine if *in vitro* assays, using a *X. laevis* kidney cell lines, could be used as a non-animal alternative to predict the risks posed by lead to aquatic life stages of amphibians.

2.2 Materials and Methods

2.2.1 Test chemical

The salt lead nitrate [$Pb(NO_3)_2$] was supplied by the company Sigma-Aldrich (Saint Louis City, USA). A stock solution of 1.66 mg/L of Pb was prepared in FETAX artificial medium (medium used to culture the organisms and perform the toxicity assays; Dawson and Bantle, 1987). All tested concentrations of lead were obtained by diluting the stock solution in FETAX medium.

2.2.2 Test species

In vivo assays were performed with embryos and tadpoles of the anuran species *X. laevis* (Daudin, 1802). This is a model species widely used for amphibian risk assessment of chemicals, as it is easy to: (i) maintain reproductive adults in the laboratory, (ii) obtain large quantities of embryos by inducing reproduction and fertilization through the injection of adults with the human hormone gonadotropin chorionic, (iii) both embryos and tadpoles are recognized to be sensitive to a large diversity of chemicals (Berzins & Bundy, 2002; Cong et al., 2006; Yologlu & Ozmen, 2015).

The test organisms were obtained from adults of *X. laevis* that were maintained in glass aquariums filled with dechlorinated tap water at 23 ºC and photoperiod 14:10 h light:dark, at the Department of Biology of the University of Aveiro. The animals were fed with pellets XE 40, which were developed specifically for this species, and larvae and adults of the mealworm *Tenebrio molitor*.

To induce reproduction of *X. laevis*, sexually mature males and females were injected with human chorionic gonadotropin (500 units for the female and 150 units for the male) and subsequently placed together in breeding tanks filled with dechlorinated water overnight. In the morning after the injection, adults were removed from the breeding tanks,

eggs collected, and the quality of the egg clutch was checked under a stereo microscope (Zeiss Stemi 508). During this procedure, viable eggs were sorted to be used in the toxicity assays. Part of the obtained eggs was immediately used for the embryonic teratogenesis assays. The remaining eggs were maintained in aquaria, with continuous aeration, filled with FETAX medium until they reach the Nieuwkoop and Faber (NF) developmental stage 45-46 (when the mouth of the tadpoles opens and they start to feed independently; Nieuwkoop and Faber, 1994). After the opening of the mouth in larvae, they were maintained for a few days in FETAX with continuous aeration and fed with fish food (TetraMin™ from Tetra™) until their used in the toxicity assays. During this period, the medium of the aquaria was changed every 48h and dead animals removed daily to avoid the degradation of the medium quality and growth of microorganisms.

2.2.3 *In vivo* toxicity assay

2.2.3.1 Embryo teratogenicity assay

The selected eggs were placed in a recipient filled with FETAX medium and their NF stage checked under a stereo microscope (Zeiss Stemi 508) before their use on the toxicity assays.

Embryos at developmental stage NF 6-8 were exposed to Pb by following the protocol for the Frog Embryo Teratogenesis Assay with minor changes (ASTM, 2012). Organisms were exposed to a control (consisting of FETAX medium) and to the following concentrations of lead: 0.15, 0.22, 0.33, 0.49, 0.74, 1.11, 1.66 mg/L Pb (selected following the results of preliminary assays). Four and three replicates were carried out for the control and each lead concentration, respectively. Each experimental condition contained fifteen embryos (selected randomly) per Petri dish (diameter of 6 cm) filled with 10 ml of the test solution. At the beginning of the assay, pH and conductivity of each experimental condition was measured, using a WTW 3410 meter, to ensure the quality of every solution. After 48h of exposure, all test media were renewed, by removing as much solution as possible, keeping the embryos in the Petri dish to avoid any physical damage, and immediately adding the corresponding freshly made solution. The pH and conductivity were also measured in these fresh solutions.

Exposure occurred for 96 h in a climatic chamber with control conditions of temperature, 23 ± 1ºC, and a photoperiod of 12:12 h light:dark. Mortality was monitored

daily and dead organisms were counted and removed from the Petri dish to avoid the growth of microorganisms and maintain the quality of the medium. In addition to mortality, the following endpoints were monitored: malformations and hatching rate.

2.2.3.2 Tadpoles toxicity assay

The toxicity assay with tadpoles was performed according to the protocol of ASTM (2002) to run assays with larvae of amphibians, with minor modifications. Tadpoles at developmental stage NF 45-46 were exposed to a control (consisting of FETAX medium) and to the following Pb concentrations: 0.15, 0.22, 0.33, 0.49, 0.74, 1.11, 1.66 mg/L Pb (selected based on results from preliminary assays). Four replicates were performed for the control and each tested Pb concentration. Each replicate consisted of a high-density plastic recipient containing 200 mL of the test solution, 0.0125 g of Tetramin™ and four tadpoles. Exposure occurred for 14 d, in a climatic chamber, under constant aeration and controlled conditions of temperature (23 ± 1ºC) and photoperiod (12:12 h light:dark). Test solutions were changed every 48 hours with the addition of 0.0125 g of TetraMin™. Tadpole mortality was checked daily and dead tadpoles were registered and removed to avoid the growth of microorganisms and deterioration of the test solutions. In addition to mortality, at the end of the assay the following endpoints were monitored: the length of snout to vent (SVL), tail (TL) and total body (TBL), weight and stage of development (NF). At the beginning of the assay 11 tadpoles were randomly selected to measure their length (average ± standard deviation: 13.08 ± 1.02 mm) and weight (average ± standard deviation: 0.019 ± 0.0033 g) to allow compute growth and weight gain rates. To perform length measurements at the end of the assay, all tadpoles were photographed and afterwards the body, tail and total length were measured in the computer by using the ImageJ software. Growth and weight gain rates were measured according to equation 1 and 2, respectively:

(1) $GR = (lnL_f - ln\bar{L}_i)/(t_2 - t_1)$, where L_f is, respectively, the final length of SVL, TL or TBL of the organisms, $\bar{L}_i$ is the average of the tadpole's weights at the start of the assay and t_2-t_1 correspond to the exposure period (in days).

(2) $WgR = (ln\bar{W}_i - ln\, W_f)/(t_2 - t_1)$, where W_f is the final weight of tadpole, $\bar{W}_i$ is the average of the tadpole's weights at the start of the assay and t_2-t_1 correspond to the exposure period (in days).

During the assay, pH, conductivity and dissolved O_2 values were measured at the fresh and old medium by using a WTW 3410 meter.

2.2.4 Cell lines cultures

The A6 cell line (XLK-WGATCC® CRL-2527™), firstly isolated from the distal kidney tubulus of *X. laevis*, were used to perform the cytotoxicity assays. This cell line was grown in Petri dish plates containing 55% of Leibovitz's L-15 medium, 35% H_2O 10% Fetal bovine serum and 1% Gentamicin and incubated at 25 ºC. After petri dishes reached around 80% of cell confluence it was used an enzyme (trypsin) to detach the cells from the petri dish. Next, it was used a cell counter to assess the number of alive and dead cells, in order to perform the assays, the number of dead cells had to be equal or inferior to 5%. Lastly, these cells were introduced in 96-well plates, at a concentration of 1 x 10^5 cells per well.

2.2.5 *In vitro* cytotoxicity assays

To assess the effects of Pb on cell viability, the A6 kidney cells of *X. laevis* were exposed, in 96-well culture plates, to a control (consisting of a medium with 55% of L-15, 35% H_2O 10% FBS and 1% Gentamicin) and to the following Pb concentrations: 0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10, 20 mg/L, dissolved in the cell culture medium. Nine 96-well plates were prepared per control and per Pb concentration, each plate was filled with four technical replicates of each experimental condition. The culture medium was renewed at 48 and 72h. To assess cell viability, the tetrazolium reduction assay protocol described in Riss et al. (2004), was used. The MTT-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide substrate was previously prepared in a physiologically balanced solution, and afterwards added to cells in the 96-well culture plates, at a final concentration of 0.5 mg/ml. The plates were placed in the cell incubator, at 25 ºC, for 2 hours. Cell viability was assessed at 24, 48, and 72h, by measuring the formation of formazan in a spectrophotometer plate reader (Multiskan Spectrum, Thermo Fisher Scientific, Waltham, USA). At each observation time, three 96-well plates were measured. Viable cells with active metabolism convert MTT into a purple colored formazan product, that holds a maximum absorbance peak near 570 nm. Dead or inviable cells lose the ability to convert

MTT into formazan. The amount of formazan metabolized by cells is measured by reading absorbance at 570 nm on a spectrophotometer.

2.2.6 Data analysis

To identify differences in the monitored parameters, both for *in vivo* and *in vitro* assays, between the controls and the Pb concentrations, one-way analysis of variance was performed followed by the post-hoc test Dunnett's test. Normality of data was checked through the Shapiro-Wilk's test and the homoscedasticity of variances through the Bartlett's test. When neither normality nor homoscedasticity of variances were met, the non-parametric Kruskal-Wallis One Way Analysis of Variance on ranks was carried out followed by the Dunn's test. These analyses were carried out in the in SigmaPlot (v12.5) software.

2.3 Results

2.3.1. *In vivo* toxicity assays

2.3.1.1 Embryo teratogenicity assay

In the control, mortality was 8%, thus meeting the validity criteria of the ASTM (2012) guideline (*i.e.* lower than 10%). In organisms exposed to Pb, 13% mortality was found in embryos exposed to 0.33 and 1.66 mg/L, and reached 25% in those organisms exposed to concentrations 0.49 mg/L and 0.74 mg/L.

In the control, after 24h of exposure, 30% of larvae had hatched whereas a higher percentage of larvae (≥ 35%) hatched in the Pb treatments (though it was only significantly different from the control at concentrations from 0.22 to 0.74mg/L and 1.66mg/L of Pb; Fig. 1; $p < 0.05$). After 48h, all alive organisms hatched, except in the treatment of 1.11 mg/L Pb, where 100% hatching was only observed after 72h of exposure. Nevertheless, at both 48h and 72h there were no significant difference in the number of hatched larvae between the control and Pb concentrations (Fig. 1; $p > 0.05$).

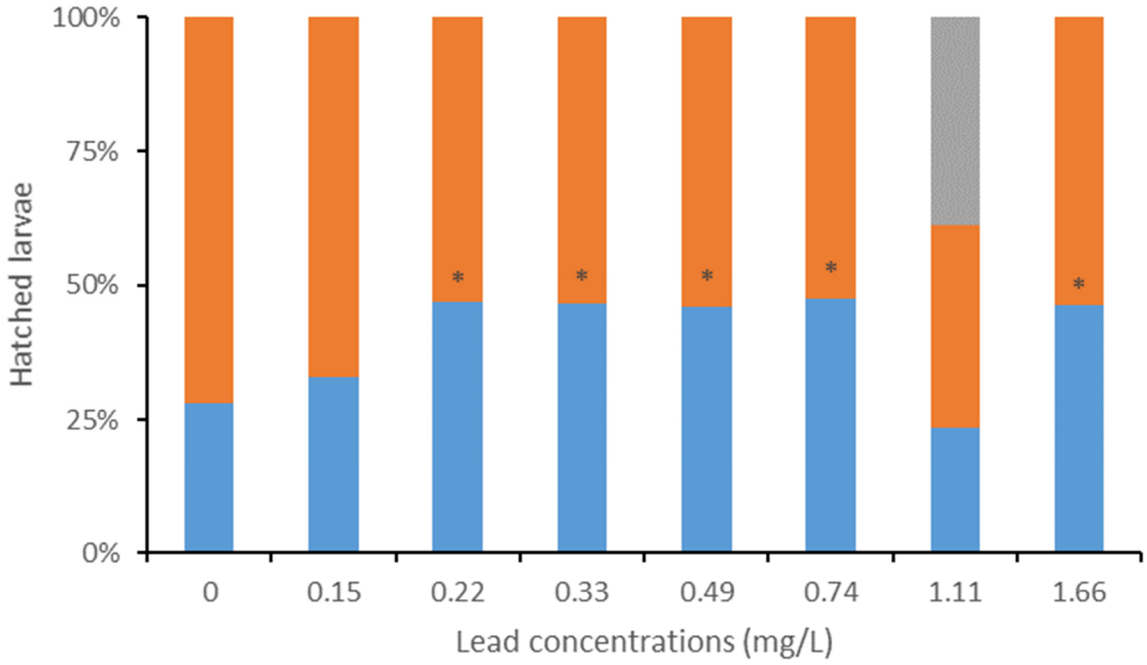

Figure 1: Hatching rate of *X. laevis* larvae after 24 h (blue), 48 h (orange) and 72 h (grey) of exposure to different lead concentrations. *denotes significant difference to the respective control ($p < 0.05$).

An increase in the number of malformations (namely the presence of edemas, tail curvature, notochord curvature, enlarged trunk, pigmentation alterations and eye malformations) were observed with increasing concentrations of Pb (Fig. 2, 3). More than 20% of the organisms exhibited malformations when exposed to Pb concentrations equal or higher than 0.22 mg/L. However, significant differences, to control, were only observed at the highest tested concentration (1.66 mg/L) which elicited 45% malformed organisms (Fig. 2; $p < 0.05$).

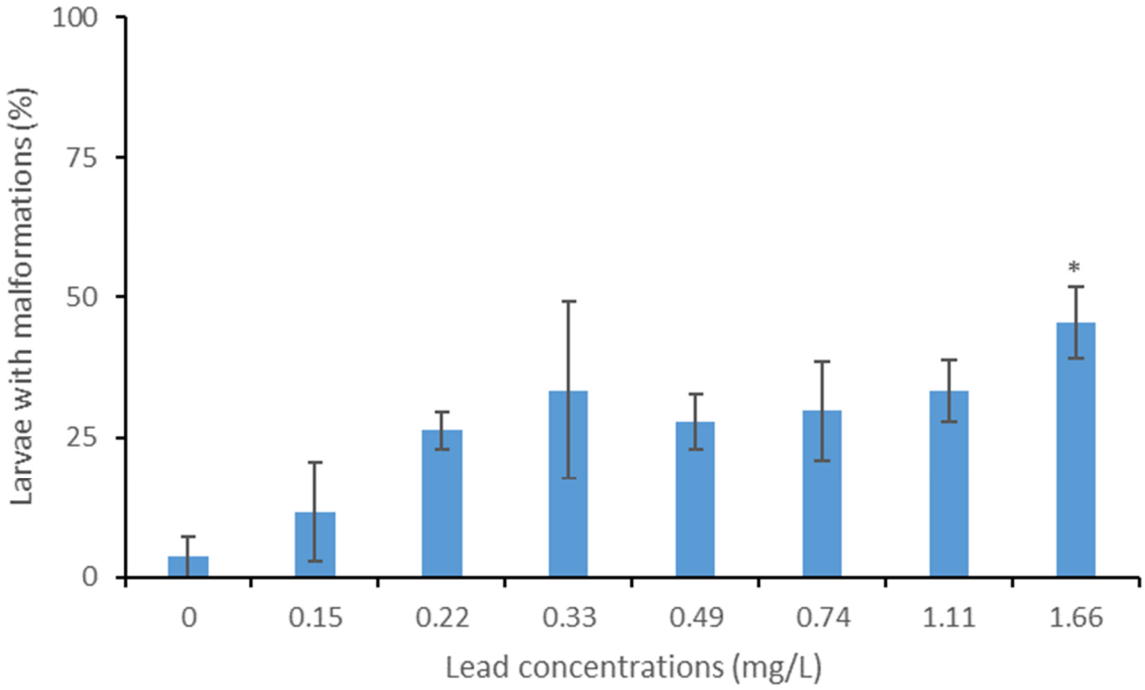

Figure 2: Percentage of *X. laevis* larvae presenting malformations, after 96 h exposure to different lead concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant differences to control ($p < 0.05$).

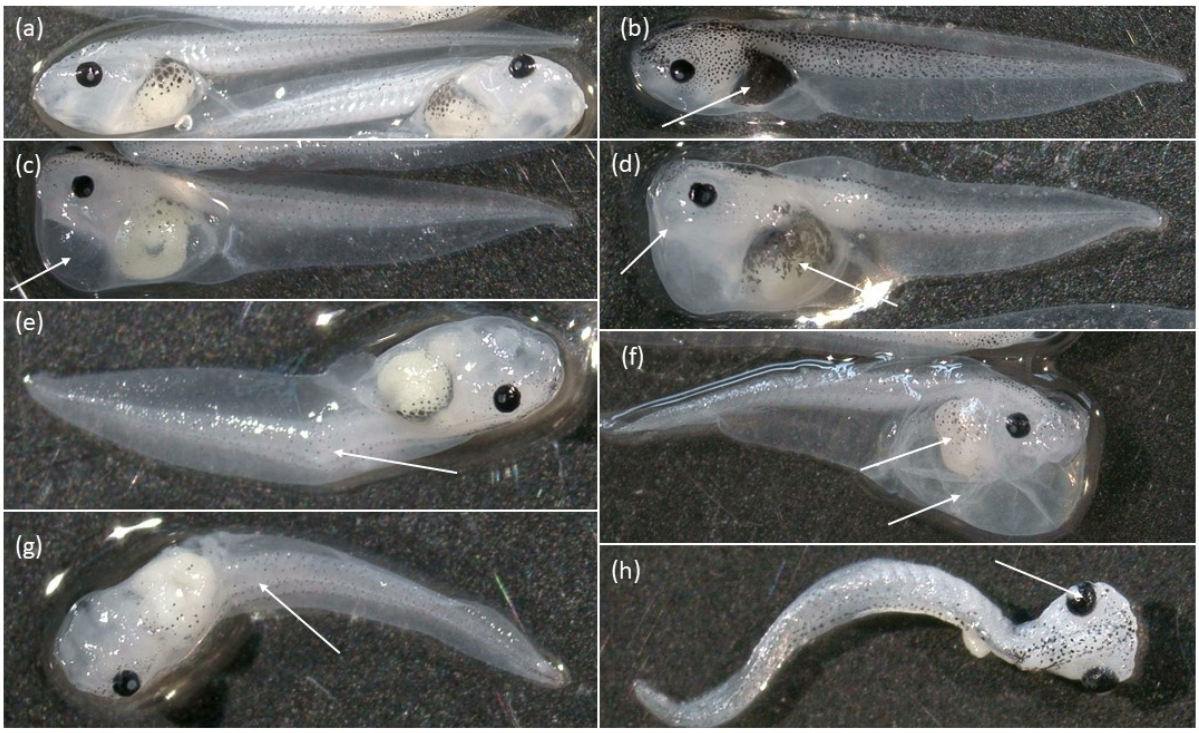

Figure 3: Larvae of *X. laevis*, after 96h exposure to lead, photographed with a digital camera (VWE VisiCam HDMI 6) under a 0.63x magnification. (a) Control larva without malformations; (b) larva

exposed to 0.15 mg/L Pb showing excess of pigmentation (hyperpigmentation); (c) larva exposed to 0.22 mg/L Pb showing a heart edema; (d) larva exposed to 0.15 mg/L Pb showing a craniofacial edema and enlarged trunk; (e) larva exposed to 0.49 mg/L Pb showing a bent tail; (f) larva exposed to 0.22 mg/L Pb showing in the upper arrow lack of pigment in the intestine (hypopigmentation) and in the lower arrow an abdomen edema; (g) larva exposed to 0.49 mg/L Pb showing a bent notochord; (h) larva from concentration 0.33 mg/L Pb, showing protruding eyes. The arrows indicate the type of malformation and their location.

The length of larvae exposed to Pb tended to decrease with the increase of metal concentrations (Fig. 4). Significant reductions in SVL, TL and TBL were observed in organisms exposed to concentrations higher than: 0.15 mg/L, 0.74 mg/L and 0.49 mg/L of Pb (Fig. 4; $p < 0.05$).

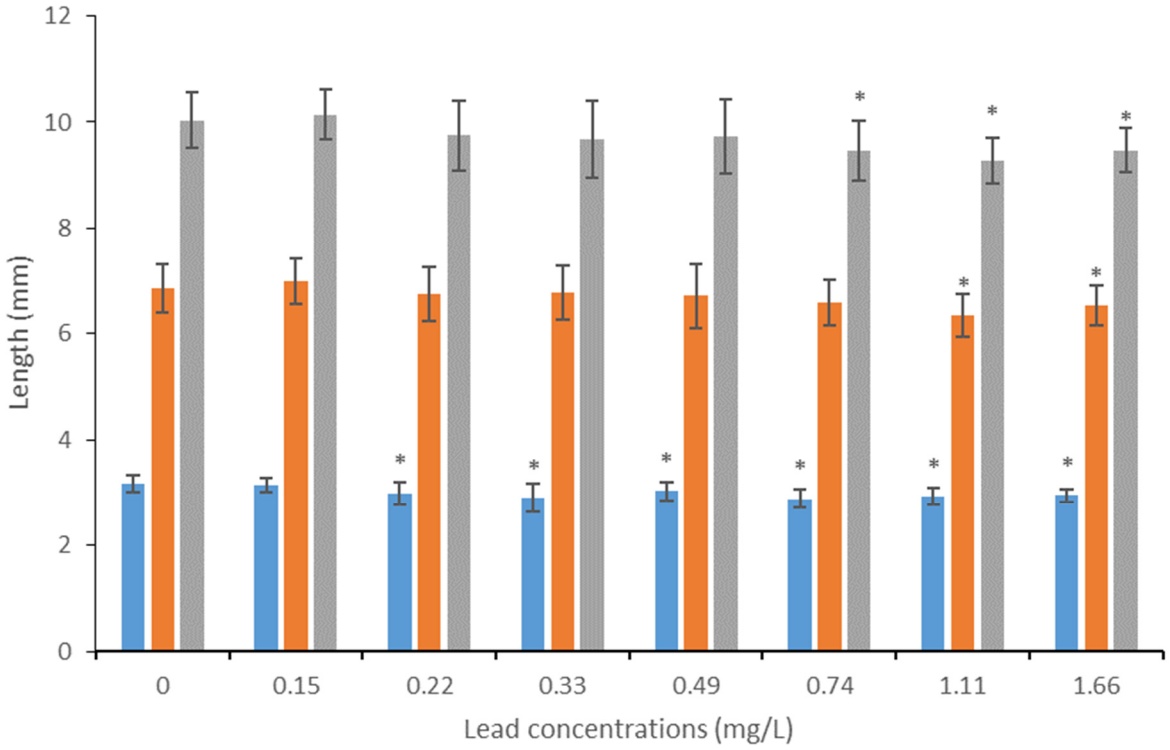

Figure 4: Snout-to-vent (blue), tail (orange) and total body (grey) length of *X. laevis* larvae exposed, for 96 h, to different lead concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant differences to the respective control ($p<0.05$).

2.3.1.2 Tadpoles toxicity assays

No tadpoles died in the control or in the following tested concentrations of Pb 0.22, 0.33 nor 0.74 mg/L. In the remaining tested concentrations of Pb, mortality was always below 20%.

The weight gain rate (WgR) of tadpoles exposed to Pb decreased with increasing concentrations of the metal (Fig. 5). A significant reduction in WgR, relatively to the control, were found in organisms exposed to concentrations higher than 0.33 mg/L of Pb (Fig. 5; $p<0.05$).

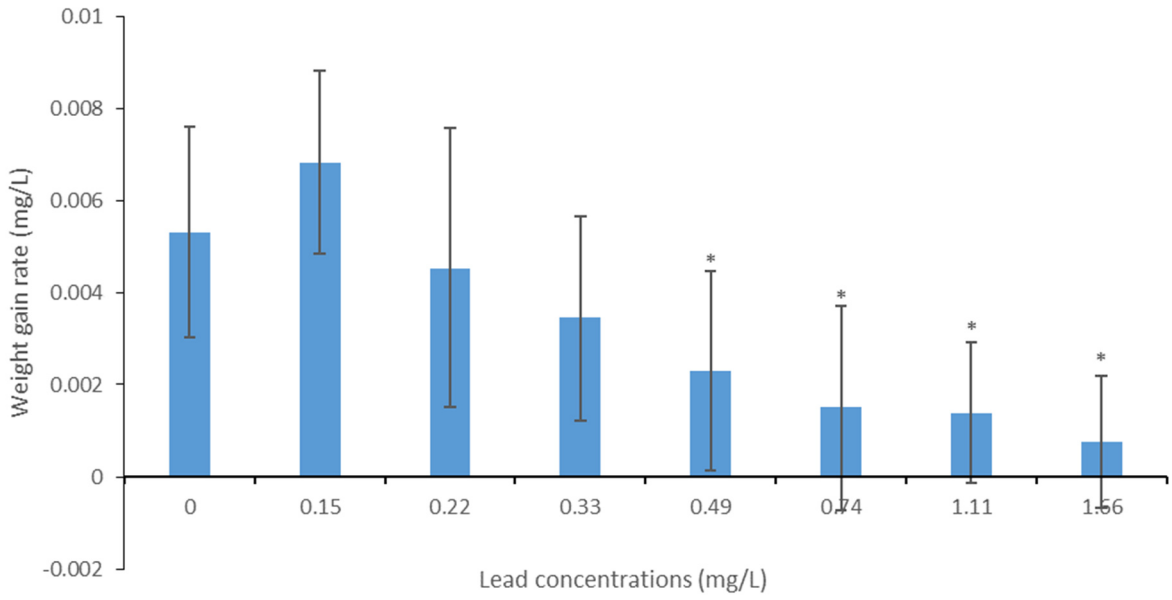

Figure 5: Weight gain rate of *X. laevis* tadpoles, after 14 d of exposure to different lead concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant differences to control ($p<0.05$).

Regarding the growth rate of SVL, TL and TBL, a decrease in these parameters was also observed in tadpoles exposed to Pb (Fig. 6). Comparing to the control, all the three length measurements exhibited a significant reduction at concentration equal or higher than 0.49 mg/L (Fig. 6; $p<0.05$).

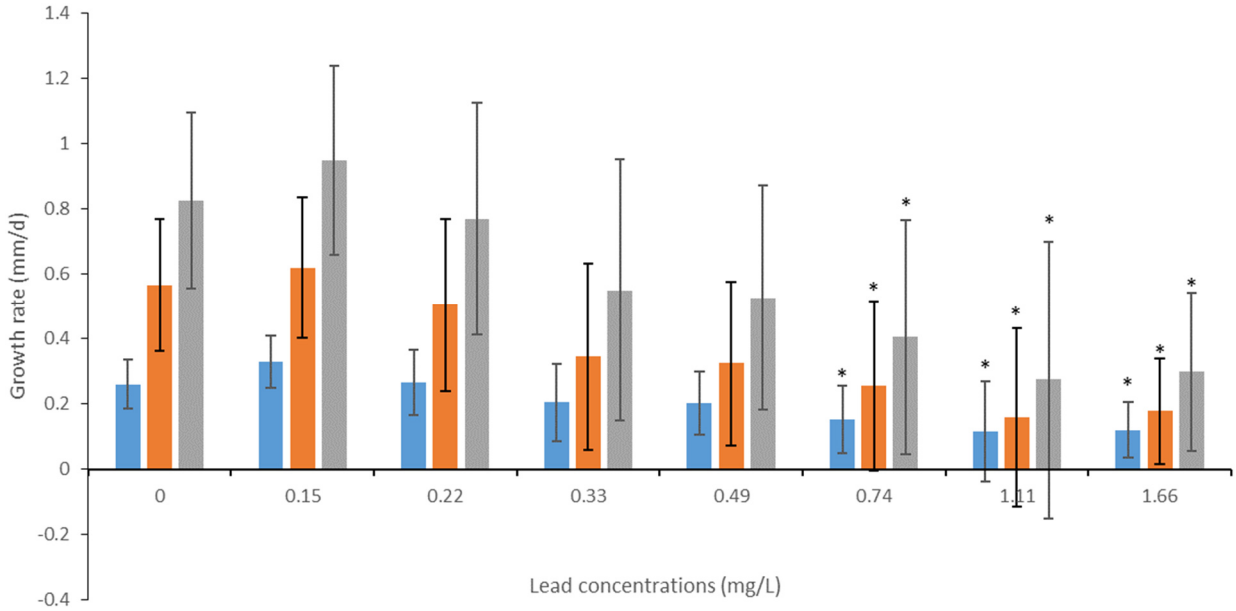

Figure 6: Snout-to-vent (blue), tail (orange) and total body (grey) length of *X. laevis* tadpoles exposed, for 14 d, to different lead concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant difference to the respective control ($p<0.05$).

Exposure to Pb delayed the development of *X. laevis* larvae (Fig. 7). Tadpoles exposed to 0.74 mg/L, 1.11 mg/L and 1.66 mg/L of Pb were on average at an earlier stage of development than control organisms (Fig. 7; $p \leq 0.05$).

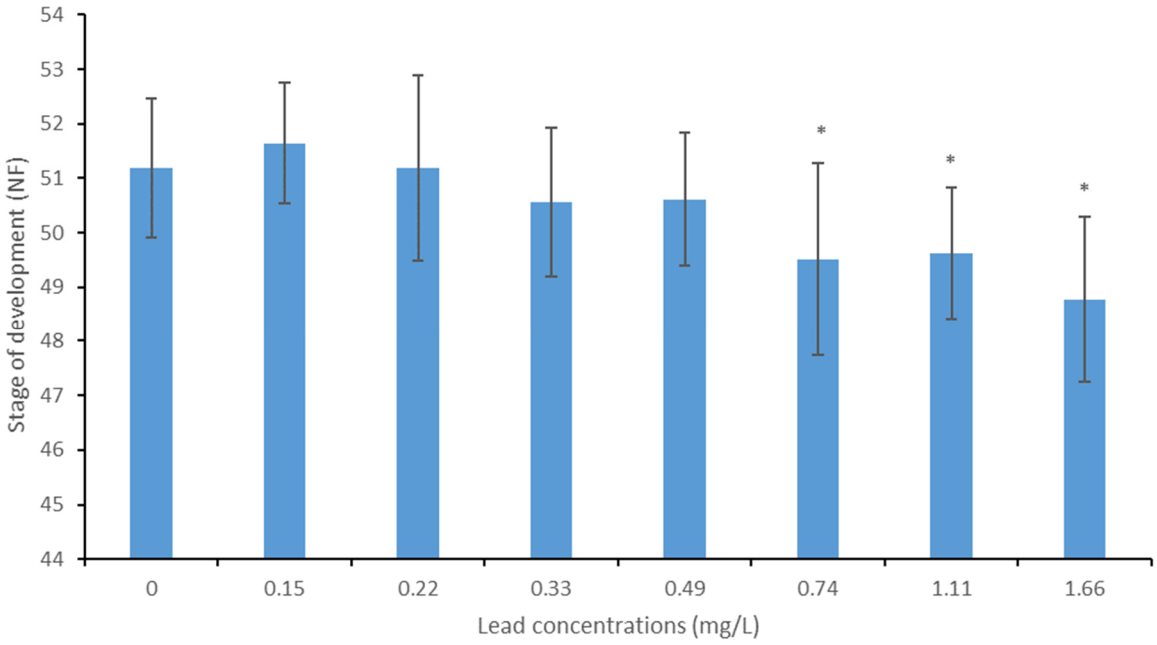

Figure 7: Developmental stage (NF) of *X. laevis* tadpoles, after 14 d of exposure to different lead concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant differences to control ($p<0.05$).

2.3.1.3 *In vitro* cytotoxicity assays

After 24h of exposure to Pb, cell viability significantly decreased at concentrations equal or higher than 2.5 mg/L (Fig. 8; $p<0.05$). However, after 48 and 72h, the negative impact of Pb was observed at all tested concentrations, except 0.039 mg/L and 20 mg/L, respectively (Fig. 8; $p<0.05$).

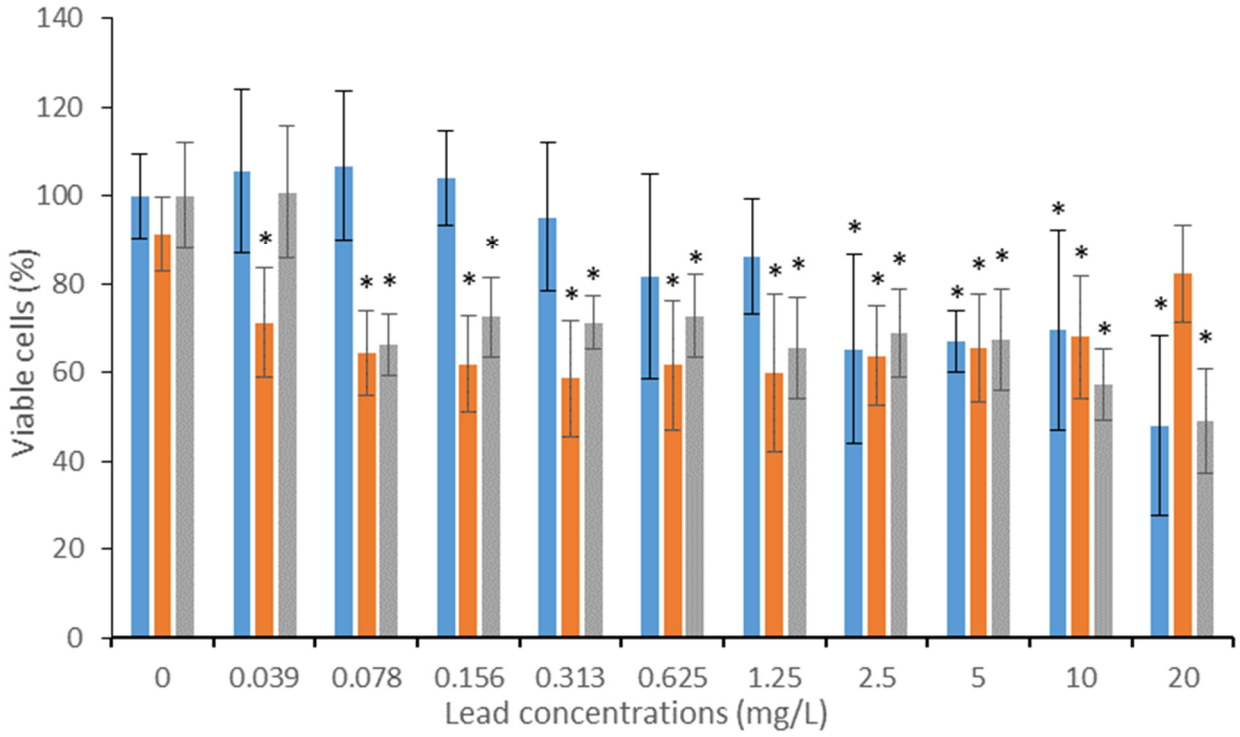

Figure 8: Cell viability of *X. laevis* kidney cells after 24h (blue), 48h (orange) and 72h (grey) exposure to different lead concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant difference to the respective control ($p<0.05$).

2.4 Discussion

2.4.1 Embryos

Exposure to Pb resulted in an increased number of malformations which included tails bent in unnatural ways, edemas, lack or excess of pigmentation, enlarged trunk and impaired eye development. Thus, lead may compromise the development of embryos, causing malformations on *X. laevis* larvae or even death when there is exposure to a high concentration. These findings are in line with those of other studies concerning the effects of lead on amphibians (e.g. Haywood et al., 2004; Mouchet et al., 2007) that established a positive relationship between the incidence of malformations and lead exposure. Mouchet et al. (2007) tested lead concentrations and even though they did not record the incidence of malformations, from 10 mg/L to 30 mg/L of Pb, *X. laevis* larvae that survived after exposure started to show sublethal effects like anemia, lower size and restricted food uptake. Haywood et al. (2004) also tested the effects of lead concentrations from 0.1 ppm to 1 ppm

(corresponding to 0.1 to 1 mg/L, within the range tested in the present work) in *X. laevis*, and found that there was a positive relationship between the incidence of malformations and lead exposure. The observed malformations (e.g. tail curvature) may have negative impacts in the swimming performance of the animals, which may impair their capacity to escape predators or search for food, and, ultimately compromise their survival. Chen et al. (2006). (2012) exposed embryos of *Rana pipiens* to 100µg/L and observed an association between the presence of tail malformation and abnormal swimming behavior. These authors reported that more than 90% of the larvae exposed to that lead concentration exhibited lateral spinal curvature, and that such spinal deformity was associated with abnormal swimming behavior.

The present study also demonstrated that Pb may compromise hatching rate at concentrations higher than 0.22 mg/L. Haywood et al. (2004) study with *X. laevis* embryos exposed to lead also found that the hatching success was negatively affected by Pb exposure at concentrations starting at 0.9 mg/L of Pb. Studies with other amphibian species, failed to demonstrate an effect of Pb on this parameter. For example, in Stansley et al. (1997) studies, with *Rana palustris* and *Rana catesbeiana*, Pb did not affect the hatching rate. These results suggest species specific sensitivity of lead and support the need to perform more studies to understand the mechanisms associated with the toxic effects of Pb two amphibians in order to adequately protect this group of organisms.

2.4.2 Tadpoles

The exposure of *X. laevis* tadpoles to Pb had a negative effect in their overall growth and development. It is clear that high concentrations of Pb result in lower body weight. These results are supported by the studies of Haywood et al. (2004) and of Sobotka & Rahwan (1995) with *X. laevis*, which found that tadpole body weight and growth was negatively related to lead concentrations (0.9mg/L and 0.5 to 3.0 mg/L, respectively). Berzins & Bundy (2002) showed that not only the tadpoles' body weight was negatively affected by Pb exposure but also their normal development. Data from the present study showed that development was delayed when exposed to higher lead concentrations. Thus, it is likely that Pb can cause sublethal effects like underdevelopment in lower concentrations leading to death in higher concentrations. These results may have relevant impacts in the

metamorphosis of these animals, as they need to attain a certain size to initiate metamorphosis.

2.4.3 Kidney Cells

The ability of Pb to impact cell viability was clearly demonstrated with cells demonstrating increased sensibility overtime. Similar results were reported by Tchounwou et al. (2001) study with human liver carcinoma (HepG2) that found a dose- and time-dependent response to Pb. Previous *in vitro* studies have reported the ability of Pb to affect the viability of human leukemia cells (HL-60 cells) as a result of DNA damage and cell cycle arrest at the G0/G1 checkpoint but also triggering the apoptosis through caspase-3 activation and nucleosomal DNA fragmentation accompanied by secondary necrosis (Yedjou, et al., 2015). The mechanisms associated with the decreased viability of A6 cell found in the present study should be clarified. Effects were detected at concentrations higher than those found in the *in vivo* study which may be associated with a protective effect of fetal bovine serum making Pb less available to cells. This aspect should also be further tested in order to increase the relevance/reliability of *in vitro* data.

2.5 Conclusion

Considering the lead concentrations that may be found at superficial waters in the environment (0.18 to 179 µg/L; Mutia et al., 2012), the results obtained in this work suggest that some risk may exist for aquatic life stages of amphibians, since significant sublethal effects (on hatching and snout-to-vent length) were observed at concentrations of 0.22 mg/L (i.e. 220 µg/L), which is close to environmental concentrations. Furthermore, the used in vitro methodology revealed a sensitivity to Pb quite similar to that observed for embryos and tadpoles, suggesting its adequacy to be used at early stages of ecological risk assessment of this metals.

Chapter III

Evaluation of the toxicity of silver to aquatic life stages and to a kidney cell line of *X. laevis*

3. Evaluation of the toxicity of silver to aquatic life stages and to a kidney cell line of *X. laevis*

3.1 Introduction

Environmental contamination by silver is of major concern since it is considered amongst the most toxic metal (EC, 2018). This type of contamination is mostly anthropogenically-driven; just in the year of 2018 the world total amount of silver demand exceeded 32,000 tonnes (Alexander et al. 2019), being associated with activities such as smelting, cloud seeding with silver iodide, mining, photo processing industry, wastewater treatment plants, textile industry, nanotechnology, among others. (Holler et al., 2015). In freshwater ecosystems, silver may occur in several forms (e.g. combined with sulfide or sulfate, bromides, chlorides), being its ionic free form considered the most toxic to freshwater biota (Schnizler et al., 2007; Adams and Kramer, 2009; Moermond and van Herwijnen, 2012). The adverse effects posed by ionic silver to biota involves diverse mechanisms, for example on bacteria, silver inhibits the activity of thiol enzymes, while in crustaceans and fish it impairs the ion regulation by blocking the uptake of Na^+ and Cl^- (Domsch et al., 1984; Morgan et al., 1996). For more than 40 years the ecotoxicity of ionic silver has been extensively studied for several freshwater taxonomic groups (Ratte, 2009; Moermond and van Herwijnen, 2012), namely: bacteria (e.g., Fulladosa et al., 2005), algae (Navarro et al., 2008), macrophytes (Oukarroum et al. 2013), crustaceans (e.g. Kolts et al. 2009; Lekamge et al., 2018), cnidaria (Lekamge et al., 2018), annelida (Khangarot, 1991), insecta (Call et al., 1999), fish (Naddy et al., 2011), amphibians (Khangarot & Ray, 1987), among other groups. Although both acute and chronic toxicity were addressed in most of the published literature, for some taxonomic groups only acute/lethal toxicity data is available (e.g. cnidaria, amphibian; review on Moermond and van Herwijnen, 2012). Though it is an important information, the acute toxicity of ionic silver does not constitute a major environmental concern because as silver enters the freshwater systems readily complexes and/or adsorbs to other anions and particulate matter, thus low concentrations of ionic silver usually occur in these environment (Rozan et al., 1995; Adams and Kramer, 2009; Moermond and van Herwijnen, 2012). Concentrations of silver in non-pristine superficial waters have been reported on the range of 10 to 100 ng/L in water bodies located near industrialized and urban areas, with some of the highest measured concentrations worldwide reaching values near 40 µg/L (Rozan et al., 1995; Howe and Dobson, 2002). Nevertheless,

chronic and sublethal exposure of biota to low ionic silver concentrations may constitute a long-term risk that must be assessed, since the continuous exposure to such low levels and the eventual accumulation of ionic silver by organisms may lead to toxic effects (Ratte, 2009). Amphibians constitute a group of vertebrates for which the effects of silver have rarely been assessed, and the few existing ecotoxicity data only focus acute and lethal toxicity (Ratte, 2009; Moermond and van Herwijnen, 2012). This is a group of organisms very sensitive to chemical contamination, mainly because their skin is highly irrigated and permeable, which is critical for gas exchange and osmoregulation (Rowe et al., 2003). Furthermore, presently amphibians are facing a global decline, making them the group of vertebrates with the highest proportion of endangered species (Monastersky, 2014). This context, highlights the need for assessing the sub-lethal effects of silver contamination on aquatic life stages of amphibians, aiming at a more accurate protection and conservation of this group of organisms. According, the present work aimed at assessing the sublethal toxicity of silver to aquatic life stages of the model species *X. laevis*. Furthermore, it was intended to evaluate the cytotoxicity of silver to a kidney cell line of *X. laevis* in order to determine if this *in vitro* methodology could be used as an adequate surrogate for animal experimentation in the risk assessment of silver to aquatic life stages of amphibians.

3.2 Materials and Methods

3.2.1 Test chemical

Silver nitrate ($AgNO_3$) was purchase to the company Sigma-Aldrich (Saint Louis City, USA). A stock solution of this metallic salt was prepared at a concentration of 10 mg/L of Ag in the artificial standard medium FETAX (this medium was used to maintain the test organisms and also to perform the toxicity assays, Dawson and Bantle, 1987). All the tested concentrations of silver were obtained through the dilution of the stock solution in FETAX medium.

3.2.2 Test species

The African clawed *X. laevis* was selected as the model species to perform the *in vivo* toxicity assays. Adults of this species were maintained in laboratories of the Department of Biology, University of Aveiro, as described in Chapter II. The procedure used to obtain the

eggs and tadpoles for the *in vivo* toxicity assays was also previously described in Chapter II (please see section 2.2.2 of that chapter for further details).

Part of the obtained eggs were immediately used for the embryonic teratogenicity assay. The remaining eggs were maintained in recipients filled with FETAX, with constant aeration, until they reached the Nieuwkoop and Faber (NF) developmental stage 45-46 (when the mouth of the tadpoles opens and they start to feed independently; Nieuwkoop and Faber, 1994). Further detailed information on the maintenance of organisms until reaching developmental stage 45-46 (time at which were used for the tadpole's toxicity assays) is described in Chapter 2.

3.2.3 *In vivo* toxicity assay

3.2.3.1 Embryo teratogenicity assay

The sorted viable eggs were placed in a recipient filled with FETAX medium and their NF stage was checked under a stereo microscope (Zeiss Stemi 508) before using them on the toxicity assays.

Embryos at developmental stage NF 6-8 were exposed to silver concentrations by following the protocol for the Frog Embryo Teratogenesis Assay with minor changes (ASTM, 2012). Organisms were exposed to a control (consisting of FETAX medium) and to the following concentrations of silver: 0.013, 0.019, 0.029, 0.044, 0.067, 0.1, 0.15 mg/L of Ag (these concentrations were chosen following the results of previous preliminary assays). Fifteen embryos (selected randomly) were introduced per Petri dish (with a diameter of 6 cm), each filled with 10 ml of the test solution. Four replicates were performed for the control and three for each Ag concentration. Exposure lasted for 96 h and the assay was carried out in a climatic chamber with control conditions of temperature (23 ± 1ºC) and with a photoperiod of 12:12 h light:dark. All the test media were renewed after 48 h of exposure. Mortality was monitored daily and dead organisms were counted and removed from the Petri dish to avoid the growth of microorganisms and maintain the quality of the medium. In addition to mortality, two other endpoints were also monitored: malformations and hatching rate.

3.2.3.2 Tadpoles toxicity assay

Tadpole toxicity assay was performed according to the protocol of ASTM (2002) to run assays with larvae of amphibians, with some minor adjustments. When tadpoles reached the development stage of NF 45-46, they were exposed to a control (consisting of FETAX medium) and to the following silver concentrations: 0.013, 0.019, 0.029, 0.044, 0.067, 0.1, 0.15 mg/L of Ag (these concentrations were selected based on results from previous preliminary assays). Four replicates were performed for the control and each tested Ag concentration. Each replicate consisted of a high-density plastic recipient containing 200 mL of the test solution, 0.0125 g of Tetramin™ and four tadpoles. Exposure lasted for 14 days, in a climatic chamber, under constant aeration and controlled conditions of temperature (23 ± 1ºC) and photoperiod (12:12 h light:dark). Every 48h all test solutions were changed and 0.0125 g of TetraMin™ was added to each replicate. Tadpole mortality was checked daily and dead tadpoles were registered and removed to avoid deterioration of the quality of test solutions and the growth of microorganisms. Besides mortality, the following endpoints were monitored at the end of the assay: the length of snout to vent (SVL,), tail (TL) and total body (TBL), weight, stage of development (NF), heartbeat rate and heart area. At the beginning of the assay 11 tadpoles were randomly selected to measure their length (average ± standard deviation: 11.78 ± 1.19 mm) and weight (average ± standard deviation: 0.018 ± 0.003 g) to allow compute growth and weight gain rates. To perform these length measurements, at the end of the assay, all tadpoles were photographed and afterwards the body, tail and total length were measured in the computer by using the ImageJ software. Growth and weight gain rates were measured according to equation 3 and 4, respectively:

(3) $GR = (lnL_f - ln\bar{L}_i)/(t_2 - t_1)$, where L_f is, respectively, the final length of SVL, TL or TBL of the organisms, $\bar{L}_i$ is the average of the tadpole's weights at the start of the assay and t_2-t_1 correspond to the exposure period (in days).

(4) $WgR = (ln\bar{W}_i - ln\, W_f)/(t_2 - t_1)$, where W_f is the final weight of tadpole, $\bar{W}_i$ is the average of the tadpole's weights at the start of the assay and t_2-t_1 correspond to the exposure period (in days).

During the assay there were three parameters measured: the pH, conductivity and dissolved O_2 values, measured at the fresh and old medium by using a WTW 3410 meter.

3.3.4 Cell lines culture

The A6 cell line (XLK-WGATCC® CRL-2527™), which was firstly isolated from the distal kidney tubulus of *X. laevis*, were selected to perform the cytotoxicity assays. This cell line was grown in Petri dish plates containing 55% of L-15, 35% H_2O 10% FBS and 1% Gentamicin and incubated at 25 ºC. Cells cultured in petri dishes were detached using an enzyme (trypsin) when cell confluence reached around 80%. Afterwards, alive and dead cells were counted using a cell counter to assess their number. In order to perform the assays, the number of dead cells had to be equal or inferior to 5%. Lastly, these cells were introduced in 96-well plates, at a concentration of 1 x 105 cells per well.

3.3.5 *In vitro* cytotoxicity assays

To assess the effects of silver in cellular viability, kidney cells of *X. laevis* (XLK-WGATCC® CRL-2527™) were exposed, in 96-well culture plates, to a control (consisting of a medium with 55% of L-15, 35% H_2O 10% FBS and 1% Gentamicin) and to the following silver concentrations: 0.0004, 0.0008, 0.0016, 0.003, 0.006, 0.01, 0.025, 0.05, 0.1, 0.2 mg/L of silver. For each control and Ag concentration were performed nine replicates, which consisted in 96-well plates with four technical replicates of each treatment. After 48 and 72 hours of exposure, the culture medium was renewed. Every 24 hours, until 72 h of exposure, three 96-well plates were read using a spectrophotometer plate reader (Multiskan Spectrum, Thermo Fisher Scientific, Waltham, USA) at an absorbance of 570 nm. To assess cell viability the MTT protocol described in Riss et al. (2004) was used.

The MTT substrate is previously prepared in a physiologically balanced solution, then it's added to the cells of the 96-well culture plates, at a final concentration of 0.5mg/ml and is incubated for 2 hours. The viable cells, with active metabolism, convert MTT into a purple colored formazan product with a maximum peak of absorbance near 570 nm. When cells die or are inviable, they lose the ability to convert MTT into formazan, therefore the color formation serves as a useful and convenient marker of cytotoxicity.

3.3.6 Data analysis

The comparison of sublethal parameters, between organisms exposed in the control and those exposed to silver was performed, by conducting a one-way analysis of variance followed by the multi-comparison Dunnett's test. Normality of data was checked through the Shapiro-Wilk's text and the homoscedasticity of variances through the Bartlett's test. When either normality and/or the homoscedasticity of variances failed to be met the Kruskal-Wallis one-way analysis of variance on ranks was performed followed by the multi-comparison Dunn's test. These analyses were carried out in the in SigmaPlot (v12.5) software.

3.4 Results

3.4.1 *In vivo* toxicity assays

3.4.1.1 Embryo teratogenicity assay

Mortality observed in the control was below 10%, thus complying with the assay validity criteria set in the guideline (ASTM, 2012). Mortality above 10% was observed at the following silver concentrations: 0.013 (13%), 0.019 (18%), 0.029 (16%), and 0.1 (14%) mg/L.

The percentage of hatching larvae, after 24 h of exposure, was higher at concentrations 0.029 mg/L and 0.44 mg/L of Ag, being above 45% (Fig. 9; $p \leq 0.001$). Nevertheless, Ag seemed, in general, to delay the hatching of larvae: after 48h exposure, 100% hatching only occurred at the control and at concentrations 0.029, 0.04 and 0.1 mg/L of Ag. At the remaining tested Ag concentrations, 100% hatching of alive organisms only occurred after 72h of exposure. However, at 48h and 72h of exposure, there was no significant difference in the number of hatched larvae when comparing control with Ag treatments (Fig. 9; $p > 0.05$).

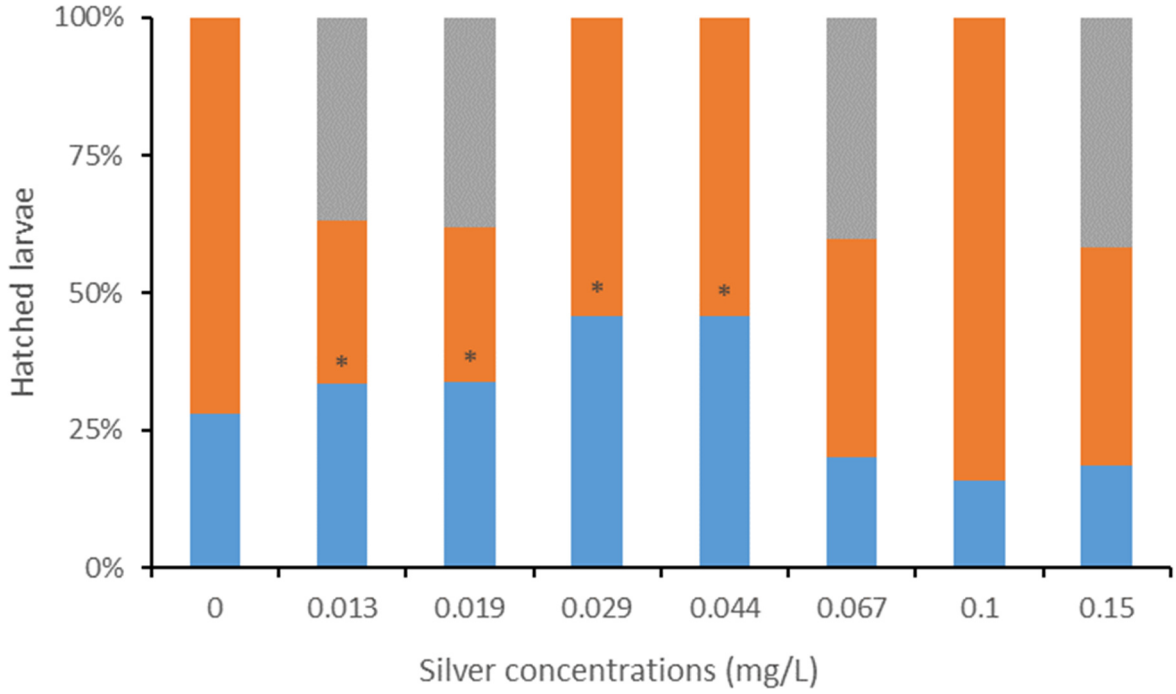

Figure 9: Hatching rate of *X. laevis* larvae exposed for 24 h (blue), 48 h (orange) and 72 h (grey) to different silver concentrations. * denotes significant differences to the respective control ($p \leq 0.001$).

At all tested concentrations of Ag, the frequency of malformations was above 40%, being significantly higher than in the control ($p \leq 0.001$; Fig. 10 and 11).

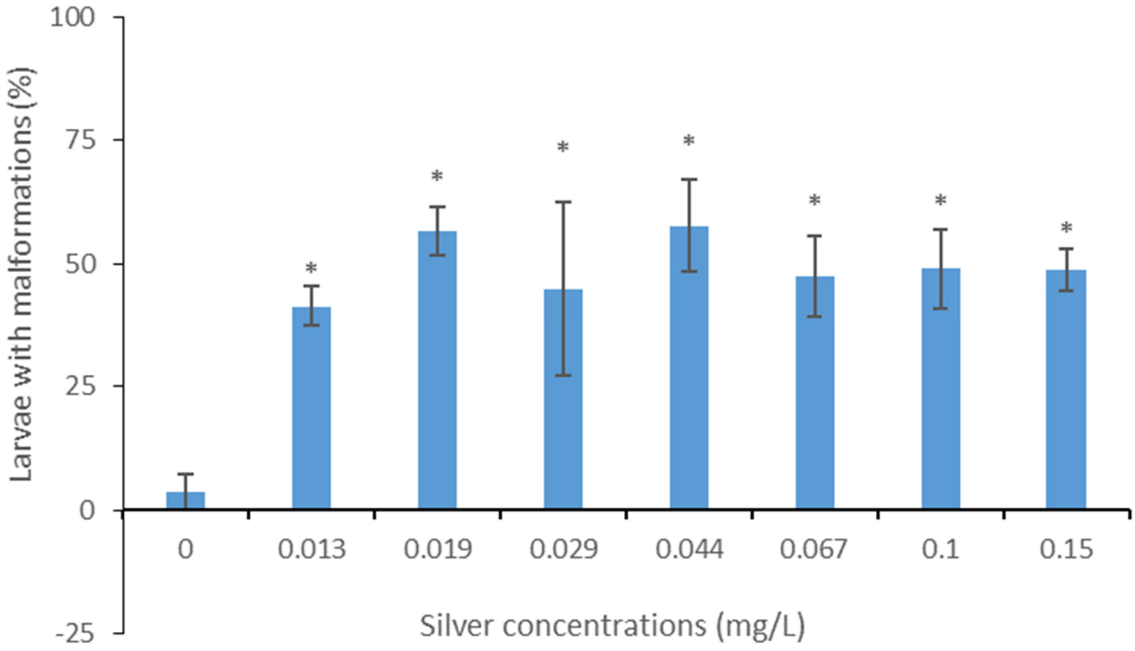

Figure 10: Percentage of *X. laevis* larvae with malformations, after 96 h exposure to different silver concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant differences to control ($p<0.05$).

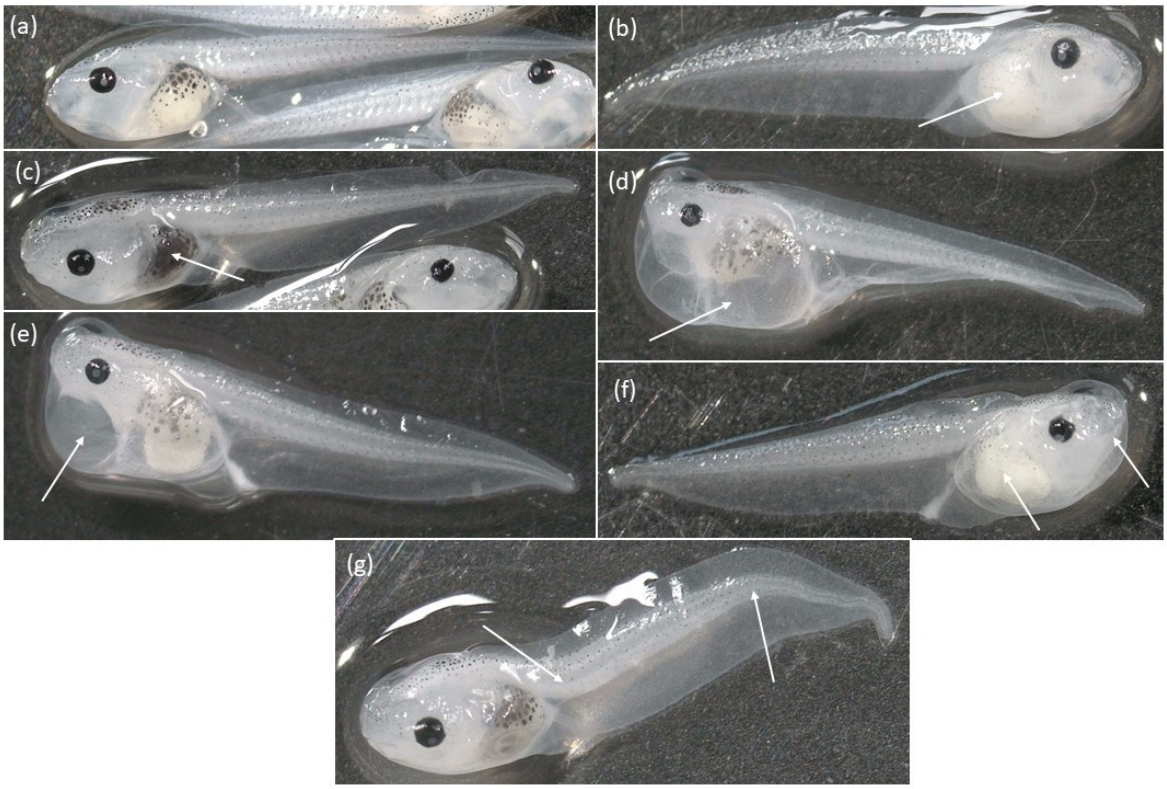

Figure 11: Larvae of *X. laevis*, after 96h exposure to silver, photographed with a digital camera (VWE VisiCam HDMI 6) under a 0.63x magnification. (a) Control larva without malformations; (b) larva exposed to 0.15 mg/L Ag, showing the lack of pigmentation (hypopigmentation); (c) larva exposed to 0.029 mg/L Ag, showing an excess of pigmentation (hyperpigmentation); (d) larva exposed to 0.1 mg/L Ag, showing an abdominal edema; (e) larva exposed to 0.067 mg/L Ag, showing a heart edema; (f) larva exposed to 0.019 mg/L Ag, the left arrow indicates the enlarged trunk and the right arrow indicates a craniofacial edema; (g) larva exposed to 0.044 mg/L Ag, the left arrow shows a bent notochord and the right arrow shows a bent tail. The arrows indicate the type of malformation and their location.

Exposure during 96 h to Ag caused a decrease in the length of larvae (Fig. 12). The SVL was significantly smaller in organisms exposed to all tested concentrations of this metal when compared to control (Fig. 12; $p<0.05$;). In terms of TL, a significant reduction ($p<0.05$) was observed in organisms exposed to 0.019, 0.10 and 0.15 mg/L Ag. Finally, TBL was significantly reduced in tadpoles exposed to 0.019 mg/L and to concentrations above 0.029mg/L of Ag compared to control (Fig. 12; $p<0.05$).

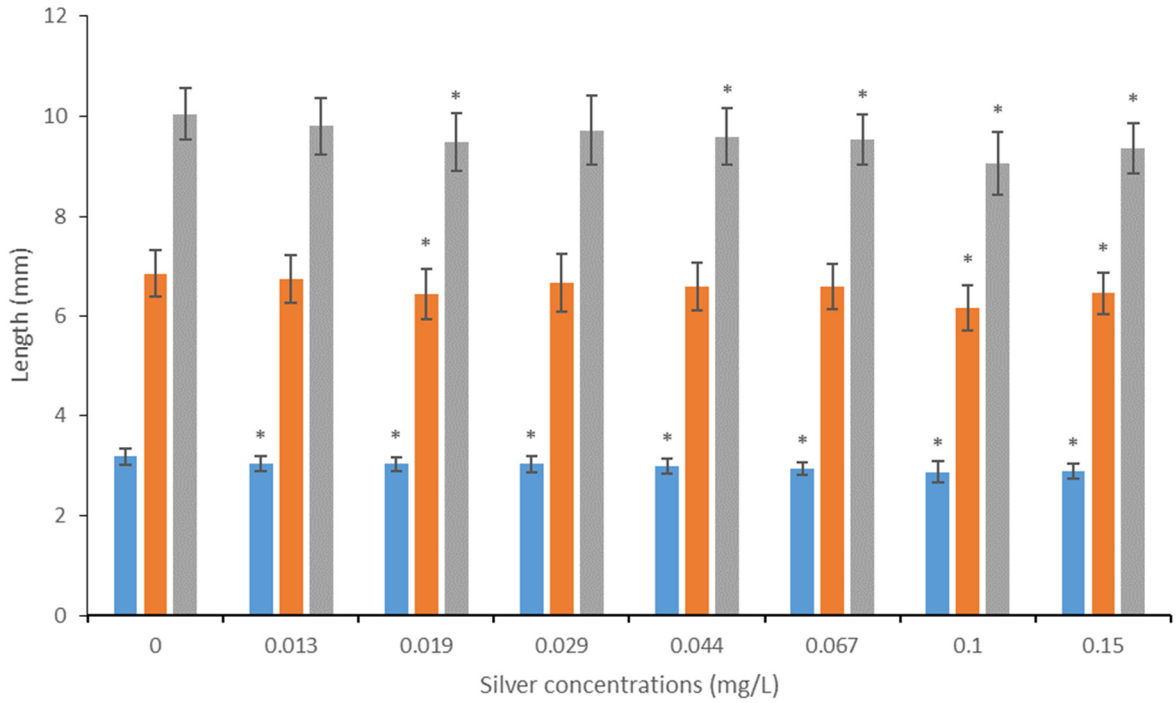

Figure 12: Snout-to-vent (blue), tail (orange) and total body (grey) length of *X. laevis* tadpole exposed, for 96 h, to different silver concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant differences to the respective control ($p<0.05$).

3.4.1.2 Tadpoles toxicity assay

Mortality in the controls was 13%. The three highest tested concentrations of Ag caused more than 90% of mortality in tadpoles (0.067: 94%; 0.1 and 0.15 mg/l: 100%), while the remaining tested concentrations induced mortality below 20%.

No significant differences in terms of weight gain rate were observed between control tadpoles and tadpoles exposed to Ag (Fig. 13; $p>0.05$). Note that in concentration 0.067 mg/L of Ag survived only one tadpole, so all data presented in the following figures, regarding this concentration, represents only one tadpole.

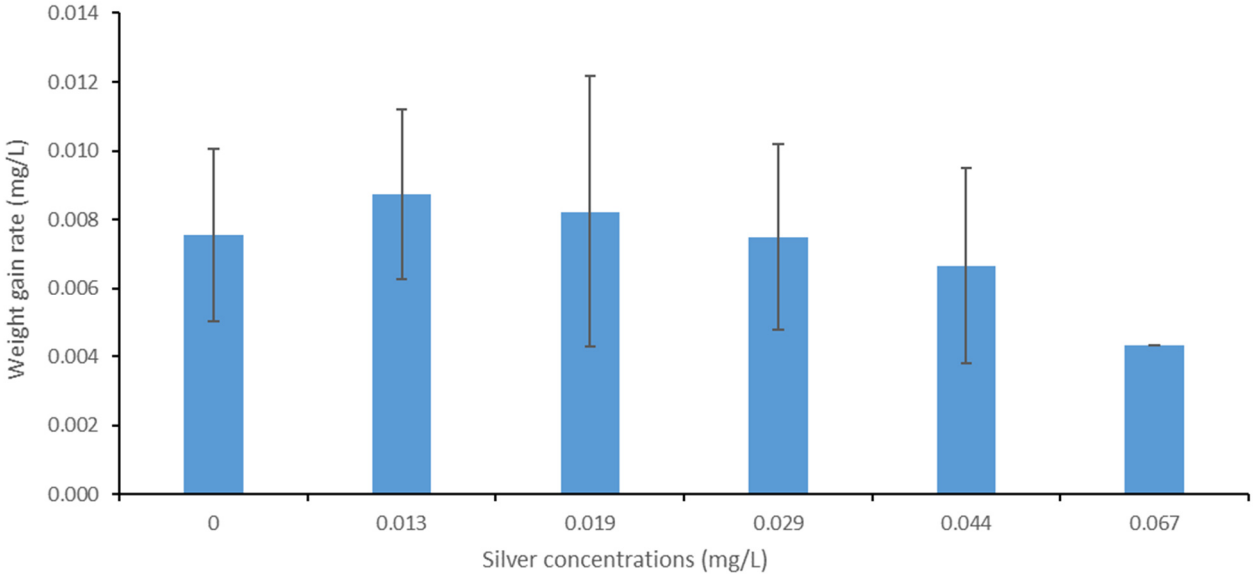

Figure 13: Weight gain rate of *X. laevis* tadpoles exposed, for 14 days, to different silver concentrations. Results are expressed as mean ± standard deviation (error bars).

Exposure to the tested Ag did not affect the SVL of tadpoles (Fig. 14). However, exposure to the intermediate tested concentrations (0.013, 0.019 and 0.029 mg/L) of Ag induced a significant increase in TL and TBL of tadpoles (Fig. 14; $p<0.05$).

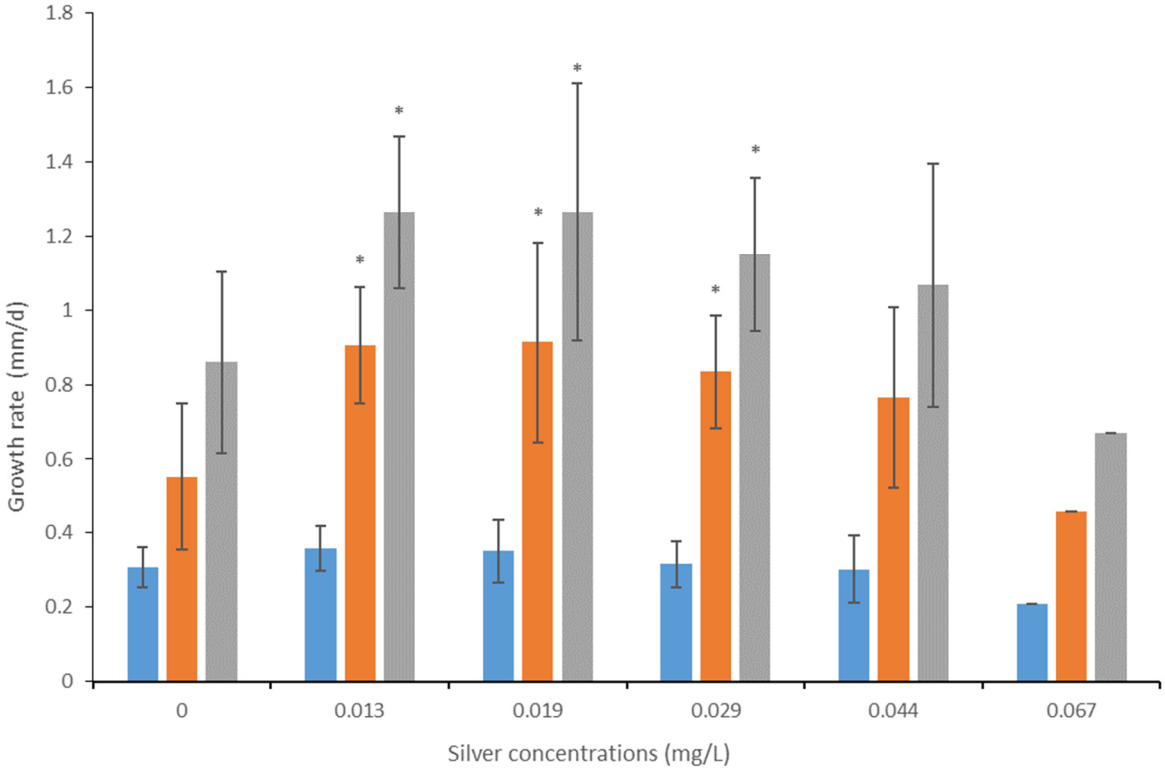

Figure 14: Length of snout-to-vent (blue), tail (orange) and total body length (grey), of *X. laevis* tadpoles after being exposed, for 14 days, to different silver concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant differences to the respective control ($p<0.05$).

Tadpoles average developmental NF stage was significantly higher in organisms exposed to 0.019 mg/L of Ag (Fig. 15; $p<0.05$).

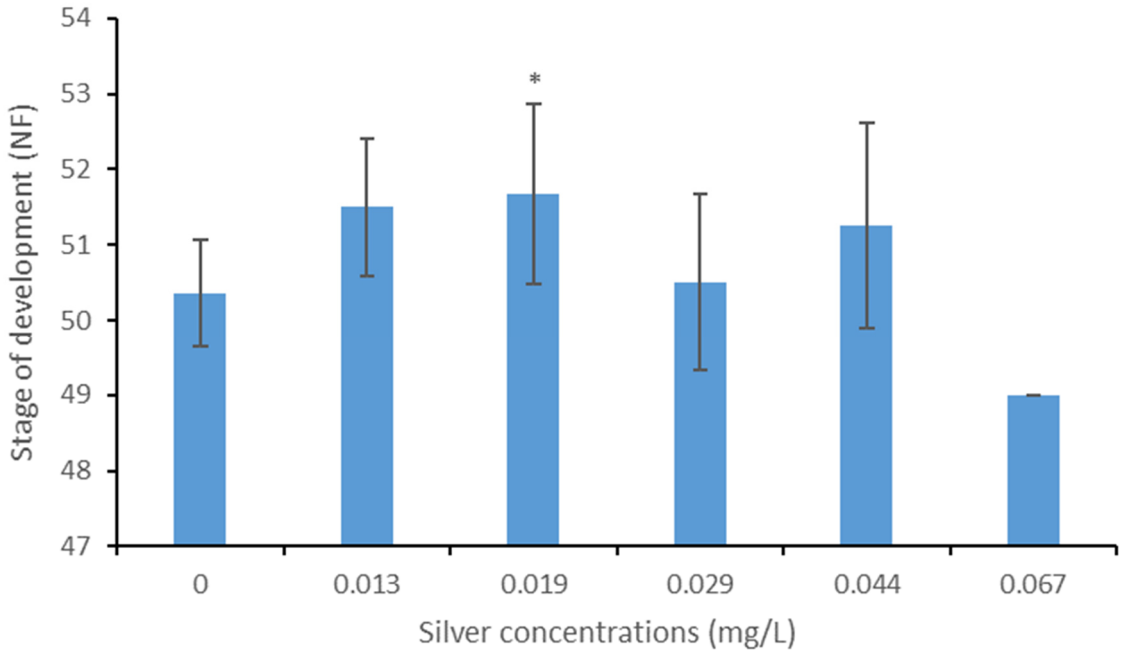

Figure 15: Developmental stage (NF) of *X. laevis* tadpoles, after 14 days exposure to different silver concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant differences to the respective control ($p<0.05$).

The heartbeats of tadpoles exposed to 0.013 and 0.019 mg/L of Ag, was significantly lower than that of tadpoles from the control (Fig. 16A; $p<0.05$). However, no significant changes were observed in the heart area of tadpoles exposed to Ag (Fig. 16B; $p>0.05$).

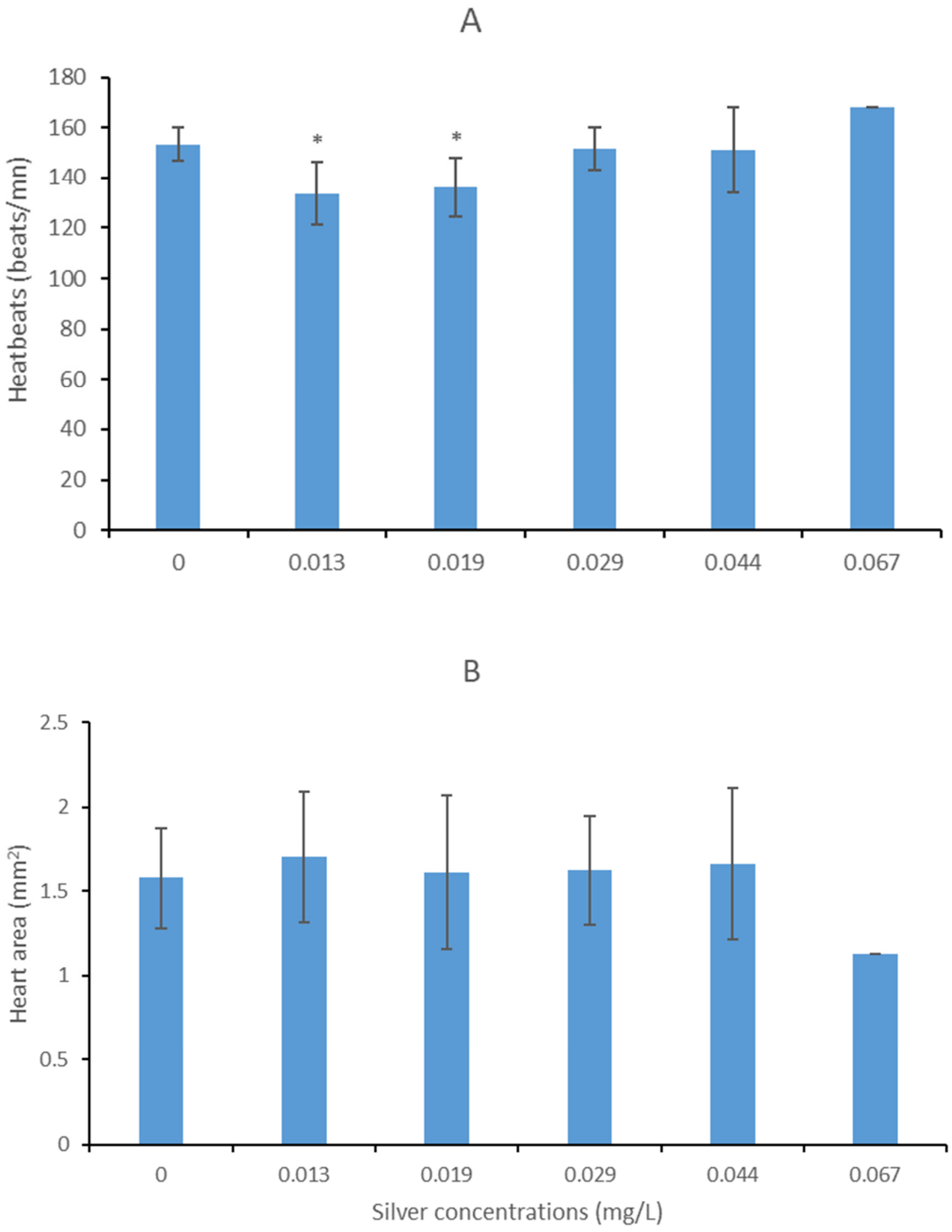

Figure 16: Heartbeats (A) and heart area (B) of *X. laevis* tadpoles exposed, for 14 days, to different silver concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant difference to the respective control ($p<0.05$).

3.4.1.3 *In vitro* cytotoxicity assays

The viability of A6 cells, after 24 and 48h of exposure to Ag, displayed no clear concentration related effect. Thus, significantly decreased viability was found after 24h exposure to 0.006 and 0.2 mg/L whereas a significantly higher viability was induced by 0.1 mg/L. After 48h, decreased viability was found after exposure to 0.003 and 0.2 mg/L (Fig. 17; $p<0.05$). At 72h, dose dependent effects were observed with significantly lower viability found at all tested concentration (Fig. 17; $p<0.05$).

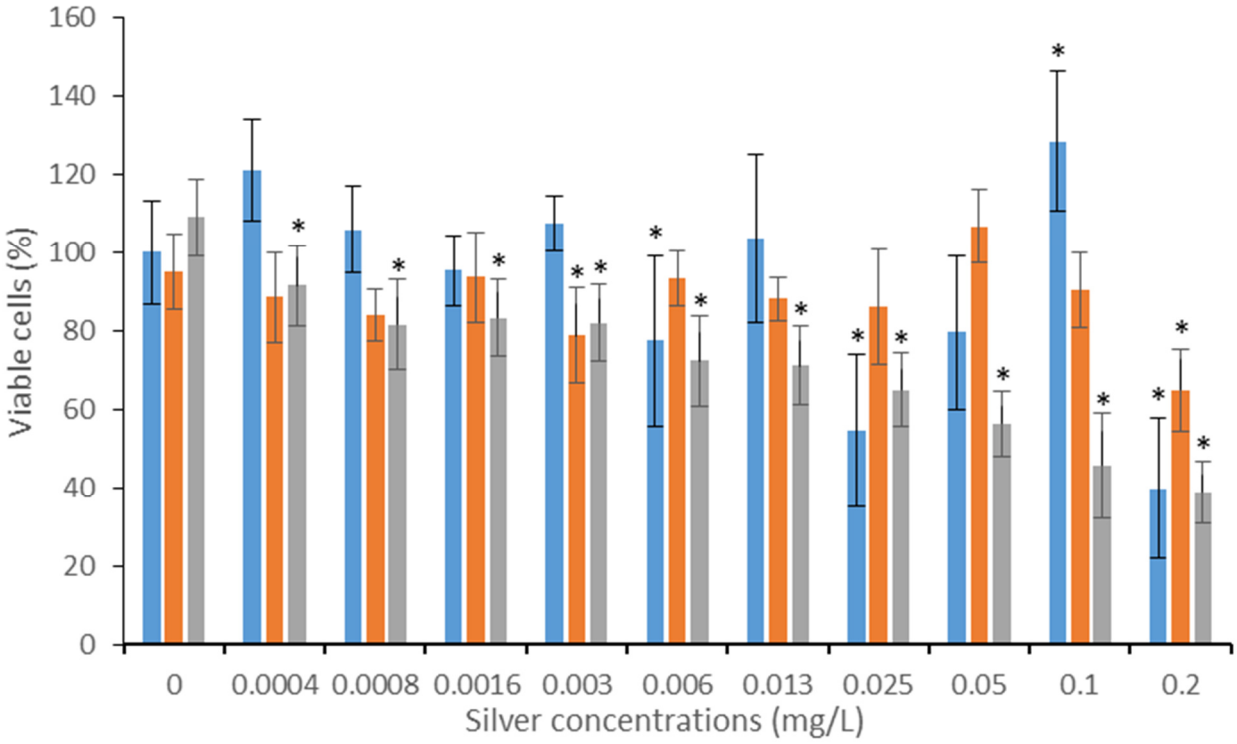

Figure 17: Cell viability of *X. laevis* kidney cells after 24h (blue), 48h (orange) and 72h (grey) exposure to different lead concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant difference to the respective control ($p<0.05$).

3.5 Discussion

3.5.1 Embryos

There is not much information about silver toxicity towards embryos of amphibians, specifically for *X. laevis*, no studies were found in the literature. For other amphibian species, only one work was found assessing the toxic effects of silver to embryos of amphibians. Birge & Zuiderveen (1995) reported values of LC_{50} for embryos of six amphibian species

ranging from 10 (*Gastrophryne caroliensis* and *Rana palustris*) to 240 (*Ambystoma opacum*) µg/L of Ag. In the present work, no significant mortality was registered for embryos of *X. laevis* exposed to silver concentrations up to 0.15 mg/L (i.e. 150 µg/L). However, sublethal effects, that may impair the fitness of the organisms, were registered at 0.013 mg/L (13 µg/L), which is a concentration similar to the ones reported by Birge & Zuiderveen (1995) to be lethal for other anuran species.

3.5.2 Tadpoles

As for embryos, there is little information available about the effects of silver on tadpoles. Two studies were found, reporting the toxicity of silver to tadpoles. Khangarot and Ray (1985, 1987) reported 96-h LC_{50} values of 4.1 µg/L for *Bufo melanostictus* and of 25.7 µg/L for *Rana hexadactyla*. In the present study, an LC_{50} could not be computed for the tadpoles of *X. laevis*, however 100% of mortality was observed at 0.067 mg/L. However, sublethal effects were registered at concentrations of 13 mg/L in growth rate and heartbeat. These effects may have implications later in the development of the organisms, for example by delaying metamorphosis. The toxic effects of silver have been mainly associated to its ionic form. Navarro et al. (2008) and Monfared & Soltani (2013) hypothesized that silver released from nanoparticles can disrupt ion uptake in the gills (Na^+, Cl^- and H^+), which initiates a complex chain of events, which, among others, may lead to a cardiovascular collapse. Actually, in the present work significant changes in the heartbeat of tadpoles was observed at the two lowest tested concentrations, which could be related to this mode of action.

3.5.3 Kidney Cells

No clear concentration Ag concentration related effect was observed in the first 48h of exposure. A concentration eliciting significant cell variability decrease at 24h, 0.025 mg/L) did not induce significant effects at 48h, suggesting that cells at this period may be trying to adapt (e.g. through the activation of scavenging molecules observed *in vivo* like reduced glutathione or metallothionein). However, a clear dose response effect was observed after 72h even at the lowest tested concentration suggesting surpassed defenses. Further

studies should be performed to be assess the mechanisms associated with the susceptibility of A6 cells to Ag. There is a clear lack of studies concerning the *in vitro* effects of silver despite the recognition that most of the silver nanoparticles effects are related with a release of the ion.

3.6 Conclusion

The lack of data on the toxicity of silver to amphibians highlights the need of generating such knowledge. The present work is a step forward in producing such type of information, highlighting that realistic environmental concentrations of silver (40 µg/L; Rozan et al., 1995; Howe and Dobson, 2002) may cause adverse effects in early stage of development of *X. laevis*. Furthermore, the high sensitivity of cell lines A6 to these metals, suggest its adequacy to be used as surrogates of *in vivo* toxicity assays that involves the exposure embryos or tadpoles.

Chapter IV

Evaluation of the toxicity of polystyrene to aquatic life stages of *X. laevis*

4. Evaluation of the toxicity of polystyrene to aquatic life stages of *X. laevis*

4.1 Introduction

Environmental contamination caused by small plastic particles has been increasingly reported in a wide range of habitats, including remote and protected areas (Sá et al., 2018). The wide range of applications of plastics and their attractive properties for different areas of human activities (e.g. construction, automobile industry, clothing, food processing and biomedical), has led to increasing production since the 1950's with and estimated world production of 348 million tons in 2017 (PlasticsEurope, 2018). The low price of these materials, which supported its high usage in single use applications like packaging, allied to a global improper management of plastic disposal is responsible for its release into the environment (Oliveira et al., 2019). Some of the characteristics that make plastics attractive (e.g. to industrial applications), like resistance to corrosion and durability, turned plastic materials into dangerous environmental contaminants. Currently, particles of different sizes may be found in the environment as a result of plastic degradation by abiotic (e.g. UV radiation and wave action) and biotic factors or by direct release of small particles associated with their use in personal care products and paints. As for other types of contaminants, the aquatic environment is considered as an important destination/target of plastic pollution.

The concerns associated with the presence of small plastic particles in the environments are related with their reported ingestion in different types of organisms (which increases with plastic particle size decrease) and their potential direct effects, effects of chemicals used in their synthesizes, and of additives used to endow plastics with particular characteristics (e.g. phthalates and bisphenol A) and their ability to adsorb other environmental (e.g. polycyclic aromatic hydrocarbons contaminants and metals) to serve as a trojan horse for their incorporation into biota used (Oliveira & Almeida, 2019). Among the reported effects of microplastics (defined as having sizes between 5 mm and 100 nm) are effects on feeding, alterations of hormone levels immune and reproductive systems, inhibition of growth, decreased predatory efficiency and even death (Sá et al., 2018). However, for smaller sizes (nanoplastics), the effects are less studied with reported altered levels of enzymatic activities associated with neurotransmission in mussels and fish and ability to affect immune system (Brandts et al., 2018). Nonetheless, more studies need to be

conducted to understand the environmental consequences of nanoplastics in the environment.

Amphibians are currently facing high risk of extinction. The presence of environmental contaminants in their natural habitats may significantly be contributing to the extinction pressure they are currently suffering. In this perspective, it becomes highly relevant to study the effects that small plastic particles may have on these organisms as they are reported ubiquitous, including in areas inhabited by amphibians (Hu et al., 2018). Thus, considering the scarce number of studies with nanoplastics and vertebrate organisms and the relevance of understanding how environmental contaminants impact amphibians, this study was designed to assess the effects of 50 nm polystyrene nanoplastics (PS NPs) on aquatic life stages of *X. laevis.*

4.2 Materials and Methods

4.2.1 Tested particles

The particles selected for this study were made of polystyrene. This polymer is among the most produced worldwide (*e.g.* for packaging - food storage, wrapping, disposable cutlery and industrial packaging). Due to its low reuse, particles of this polymer are commonly found in the aquatic environment with reports of formation of nanoplastics. The particles used in this study, were synthesized at the Aveiro Institute of Materials (CICECO), using the procedures described by Rabelero et al. (1997) and Tang et al. (1984). Particles characteristics like shape, diameter, size dispersion (average size) were analyzed by scanning electron microscopy (SEM - Hitachi SU-70). The hydrodynamic size was analyzed using a Malvern zeta sizer Nano ZS equipment.

Polystyrene nanoparticles (PS NPs) presented a round shape and normal distribution and an average size of 22 nm in ultra-pure water. The hydrodynamic size, in ultrapure water, was 59.25 nm.

4.2.2 Test species

The amphibian model species selected to perform the *in vivo* toxicity assays was the African clawed (*X. laevis*). Adults of this species were maintained in laboratories of the Department of Biology, University of Aveiro (as described in Chapter II). To obtain the eggs

and tadpoles that were used in the *in vivo* toxicity assays, the same procedure described in Chapter II was used (please see section 2.2.2 of that chapter for further details).

A part of the obtained eggs was immediately used for the embryonic teratogenicity assay. The remaining eggs were maintained in recipients filled with the artificial medium FETAX, under constant aeration, until they reach the Nieuwkoop and Faber (NF) developmental stage 45-46 (when the mouth of the tadpoles opens and they start to feed independently) (Nieuwkoop and Faber, 1994).

4.2.3 *In vivo* toxicity assay

4.2.3.1 Embryo teratogenicity assays

The viable embryos were placed in a recipient filled with FETAX medium, and before their use on the toxicity assays, the NF stage was checked under a stereo microscope (Zeiss Stemi 508).

Embryos at developmental stage NF 6-8 were exposed to polystyrene by following the protocol for the Frog Embryo Teratogenesis Assay with minor changes (ASTM, 2012). Organisms were exposed to a control (consisting of FETAX medium) and to the following concentrations of polystyrene: 3.95, 5.95, 8.89, 13.33, 20, 30 mg/L of polystyrene (these concentrations were chosen following the results of previous preliminary assays). Four and three replicates were carried out for the control and each polystyrene concentration, respectively. Fifteen embryos (selected randomly) were introduced per Petri dishes (diameter of 6 cm) filled with 10 ml of the test solution. Exposure occurred for 96 h in a climatic chamber with control conditions of temperature (23 ± 1ºC) and a photoperiod of 12:12 h light:dark. Medium of all test solutions was changed after 48h of exposure. Mortality was monitored daily and dead organisms were counted and removed from the Petri dish to avoid the growth of microorganisms and maintain the good quality of the medium. In addition to mortality, there were two other endpoints monitored: malformations and hatching rate.

4.2.3.2 Tadpoles toxicity assay

The toxicity assay with tadpoles was performed by following to the protocol of ASTM (2002) to run assays with larvae of amphibians, with some minor adjustments. When tadpoles reached the developmental stage NF 45-46 were exposed to a control (consisting of

FETAX medium) and to the following polystyrene concentrations: 3.95, 5.95, 8.89, 13.33, 20, 30 mg/L of polystyrene (these concentrations were selected based on results from previous preliminary assays). Four replicates were performed for the control and each tested polystyrene concentration. Each replicate consisted of a high-density plastic recipient containing 200 mL of the test solution, 0.0125 g of TetraminTM and four tadpoles. Exposure lasted for 14 days, in a climatic chamber, under constant aeration and controlled conditions of temperature (23 ± 1ºC) and photoperiod (12:12 h light:dark). Test solutions were changed every 48 hours with the addition of 0.0125 g of TetraMin™. Tadpole mortality was checked daily and dead tadpoles were registered and removed to avoid any growth of microorganisms and to avoid deterioration of the quality of test solutions. In addition to mortality the following endpoints were also monitored at the end of the assay: the length of snout to vent (SVL), tail (TL) and total body (TBL), weight and stage of development (NF). At the beginning of the assay 11 tadpoles were randomly selected to measure their length (average ± standard deviation: 11.78 ± 1.19 mm) and weight (average ± standard deviation: 0.018 ± 0.003 g) to allow compute growth and weight gain rates. At the end of the assay all length measurements were performed by photographing all tadpoles and afterwards their body, tail and total length were measured in the computer by using the ImageJ software. Growth and weight gain rates were measured according to equation 5 and 6, respectively:

(5) $GR = (lnL_f - ln\bar{L}_i)/(t_2 - t_1)$, where L_f is, respectively, the final length of SVL, TL or TBL of the organisms, $\bar{L}_i$ is the average of the tadpole's weights at the start of the assay and t_2-t_1 correspond to the exposure period (in days).

(6) $WgR = (ln\overline{W}_i - ln\, W_f)/(t_2 - t_1)$, where W_f is the final weight of tadpole, $\overline{W}_i$ is the average of the tadpole's weights at the start of the assay and t_2-t_1 correspond to the exposure period (in days).

During the assay the following parameters were measured: the pH, conductivity and dissolved O_2 values were measured at the fresh and old medium by using a WTW 3410 meter.

4.2.4 Data analysis

To identify significant differences in the monitored responses between organisms exposed to the control and NP concentrations, a one-way analysis of variance was carried out followed by the multi-comparison Dunnett's test. Normality of data was checked through the Shapiro-Wilk's text and the homoscedasticity of variances through the Bartlett's test. Whenever normality of data or homoscedasticity of variances failed to be met, the Kruskal-Wallis one-way analysis of variance on ranks was performed followed by the Dunn's test. These analyses were carried out in the in SigmaPlot (v12.5) software.

4.3 Results

4.3.1. *In vivo* toxicity assays

4.3.1.1 Embryo teratogenicity assay

Mortality in the control was 10% whereas in the PS NPs treatment it was below 20% for all the tested concentrations of polystyrene.

After 24 hours of exposure, all embryos from the control and from concentrations 5.93, 13.33 and 20 mg/L PS NPs had already hatched (Fig. 18). However, at 3.95, 8.89 and 20 mg/L PS NPs, only 50% of larvae hatched after 24 h, the remaining only hatched after 48 h (Fig. 18), though no significant difference were observed relatively to the control (Fig. 18; $p>0.05$).

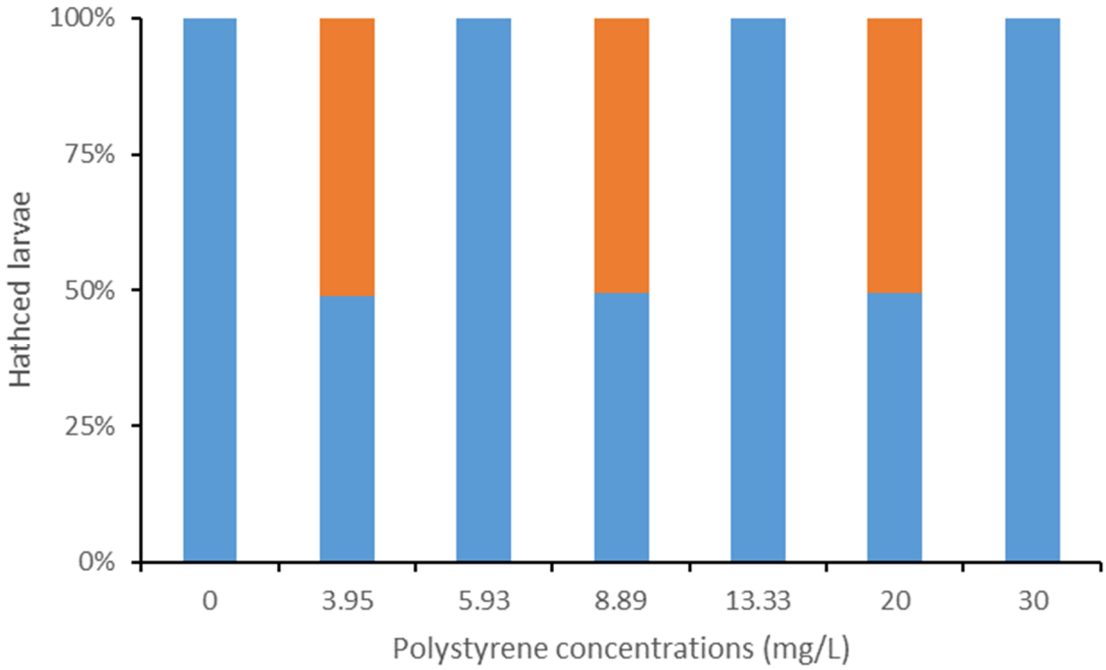

Figure 18: Hatched larvae of *X. laevis* exposed for 24 h (blue) and 48 h (orange) to different polystyrene nanoparticles concentrations.

Although an increase in the number of tadpoles exhibiting malformations was registered at PS NPs concentrations, they were not significantly different from the control (Fig. 19, 20; $p>0.05$).

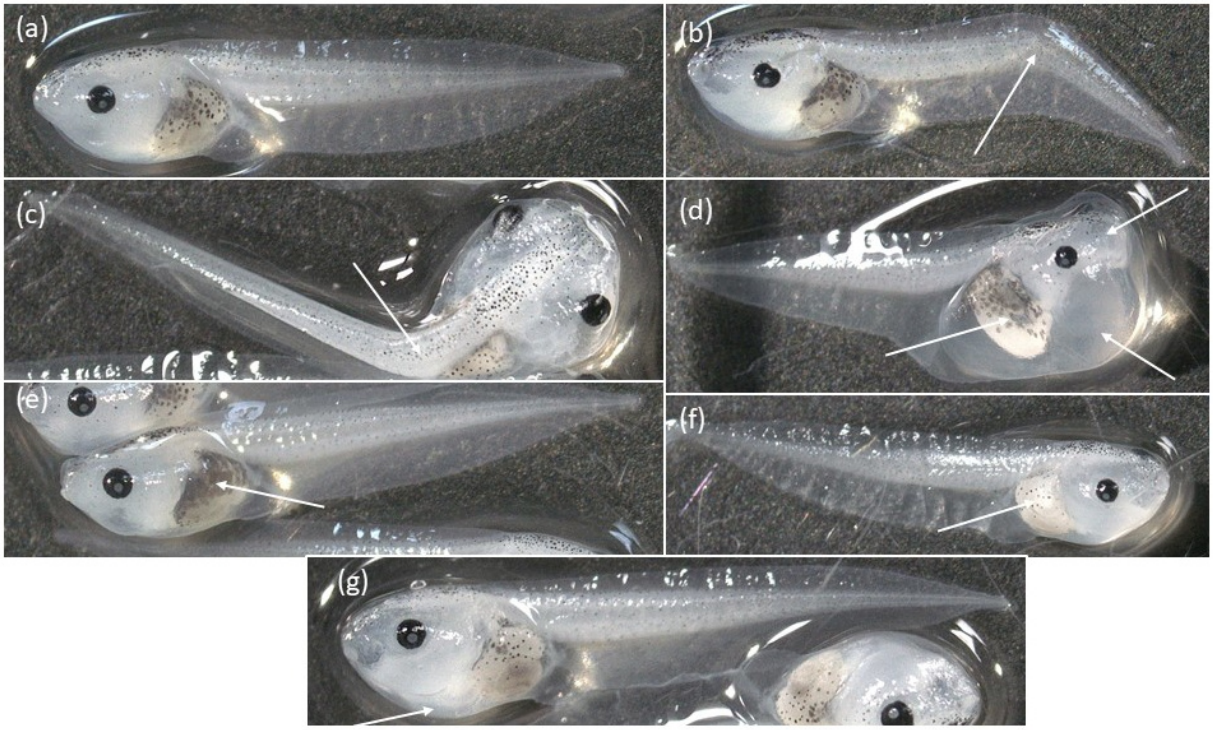

Figure 19: Larvae of *X. laevis* after 96h exposure to polystyrene nanoparticles (PS NP), photographed with a digital camera (VWE VisiCam HDMI 6) under a 0.63x magnification. (a) Control larva without malformations; (b) larva exposed to 5.93 mg/L of PS NP, showing a bent tail; (c) larva exposed to 13.33 mg/L of PS NP, showing a bent notochord; (d) larva exposed to 20 mg/L of PS NP, the upper arrow shows a craniofacial edema, the left arrow shows an enlarged trunk and the bottom arrow shows a heart edema; (e) larva exposed to 3.95 mg/L of PS NP, showing an excess of pigmentation (hyperpigmentation); (f) larva exposed to 30 mg/L of PS NP, shows a lack of pigmentation (hypopigmentation); (g) larva exposed to 13.33 mg/L of PS NP, shows a edema in the abdomen. The arrows indicate the type of malformation and their location.

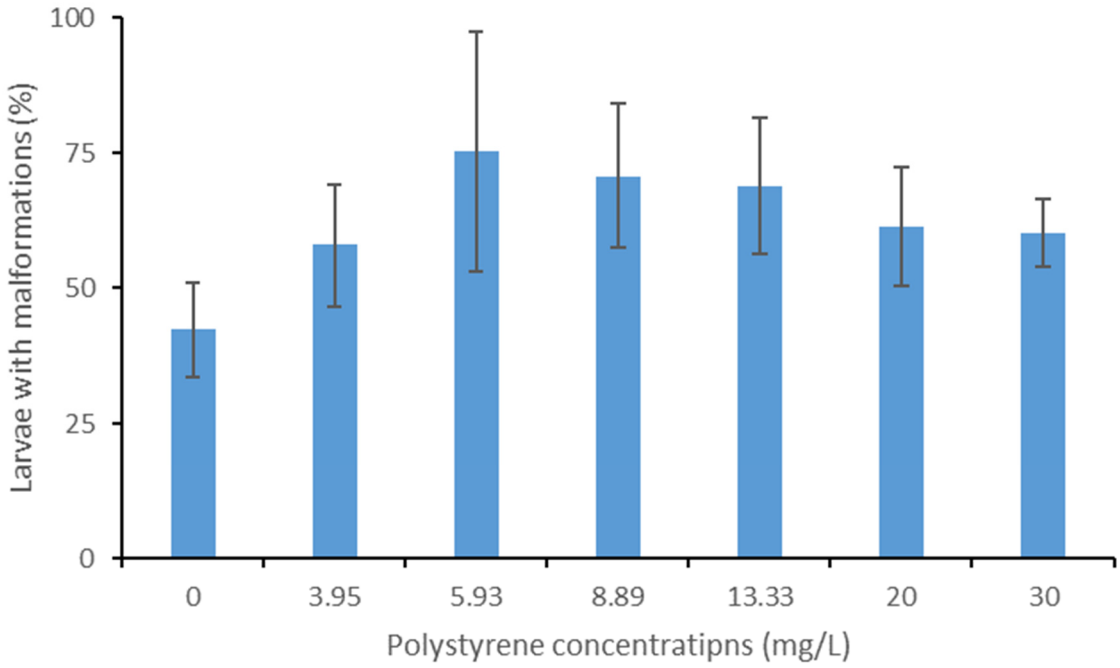

Figure 20: Average of the percentage of alive larvae of *X. laevis* with malformations, after 96 h exposure to different polystyrene nanoplastic concentrations. Results are expressed as mean ± standard deviation (error bars).

Tadpoles exposed to different PS NPs concentrations elicited significant changes in the length of tadpoles (Fig. 21; $p<0.05$). For SVL, a significant reduction occurred at 3.95 mg/L and 30 mg/L of PS NPs (Fig. 21; $p<0.05$), when compared with the control. Both for the TL and TBL, a significant decrease relatively to the control was only observed at 3.95 mg/L of PS NPs (Fig. 21; $p>0.05$).

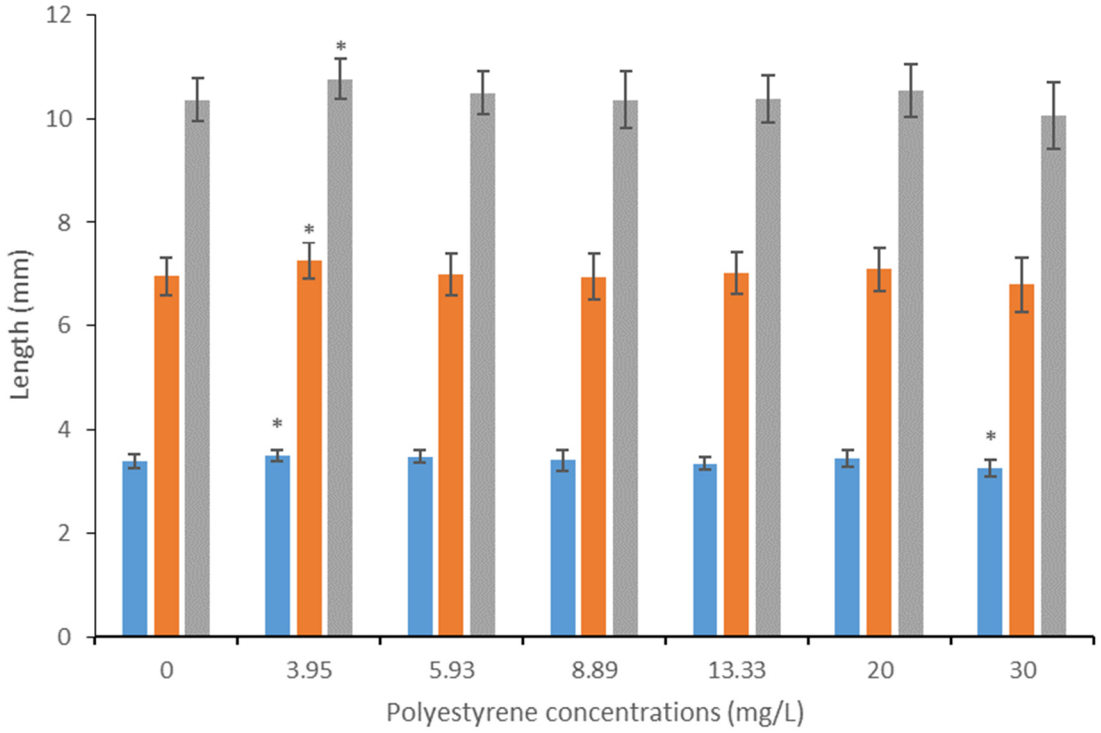

Figure 21: Sout-to-vent (blue), tail (orange) and total length (grey), of *X. laevis* tadpoles exposed for 96 h to different polystyrene nanoplastics concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes significant difference to the respective control ($p<0.05$).

4.3.1.2 Tadpoles toxicity assays

Mortality in the controls was 13% and in PS NPs treatments was below 20%, except for 3.95 mg/L, that presented a 25% of mortality. In the other tested concentrations of PS NPs, mortality was 6% for 5.95 mg/L, 8.89 mg/L and 30 mg/L and 13% for 13.33 mg/L and 20 mg/L.

Weight gain rate (Fig. 22) of tadpoles exposed to PS NPs was slightly lower than that of tadpoles exposed to the control, though no significant differences were observed (Fig. 22; $p>0.05$).

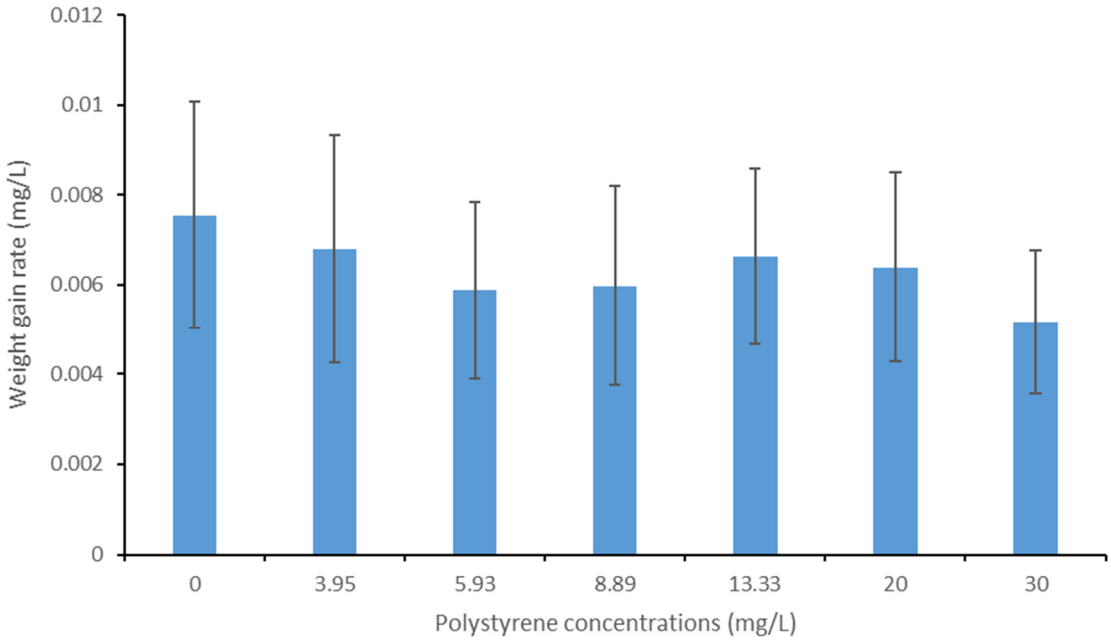

Figure 22: Weight gain rate of *X. laevis* tadpoles, after 14 d of exposure to different polystyrene nanoplastics concentrations. Results are expressed as mean ± standard deviation (error bars).

In terms of length measurements (SVL, TL and TBL), exposure of tadpoles to PS NPs concentrations induced no significant changes in these parameters, relatively to the control (Fig. 23; $p>0.05$).

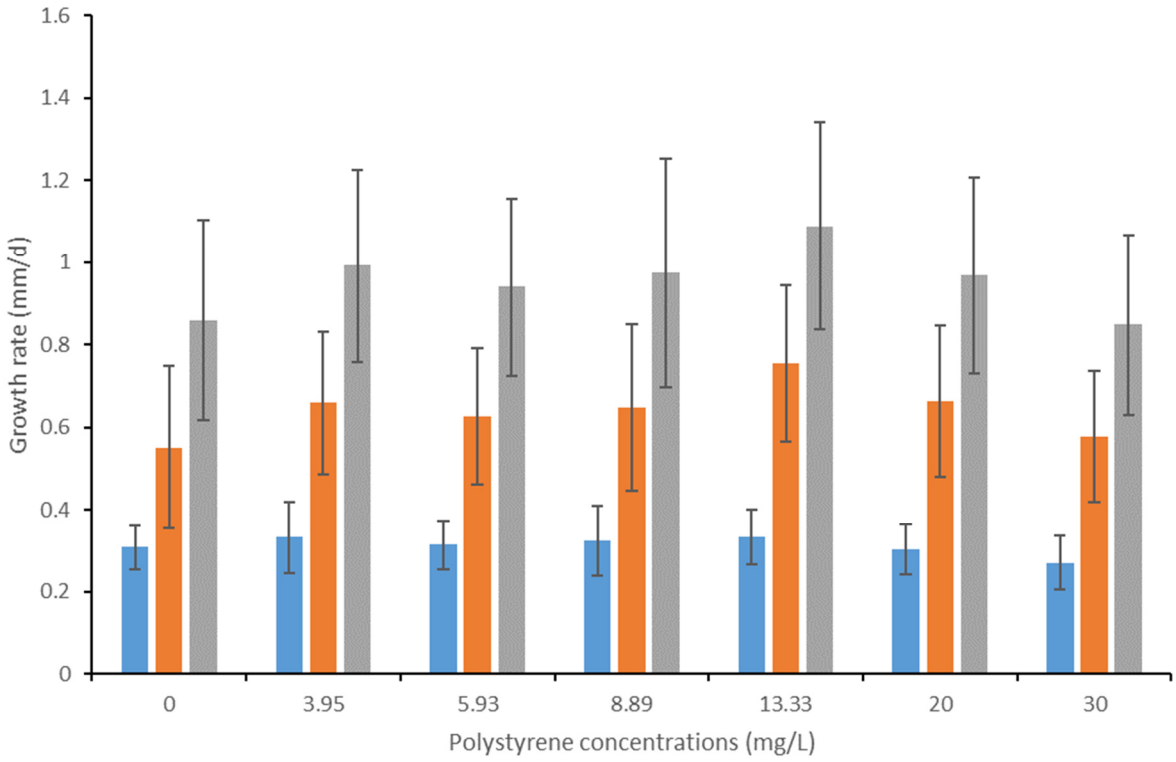

Figure 23: Growth rate of snout to vent (blue), tail (orange) and total length (grey) of *X. laevis* tadpoles exposed, for 14 d, to different polystyrene nanoparticle concentrations. Results are expressed as mean ± standard deviation (error bars).

In terms of development stage, no significant differences were observed between tadpoles exposed in the control and to the PS NP concentrations (Fig. 24; $p>0.05$).

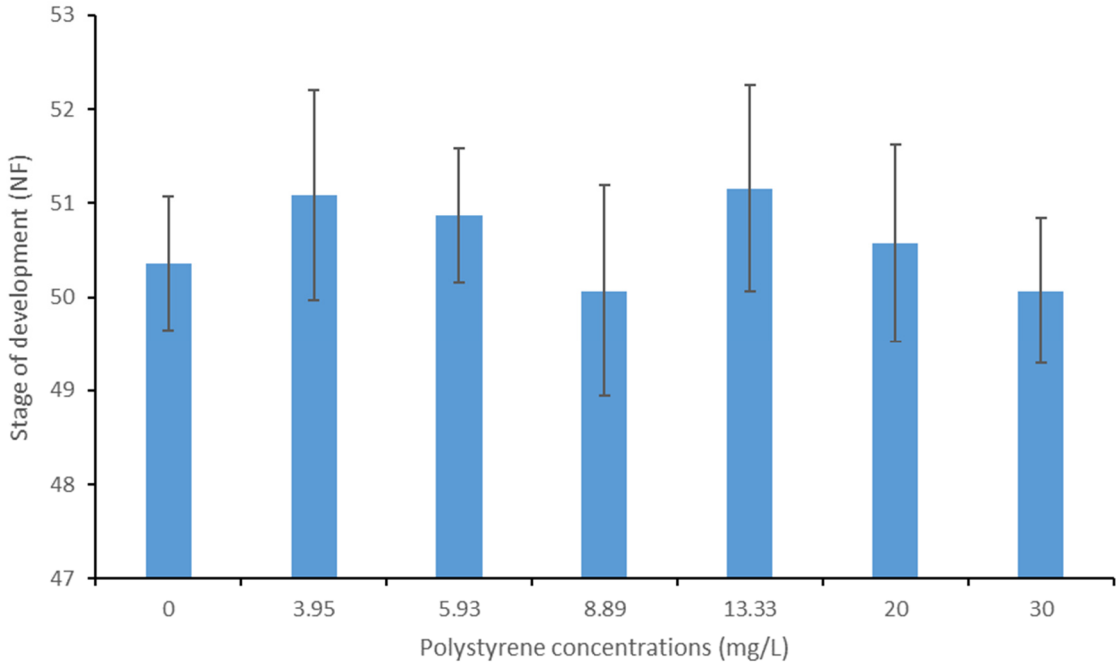

Figure 24: Developmental stage (NF) of *X. laevis* tadpoles exposed, for 14 d, to different polystyrene nanoparticles concentrations. Results are expressed as mean ± standard deviation (error bars).

Regarding the number of heartbeats, a significant decrease, relatively to the control, was measured in tadpoles exposed to 13.33 and 20 mg/L (Fig. 25A; $p<0.05$). For the heart area measurements, PS NPs elicited no significant effects when compared to control (Fig. 25B; $p>0.05$).

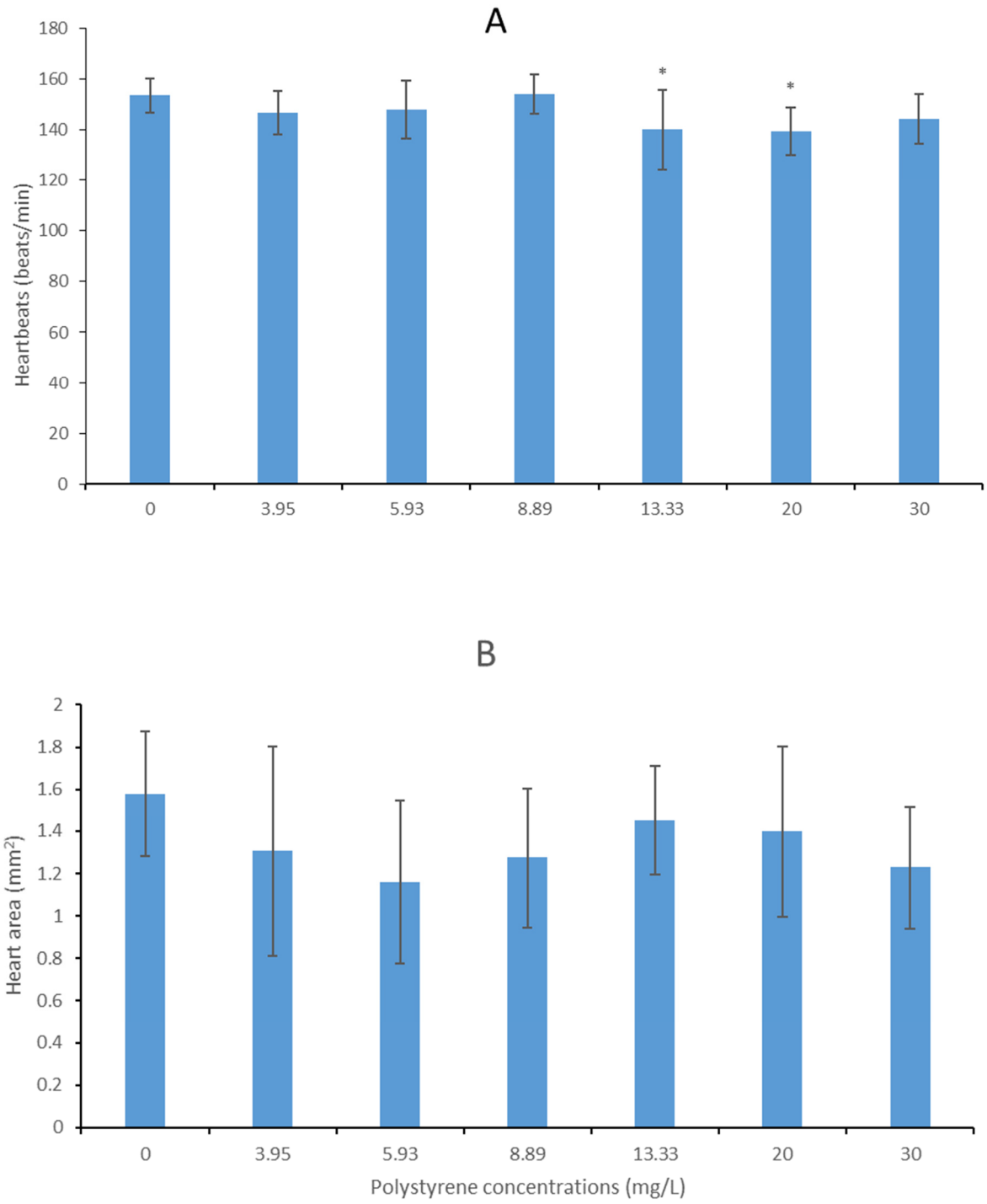

Figure 25: Heartbeats (A) and heart area (B) of *X. laevis* exposed, for 14 d, to different polystyrene nanoparticle concentrations. Results are expressed as mean ± standard deviation (error bars). * denotes means significant difference to the respective control ($p<0.05$).

4.4 Discussion

4.4.1 Embryos

There is little information about the effects of polystyrene nanoplastics on amphibians. Data from the present study shows that when embryos were exposed to polystyrene nanoplastics no pernicious effects were found. However Tussellino et al. (2015) study reported that *X. laevis* embryos exposed to 9 and 18 mg/L of 50 nm polystyrene nanoparticles presented tail malformations with regard to length and shape, head malformations and edemas. The contradictory finding may be related with size specific effects as the effects of nanoparticles have been reported to vary considerably with the size of the particles, the surrounding environment and their agglomeration behavior that may compromise their bioavailability.

4.4.2 Tadpoles

In the present study, when tadpoles were exposed to polystyrene nanoplastics there were some effects namely in terms of a delay of the stage of development and on tadpoles' heartbeats frequency. Such effects were not previously reported in any other study.

In Hu et al., 2016 study, *X. laevis* tadpoles were exposed for 48h to polystyrene microparticles (1 and 10 μm) and particles were found present in gills, alimentary canal, stomach and intestine. The presence of particles inside the organisms, in addition to other potential pernicious effects lay lead to blockage, physical injury and altered feeding which may lead to a sublethal effect like different growth.

De Felice et al. (2018) proved that even though *X. laevis* tadpoles may ingest polystyrene microplastics in early life stages it does not affect their survival, body growth or swimming ability. The particles used in this study were considerably smaller and the potential toxic effects may be associated with other mechanisms as supported by the lack of effect in body weight or length measurements. Nonetheless, more studies should be performed assessing endpoints (e.g. associated with neurotransmission reported to be affected by polystyrene nanoplastics) (Brandts et al., 2018).

4.5 Conclusion

There is a considerable lack of knowledge in terms of the effects of nanoplastics to vertebrate organisms. The endpoints assessed in the present study shoe that effects may not involve altered growth or development. Nonetheless, effects like alterations on heartbeat justify further studies on this type of contaminant to amphibians.

Chapter V
Final considerations and future perspectives

5. Final considerations and future perspectives

This study had as main objectives to assess the effects of lead (Chapter II), silver (Chapter III) and 22 nm polystyrene nanoplastics (Chapter IV) on different life stages of *X. laevis* as well as to evaluate the potential value of A6 (XLK-WGATCC® CRL-2527™) cell lines (established from X laevis kidney) as a model to assess the effects these contaminants.

Overall, for lead, effects were detected at concentrations that may be higher than those reported for freshwater environments that are within 0.18 to 179 µg/L (Mutia et al., 2012). Nonetheless, lead has demonstrated the ability to promote an earlier hatching of these organisms, in general at concentrations higher than 0.22 mg/L (Chapter II). The ability to induce significant developmental alterations was also observed at concentrations higher than 1.66 mg/L. The observed lead induced malformations included edemas, bent notochord and tails and protruding eyes. In addition, embryos growth was impaired at concentrations higher than 0.22 mg/L for head, 1.11 mg/L for tail and 0.74 mg/L for total length.

Exposure of animals at the tadpole stage, revealed that this stage is slightly less sensitive to lead than embryos. Thus, effects on weight gain were detected at concentrations starting at 0.49 mg/L whereas, for growth rate, effects were detected for concentrations higher than 0.74 mg/L. There was a general trend for a delayed development stage with increasing lead concentrations although only significantly for concentration 0.74 mg/L and higher.

Silver (Chapter III) induced a similar response trend in terms of hatching rate, promoting earlier hatching, but at lower concentrations (0.013, 0.019, 0.029,0.044 mg/L). However, this effect was not observed for higher concentrations. Malformations were detected at all tested concentrations (0.013-0.15 mg/L), and included the same types except protruding eyes. Considering embryos growth, this parameter was also more sensitivity to silver than lead. Head was the most affected with significant effects detected at all tested concentration but, in terms total length effects were detected only at concentrations higher than 0.019 mg/L (except 0.029 mg/L).

Tadpoles, as observed for lead, were less sensitive than embryos. However, unlike the previously mentioned parameters, in terms of weight gain, and growth rate organisms were less sensitive to silver with no effects detected on weight gain in the tested concentrations and for growth, no significant effects were detected in terms of head and for tails and total length, effects were only found for 0.013, 0.019 and 0.029 mg/L. In terms of development stage, a single concentration induced effects when compared to control (0.019 mg/L which promoted faster development). For silver, heart beat and heart area were also assessed revealing the ability of this element to induce bradycardia at 0.013 and 0.018 mg/L with no effects in terms of area.

The tested nanoplastics revealed an overall lower toxicity to the tested amphibians. Thus, no effects on hatching rate were found in the tested concentrations (3.95-30 mg/L). Despite a trend to increase malformations, no significant effects were found whereas in terms of growth, effects were only found at the lowest and highest tested concentrations which induced increased sized head.

For tadpoles no effects were found in terms of weight gain, growth rate, stage of development. However, in terms of effects on the heart 13.33 and 20 mg/L induced bradycardia without any effects on the heart area.

Overall, the data from this study suggests that exposure to these contaminants alone may have little effects on amphibian population considering that effects were observed at concentrations considerably higher than those reported in the environment. However, considering that amphibians live in areas contaminated with several contaminants, the potential toxic effects of combined exposures should be taken into consideration.

For the assessed endpoints, embryos were more sensitive stage and hatching proved the most sensitive endpoint. Thus, for *in vivo* exposures, based on the obtained data, the use of embryos should be promoted based on sensitivity as well as ethical considerations associated with the use of animals.

For cells, these biological models demonstrated to be less sensitive to the tested contaminants, in terms of lethality (cells *versus* organisms). However, it has to be emphasized that only cell viability (through mitochondrial metabolism), was assessed in the present study. Other biological responses (e.g. gene expression or enzymatic activities)

should be assessed. The reason for the lower sensitivity of the cell models should be investigated but may be associated with the cell culture media components (e.g. serum bovine albumin) that may bind the tested contaminants making them less available to induce toxic effects on cells. Additionally, the toxicity of contaminants is also influenced by the type of cells which have different metabolic activities and defenses. Other cell lines should also be considered in the assessment of the *in vitro* effects of environmental contaminants.

In terms of plastics, the obtained data suggest low toxicity of the tested nanoplastics. These results do not allow us to infer a lack of toxicity of nanoplastics to these organisms. For nanoplastics, different factors may lead altered bioavailability (e.g. altered size). Further studies should be performed with nanoplastics of different sizes studying the behavior of particles in the test media and its link with the effects on the different stages of organisms. Considering that the ability of nanoplastics to aggregate and this become less bioavailable increases with concentration increase (i.e. the probability of particles to collide increases with concentration), the use of lower concentrations should also be considered (Silva et al., 2020). Furthermore, concerns associated with the presence of plastic particles in the environment are based on the ability of the particles to adsorb environmental contaminants and serve as a trojan horse for their entrance on biota as well as release of additive used to endow plastics with specific characteristics, which are often highly toxic (Oliveira & Almeida, 2019). Thus, despite the lack of effects detected in the individual exposure, the tested nanoplastics may have a considerable impact in complex environments.

Final considerations and future perspectives

In order to understand the mechanisms associated with the observed effects, the used of biochemical and molecular biomarkers should be considered. This approach should also involve the quantification of the contaminants present in the tissues, as well as, for the nanoplastics the study of the behavior in the tested media (e.g. assessing size and surface charge, known to modulate toxic effects).

Based on the data obtained in the present work, future studies should be performed with combined exposures to the tested contaminants, at environmentally relevant concentrations, a more realistic exposure scenario.

Considering nanoplastics, different sizes of polystyrene and other polymeric particles should be tested. This approach is considerably important given the need to assess the toxicity of these ubiquitous environmental contaminants that may have unforeseen effects on the long term.

Taking into account that amphibians are under the stress of population decline, the validation/development of cell lines should be promoted. In this perspective, more endpoints should be assessed and the use of bovine serum albumin in the toxicity testing validated (i.e. assessment of cell viability under different concentrations of serum which can serve protect the cells from the toxic effects of the tested contaminants) (Barreto et al., 2015).

References

T. M. Mutia • M. Z. Virani • W. N. Moturi • B. Muyela • W. J. Mavura • J. O. Lalah. 2012. Copper, lead and cadmium concentrations in surface water, sediment and fish, C. Carpio, samples from Lake Naivasha: effect of recent anthropogenic activities. Environ Eartj Sci. DOI 10.1007/s12665-012-1556-8.

Melek Türker Saçan, Fusun Oztay, Sehnaz Bolkent. 2007. Exposure of Dunaliella tertiolecta to Lead and Aluminum: Toxicity and Effects on Ultrastructure. Biological Trace Element Research. 120: 264–272

Naomi L.Cooper, Joseph R.Bidwell, Anu Kumar. 2009. Toxicity of copper, lead, and zinc mixtures to Ceriodaphnia dubia and Daphnia carinata. Ecotoxicology and Environmental Safety 22: 1523-1528.

Martin Grosell, Robert M. Gerdes, Kevin V. Brix. 2006. Chronic toxicity of lead to three freshwater invertebrates—Brachionus calyciflorus, Chironomus tentans, and Lymnaea stagnalis. Environmental Toxicology and Chemistry 25.

Anne E. Keller, Stephen G. Zam. 1991. The acute toxicity of selected metals to the freshwater mussel, Anodonta imbecilis. Environmental Toxicology and Chemistry 10.

NilantikaPaul, SamujjwalChakraborty, MahuyaSengupta. 2014. Lead toxicity on non-specific immune mechanisms of freshwater fish Channa punctatus. Aquatic Toxicology 152: 105-112.

Hayes, T.B. et al. 2010. The cause of global amphibian declines: a developmental endocrinologist's perspective. J.Exp. Biol. 213: 921–933.

Andrew R. Blaustein,1 Barbara A. Han,2 Rick A. Relyea,3 Pieter T.J. Johnson,4 Julia C. Buck,1 Stephanie S. Gervasi,1 and Lee B. Kat. 2011. The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. Ann. N.Y. Acad. Sci. 1223:108-119

Author links open overlay panelJ.Herkovits, CS.Pérez-Coll. 1991. Antagonism and synergism between lead and zinc in amphibian larvae. Environmental Pollution 2-3: 217-221.

F. Mouchet, S. Cren, C. Cunienq, E. Deydier, R. Guilet, L. Gauthier. 2006. Assessment of lead ecotoxicity in water using the amphibian larvae (*Xenopus laevis*) and preliminary study of its immobilization in meat and bone meal combustion residues. BioMetals 20:113–127.

Gloria L. Yeung. 1978. Influence of Lead, an Environmental Pollutant on Metamorphosis of Rana utricularia (Amphibia: Ranidae). Journal of the Arkansas Academy of Science, 32:83-86.

Quaranta A., Bellantuono V., Cassano G., Lippe C. Why amphibians are more sensitive than mammals to xenobiotics. PLoS ONE [Internet] 2009;4(11)

Helmer P., Whiteside D. Amphibian anatomy and physiology. In: O'Malley B., editor. Clinical Anatomy and Physiology of Exotic Species. Elsevier Saunders; Edinburgh: 2005. pp. 3–14.

Moermond CTA, van Herwijnen R.212. Environmental risk limits for silver in water. A proposal for water quality standards in accordance with the Water Frmework Directive. RIVM report 601714023/2012. National Institute for Public Health and the Environment. The Netherlands.

Domsch KH. 1984. Effects of pesticides and heavy metals on biological processes in soil. Plant Soil 76: 367–378.

Ratte HT. 2009. Bioaccumulation and toxicity of silver compounds: a review. Environmental Toxicity and Chemistry 18.

Fulladosa E, Murat JC, Martínez M, Villaescusa I. 2005. Paterns of metals and arsenic posion in Vibrio fischeri bacteria. Chemosphere 60:43-48.

Kolts JM, Boese CJ, Meyer JS. 2009. Effects of dietborne and silver on reproduction by Ceriodaphnia dubia. Environmental Toxicology and Chemistry 28.

Rozan TF, Hunter KS, Benoit G. 1995. Silver in fresh water: Sources, transport and fate in Connecticut rivers. Proceedings, 3rd Argentum International Conference on the Transport, Fate, and Effects of Silver in the Environment, Washington, DC, August 6–9, pp 181–184.

EC. 2018. Substance Evaluation Conclusion as required by REACH Article 48 and Evaluation Report for Silver. EC Nº 231-131-3. The Netherlands.

Holler, J. S., Fowler, B. A., & Nordberg, G. F. (2015). Silver. Handbook on the Toxicology of Metals, 1209–1216. doi:10.1016/b978-0-444-59453-2.00053-6.

Adams NWH, Kramer JR. 2009. Silver speciation in wastewater effluent, surface waters, and pore waters. Environmental Toxicology and Chemistry 18.

Mr P.D. Howe and Dr S. Dobson. 2002. Silver and silver compounds: environmental aspectsWorld Health Organization, Geneva, Switzerland.

Rowe CL Hopkins WA Bridges C. 2003. Physiological ecology of amphibians in relation to susceptibility to natural and anthropogenic factors. In: Linder G Krest S Sparling D, eds. Amphibian Decline: An Integrated Analysis of Multiple Stressor Effects. Pensacola: Society of Environmental Toxicology and Chemistry Press. p 958.

Morgan, I. J., Henry, R. P., & Wood, C. M. (1997). The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (Oncorhynchus mykiss) is inhibition of gill Na+ and Cl−1 transport. Aquatic Toxicology, 38(1-3), 145–163. doi:10.1016/s0166-445x(96)00835-1.

Rabelero, M., Zacarias, M., Mendizabal, E., Puig, J. E., Dominguez, J. M., & Katime, I. (1997). High-content polystyrene latex by microemulsion polymerization. Polymer Bulletin, 38(6), 695–700. https://doi.org/10.1007/s002890050107.

Tang, H. I., Johnson, P. L., & Gulari, E. (1984). Styrene polymerized in an oil-in-water

microemulsion. Polymer, 25(9), 1357–1362. https://doi.org/10.1016/0032-3861(84)90391-4.

Khangarot BS, Sehgal A, Bhasin MK (1985) Man and the biosphere, studies on the Sikkim Himalayas. Acute toxicity of selected heavy metals on the larvae of Rana hexadactyla. Act Hydroche Hydrobiol 13:259–263

M. Oliveira, M. Almeida and I. Miguel Trends Anal. Chem., 112 (2019), pp. 196-200, 10.1016/j.trac.2019.01.005.

Barreto, Â., Luis, L. G., Girão, A. V., Trindade, T., Soares, A. M. V. M., & Oliveira, M. (2015). Behavior of colloidal gold nanoparticles in different ionic strength media. *Journal of Nanoparticle Research*, *17*(12), 1–13. https://doi.org/10.1007/s11051-015-3302-0

Benvindo-Souza, M., Santos Oliveira, E. A., Assis, R. A., Araújo Santos, C. G., Borges, R. E., de Melo e Silva, D., & Raquel de Souza Santos, L. (2020). Micronucleus test in tadpole erythrocytes: Trends in studies and new paths. *Chemosphere*, *240*, 124910. https://doi.org/10.1016/j.chemosphere.2019.124910

Berzins, D. W., & Bundy, K. J. (2002). Bioaccumulation of lead in *Xenopus laevis* tadpoles from water and sediment. *Environment International*, *28*(1–2), 69–77. https://doi.org/10.1016/S0160-4120(02)00006-5

Brandts, I., Teles, M., Gonçalves, A. P., Barreto, A., Franco-Martinez, L., Tvarijonaviciute, A., … Oliveira, M. (2018). Effects of nanoplastics on Mytilus galloprovincialis after individual and combined exposure with carbamazepine. *Science of the Total Environment*, *643*, 775–784. https://doi.org/10.1016/j.scitotenv.2018.06.257

Burlibaşa, L., & Gavrilacaron;, L. (2011). Amphibians as model organisms for study environmental genotoxicity. *Applied Ecology and Environmental Research*, *9*(1), 1–15. https://doi.org/10.15666/aeer/0901_001015

Call, D. J., Polkinghorne, C. N., Markee, T. P., Brooke, L. T., Geiger, D. L., Gorsuch, J. W., & Robillard, K. A. (1999). Silver toxicity to Chironomus tentans in two freshwater sediments. *Environmental Toxicology and Chemistry*, *18*(1), 30–39.

https://doi.org/10.1897/1551-5028(1999)018<0030:STTCTI>2.3.CO;2

Chen, T. H., Gross, J. A., & Karasov, W. H. (2006). Sublethal effects of lead on northern leopard frog (Rana pipiens) tadpoles. *Environmental Toxicology and Chemistry*, *25*(5), 1383–1389. https://doi.org/10.1897/05-356R.1

Cong, L., Qin, Z., Jing, X., Yang, L., Zhou, J., & Xu, X. (2006). *Xenopus laevis* is a potential alternative model animal species to study reproductive toxicity of phytoestrogens, *77*, 250–256. https://doi.org/10.1016/j.aquatox.2005.12.014

De Felice, B., Bacchetta, R., Santo, N., Tremolada, P., & Parolini, M. (2018). Polystyrene microplastics did not affect body growth and swimming activity in *Xenopus laevis* tadpoles. *Environmental Science and Pollution Research*, *25*(34), 34644–34651. https://doi.org/10.1007/s11356-018-3408-x

Gonçalves, M. W., Vieira, T. B., Maciel, N. M., Carvalho, W. F., Lima, L. S. F., Gambale, P. G., … Silva, D. M. (2015). Detecting genomic damages in the frog Dendropsophus minutus: preserved versus perturbed areas. *Environmental Science and Pollution Research*, *22*(5), 3947–3954. https://doi.org/10.1007/s11356-014-3682-1

Haywood, L. K., Alexander, G. J., Byrne, M. J., & Cukrowska, E. (2004). *Xenopus laevis* embryos and tadpoles as models for testing for pollution by zinc, copper, lead and cadmium. *African Zoology*, *39*(2), 163–174. https://doi.org/10.1080/15627020.2004.11657213

Hu, L., Chernick, M., Hinton, D. E., & Shi, H. (2018). Microplastics in Small Waterbodies and Tadpoles from Yangtze River Delta, China. *Environmental Science and Technology*, *52*(15), 8885–8893. https://doi.org/10.1021/acs.est.8b02279

Hu, L., Su, L., Xue, Y., Mu, J., Zhu, J., Xu, J., & Shi, H. (2016). Uptake, accumulation and elimination of polystyrene microspheres in tadpoles of *Xenopus tropicalis*. *Chemosphere*, *164*, 611–617. https://doi.org/10.1016/j.chemosphere.2016.09.002

Khangarot, B. S. (1991). Toxicity of metals to a freshwater tubificid worm, Tubifex tubifex (Muller). *Bulletin of Environmental Contamination and Toxicology*, *46*(6), 906–912. https://doi.org/10.1007/BF01689737

Khangarot, B. S., & Ray, P. K. (1987). Sensitivity of toad tadpoles, Bufo melanostictus

(Schneider), to heavy metals. *Bulletin of Environmental Contamination and Toxicology*, *38*(3), 523–527. https://doi.org/10.1007/BF01606623

Lekamge, S., Miranda, A. F., Abraham, A., Li, V., Shukla, R., Bansal, V., & Nugegoda, D. (2018). The toxicity of silver nanoparticles (AgNPs) to three freshwater invertebrates with different life strategies: Hydra vulgaris, Daphnia carinata, and Paratya australiensis. *Frontiers in Environmental Science*, *6*(DEC), 1–13. https://doi.org/10.3389/fenvs.2018.00152

Monastersky, R. (2014). Quickly — But Researchers Are.

Monfared, A. L., & Soltani, S. (2013). Effects of silver nanoparticles administration on the liver of rainbow trout (Oncorhynchus mykiss): histological and biochemical studies. *Pelagia Research Library*, *3*(2), 285–289.

Mouchet, F., Cren, S., Cunienq, C., Deydier, E., Guilet, R., & Gauthier, L. (2007). Assessment of lead ecotoxicity in water using the amphibian larvae (*Xenopus laevis*) and preliminary study of its immobilization in meat and bone meal combustion residues. *BioMetals*, *20*(2), 113–127. https://doi.org/10.1007/s10534-006-9019-x

Naddy, R. B., McNerney, G. R., Gorsuch, J. W., Bell, R. A., Kramer, J. R., Wu, K. B., & Paquin, P. R. (2011). The effect of food on the acute toxicity of silver nitrate to four freshwater test species and acute-to-chronic ratios. *Ecotoxicology*, *20*(8), 2019–2029. https://doi.org/10.1007/s10646-011-0745-7

Navarro, E., Piccapietra, F., Wagner, B., Marconi, F., Kaegi, R., Odzak, N., … Behra, R. (2008). Toxicity of silver nanoparticles to Chlamydomonas reinhardtii. *Environmental Science and Technology*, *42*(23), 8959–8964. https://doi.org/10.1021/es801785m

Oliveira, M., & Almeida, M. (2019). The why and how of micro(nano)plastic research. *TrAC - Trends in Analytical Chemistry*, *114*, 196–201. https://doi.org/10.1016/j.trac.2019.02.023

Oliveira, M., Almeida, M., & Miguel, I. (2019). A micro(nano)plastic boomerang tale: A never ending story? *TrAC - Trends in Analytical Chemistry*, *112*, 196–200. https://doi.org/10.1016/j.trac.2019.01.005

Oukarroum, A., Gaudreault, M. H., Pirastru, L., & Popovic, R. (2013). Alleviation of silver

toxicity by calcium chloride (CaCl2) in Lemna gibba L. *Plant Physiology and Biochemistry*, *71*, 235–239. https://doi.org/10.1016/j.plaphy.2013.07.019

PlasticsEurope. (2018). Plastics – the Facts. *Plastics – the Facts 2018*, 38.

Riss, T. L., Moravec, R. A., Niles, A. L., Duellman, S., Benink, H. A., Worzella, T. J., & Minor, L. (2004). Cell Viability Assays. *Assay Guidance Manual*, 1–31. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23805433

Shafiuddin Ahmed, A. S., Sultana, S., Habib, A., Ullah, H., Musa, N., Belal Hossain, M., … Shafiqul Islam Sarker, M. (2019). Bioaccumulation of heavy metals in some commercially important fishes from a tropical river estuary suggests higher potential health risk in children than adults. *PLoS ONE*, *14*(10), 1–21. https://doi.org/10.1371/journal.pone.0219336

Silva, M. S. S., Oliveira, M., Lopéz, D., Martins, M., Figueira, E., & Pires, A. (2020). Do nanoplastics impact the ability of the polychaeta Hediste diversicolor to regenerate? *Ecological Indicators*, *110*(November 2019), 105921. https://doi.org/10.1016/j.ecolind.2019.105921

Slaby, S., Marin, M., Marchand, G., & Lemiere, S. (2019). Exposures to chemical contaminants: What can we learn from reproduction and development endpoints in the amphibian toxicology literature? *Environmental Pollution*, *248*, 478–495. https://doi.org/10.1016/j.envpol.2019.02.014

Sobotka, J. M., & Rahwan, R. G. (1995). Teratogenesis induced by short-and long-term exposure of *Xenopus laevis* progeny to lead. *Journal of Toxicology and Environmental Health*, *44*(4), 469–484. https://doi.org/10.1080/15287399509531975

Sparling, D., Linder, G., Bishop, C., & Krest, S. (2010). Recent Advancements in Amphibian and Reptile Ecotoxicology. *Ecotoxicology of Amphibians and Reptiles, Second Edition*, 1–11. https://doi.org/10.1201/ebk1420064162-c1

Sparling, D. W., Krest, S., & Ortiz-Santaliestra, M. (2006). Effects of lead-contaminated sediment on Rana sphenocephala tadpoles. *Archives of Environmental Contamination and Toxicology*, *51*(3), 458–466. https://doi.org/10.1007/s00244-005-0243-0

Srivastava, N. K. and C. B. M. (2008). Adsorbents Biofiltration Methods for the Treatment

of Heavy Metals From Industrial Wastewater. *Novel Advances in Environmental Research ,Journal of Hazardous*, *6*(533), 40.

Stansley, W., Kosenak, M. A., Huffman, E., & Roscoe, D. E. (1997). Effects of L E a D - C O N T a M I N a T E D Surface Water From a Trap a N D Skeet R a N G E on Frog H a T C H I N G a N D Development, *96*(1), 69–74.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., & Waller, R. W. (2003). Stuart2004, *8157*(2002). https://doi.org/10.1126/science.1103538

Tchounwou, P. B., Wilson, B. A., Ishaque, A. B., Schneider, J., & Toxicology, M. (2001). Transcriptional Activation of Stress Genes and Cytotoxicity in Human Liver Carcinoma Cells, 209–216.

Tussellino, M., Ronca, R., Formiggini, F., Marco, N. De, Fusco, S., Netti, P. A., & Carotenuto, R. (2015). Polystyrene nanoparticles affect *Xenopus laevis* development. *Journal of Nanoparticle Research*, *17*(2). https://doi.org/10.1007/s11051-015-2876-x

Venne, L. S., Cobb, G. P., Coimbatore, G., Smith, L. M., & McMurry, S. T. (2006). Influence of land use on metal concentrations in playa sediments and amphibians in the Southern High Plains. *Environmental Pollution*, *144*(1), 112–118. https://doi.org/10.1016/j.envpol.2006.01.003

Venturino, A., Rosenbaum, E., Caballero De Castro, A., Anguiano, O. L., Gauna, L., Fonovich de Schroeder, T., & Pechen de D'Angelo, A. M. (2003). Biomarkers of effect in toads and frogs. *Biomarkers*, *8*(4/3), 167–186. https://doi.org/10.1080/1354700031000120116

Wagner, N., & Lötters, S. (2013). Effects of water contamination on site selection by amphibians: Experiences from an arena approach with European frogs and newts. *Archives of Environmental Contamination and Toxicology*, *65*(1), 98–104. https://doi.org/10.1007/s00244-013-9873-9

Whiles, M. R., Lips, K. R., Pringle, C. M., Kilham, S. S., Bixby, R. J., Brenes, R., … Peterson, S. (2006). Whiles_et_al-2006-Frontiers_in_Ecology_and_the_Environment.

Yedjou, C. G., Tchounwou, H. M., & Tchounwou, P. B. (2015). DNA damage, cell cycle

arrest, and apoptosis induction caused by lead in human leukemia cells. *International Journal of Environmental Research and Public Health*, *13*(1). https://doi.org/10.3390/ijerph13010056

Yologlu, E., & Ozmen, M. (2015). Low concentrations of metal mixture exposures have adverse effects on selected biomarkers of *Xenopus laevis* tadpoles. *Aquatic Toxicology*, *168*, 19–27. https://doi.org/10.1016/j.aquatox.2015.09.006

Dawson, D. A., & Bantle, J. A. (1987). Development of a reconstituted water medium and preliminary validation of the Frog Embryo Teratogenesis Assay—Xenopus (FETAX). Journal of Applied Toxicology, 7(4), 237–244. https://doi.org/10.1002/jat.2550070403

ASTM, 2012. Standard Guide for Conducting the Frog Embryo Teratogenesis AssayXenopus (FETAX). ASTM International, West Conshohocken, PA, USA.

ASTM. (2002). Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fish, Macroinvertebrates, and Amphibians. ASTM E729-96, ASTM International. West Conshohocken, PA