Universidade de Aveiro
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Efeitos de cinzas de incêndios florestais de diferentes coberturas vegetais nos estados de desenvolvimento aquático de anfíbios

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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, investigadora Principal do Centro de Estudos do Ambiente e do Mar e do Departamento de Biologia da Universidade de Aveiro, e do Doutor Nelson José Cabaços Abrantes, investigador auxiliar do Centro de Estudos do Ambiente e do Mar e do Departamento de Ambiente e Ordenamento da Universidade de Aveiro.

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o júri

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palavras-chave Florestas de eucalipto e pinheiro, incêndios florestias, Ecotoxicidade, Anura, Estágios iniciais de desenvolvimento

resumo

As alterações climáticas têm provocado um aumento global das temperaturas da superfície da terra. Este fenómeno potenciou um aumento da frequência e intensidade de eventos extremos, aos quais está associado o aumento da ocorrência de incêndios florestais. Os Países localizados na bacia Mediterrânea são severamente afetados por estes tipo de ocorrências por apresentarem verões secos. A situação dos incêndios em Portugal é especialmente critica, porque para além dos verões quentes e secos, a sua floresta tem sido substituída por plantações mono específicas que facilitam a propagação dos incêndios. A ocorrência de incêndios, bem como a subsequente formação de cinzas pode ter um grande impacto ecológico. A formação de cinzas é um processo que converte combustíveis numa matriz composta por compostos orgânicos e inorgânicos, alguns potencialmente tóxicos. Contudo, a sua composição é condicionada pela cobertura vegetal. Durante os primeiros eventos de chuva, após a ocorrência de um incêndio, estas cinzas são arrastadas por escorrência superficiais para os corpos de água adjacentes, levando consigo elementos que podem ser tóxicos para os organismos aquáticos. Os estádios de desenvolvimento aquático de anfíbios (embriões e girinos) podem ser particularmente sensíveis a estas cinzas e aos compostos a elas adsorvidos. Deste modo, o presente estudo pretendeu avaliar a toxicidade de cinzas oriundas de incêndios de florestas com diferentes coberturas vegetais (*Eucaliptus globulus* e *Pinus pinaster*) para embriões e girinos de duas espécies de anfíbios – *Xenopus laevis* e *Pelophylax perezi*. Para tal, embriões de *X. laevis* e girinos de ambas as espécies foram expostos durante 96 h e 14 dias, respetivamente, a concentrações (26.9% - 100%) de extratos aquosos de cinzas (AEA, com 10g/L de cinzas) obtidas de incêndios de florestas de eucalipto (ELS) e de pinheiro (PLS). No final dos ensaios, foram analisados os seguintes parâmetros: mortalidade, estádio de desenvolvimento, comprimento corporal (total, rostro à cloaca, cauda) e peso (apenas em girinos). Efeitos a um nível sub-individual também foram monitorizados para stress oxidativo (catalase-CAT, glutationa-S-transferase-GST, glutationa total-TG, e peroxidação lipídica-LPO), neurotoxicidade (acetilcolinesterase-aChE), e metabolismo energético (consumo de oxigénio pelo sistema de transporte de eletrões-ETS, e conteúdos de carbohidratos, lípidos e proteína). A caracterização química das cinzas e dos AEA também foi efetuada. O AEA de ELS apresentou concentrações mais elevadas de As, Cd, Co, Cr, Pb e V, enquanto o de PLS de Cu, Mn, Ni e Zn. Embora tenha ocorrido uma mortalidade superior a 20% em embriões de *X. laevis* expostos a AEA de eucalipto e pinheiro, não foi significativamente diferente do controlo. Efeitos ao nível sub-letal foram apenas observados em embriões expostos a PLS: os quais apresentaram um conteúdo baixo de carbohidratos e elevado consumo de oxigénio. A exposição de girinos de *X. laevis* a ELS e PLS entre 10 e 35% de mortalidade, que não foi significativamente diferente do controlo, e um atraso nos estádios de desenvolvimento. Todas as concentrações testadas de ambos os AEA reduziram significativamente os comprimentos e pesos corporais de girinos de *X. laevis*. A um nível sub-individual, registou-se o decréscimo da atividade das enzimas CAT e GST e nos conteúdos de carbohidratos e lípidos. Um aumento no consumo de oxigénio foi apenas observado em girinos expostos a PLS. Quanto aos girinos de *P. perezi*, a exposição a ELS não induziu efeitos significativos nos organismos. No entanto, a exposição a PLS causou uma redução no comprimento corporal, peso, atividade de GST e um aumento no consumo de oxigénio. De um modo geral, PLS apresentou maior toxicidade para ambas as espécies, o que sugere que a cobertura vegetal é um dos fatores que afeta as propriedades físico-químicas e por consequência a sua toxicidade. Os girinos de *X.laevis*

demonstraram uma sensibilidade mais elevada aos AEA que os respetivos embriões e girinos de *P. perezi*, sugerindo que é um modelo adequado para avaliação de risco de cinzas originarias de incêndios florestais em anfíbios. Mais ainda, os resultados obtidos demonstraram que uma exposição de curta duração a escorrências de cinzas de eucalipto ou pinheiro podem comprometer as populações de anfíbios.

keywords Forest tree cover, Aqueous extracts of ashes, Ecotoxicity, Anura, Early-life stages

abstract

Climate change has led to a global increase of earth surface temperatures. This phenomenon triggered the increase of frequency and intensity of extreme events such as wildfires. Mediterranean countries are severely affected by these disturbances because of their especially dry summers. Wildfire situation in Portugal is especially critical, because besides the hot and dry summers its native forests have been replaced with monospecific plantations that facilitate wildfires. Wildfires, along with the ashes formed, may cause environmental impacts. Formation of the ashes is a process that converts fuels (organic matter) into a matrix composed of organic and inorganic compounds. Plant coverage can alter the ashes' chemical and physical properties, especially in ashes with a high organic fraction. During the first rain events post-wildfires, these ashes, with the adsorbed chemicals, are transported into nearby waterbodies, where they can induce adverse effects to aquatic biota. Early-life stages of amphibian (embryos and tadpoles) may be particularly susceptible to these chemicals and ashes. According to the above, this study intended to assess how ashes originated from wildfires of forests with different plant coverages (*Eucalyptus globulus* and *Pinus pinaster*) affect embryos or tadpoles of two species of amphibians – *Xenopus laevis* and *Pelophylax perezi*. In order to do so, embryos of *X. laevis* and tadpoles of the two species of amphibians were exposed for 96 h and 14 days, respectively, to serial concentrations (26.9% - 100%) of aqueous extracts of ashes (AEA, with 10 g/L of ashes) containing Eucalypt (ELS) and Pine (PLS) ashes. At the end of the assays, the following endpoints were measured: mortality, developmental stage, body length (total, snout-to-vent-SV, tail) and weight. Effects at sub-individual level were also monitored for oxidative stress (catalase-CAT, glutathione-S-transferase-GST, total glutathione-TG, and lipidic peroxidation-LPO), neurotoxicity (acetylcholinesterase-aChE), and energy metabolism (oxygen consumption through the electron transfer system-ETS, and carbohydrate, lipid and protein contents). Chemical characterization of ashes and AEA was also performed. The AEA of ELS showed higher concentrations of As, Cd, Co, Cr, Pb and V, while PLS showed higher concentrations of Cu, Mn, Ni and Zn. Though a mortality above 20% occurred in embryos of *X. laevis* exposed to AEA, it was not significantly different from the control. Effects at sublethal level were only observed for embryos exposed to PLS; which showed lower carbohydrates content and higher oxygen consumption. Exposure of tadpoles of *X. laevis* to ELS and PLS AEA caused some mortality, that was not significantly different from the control, and a delay in developmental stages. All tested concentrations of the two AEA significantly decrease the body lengths and body weight of *X. laevis* tadpoles. At the sub-individual level, a decrease in the activity of the enzymes CAT and GST and in carbohydrates and lipid contents were observed in these organisms. An increase in oxygen consumption was only registered for tadpoles exposed to PLS. As for tadpoles of *P. perezi*, exposure to ELS induced no significant effects in the organisms. Though exposure to PLS caused a reduction in body lengths, weight, GST activity and an increase in oxygen consumption.

Overall, PLS AEA induced more effects in the two species, suggesting an influence of vegetation cover in the toxicity of ashes. Tadpoles of *X. laevis* showed a higher sensitivity to the AEA than the respective embryos and than of *P. perezi*, suggesting to be an adequate model organism for the risk assessment of ashes derived from wildfire to amphibians. Further, the obtained results showed that short-term exposure to ash-load runoffs from eucalypt or pine forests may compromise amphibian's populations.

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Introduction

In the last century, the global earth surface temperature has increased significantly, with each of the last three decades warmer than the previous one. Within this time interval, the land and ocean surface temperature showed a warming of 0.85°C. In order to find a warmer 30 year period than the one between 1983 and 2012 in the Northern hemisphere, we would have to date back 1400 years (IPCC, 2014). Most of the observed increase in global surface temperature is caused by anthropogenic emissions of greenhouse gases (GHG), with an increase of 0.5°C to 1.3°C being due to these emissions over the period of 1951 and 2010 (IPCC, 2014).

There are many ways in which climate change can be linked to human activities. It is very likely that anthropogenic influences contributed to the melting of glaciers in the Arctic sea and also affected the water cycle, leading to increased atmospheric moisture, changes in precipitation patterns and intensification of heavy rain patterns in some regions (IPCC, 2014).

With the impacts of climate change comes an increasing frequency of extreme events such as floods and droughts. In some countries from the northern hemisphere – Europe and North America – the number of heavy precipitation events has increased. Although, due to a lack of long-term data, there is a low confidence regarding anthropogenic climate change's influence on the increase of fluvial floods and droughts. In spite of these uncertainties, climate related extremes, such as the ones mentioned above, have a high negative impact on ecosystems and human systems (IPCC, 2014).

Climate change has a deep impact on forests in terms of the ecosystem (Hansen *et al.*, 2001) and forest processes (Aber *et al.*, 2001). Besides ecological impacts, climate change also affects forests on a socioeconomic level (IRLAND *et al.*, 2001; Kirilenko and Sedjo, 2007). These impacts can be direct consequences of climate change (Kirschbaum, 2004; Boisvenue and Running, 2006) or indirect disturbance events such as wildfires or droughts (Carvalho *et al.*, 2019).

Disturbance is a natural occurring phenomenon and can be beneficial for ecosystems. Biomes characterised by certain disruptions go through succession events, with some species only inhabiting these disturbed habitats, and others being adapted enough to resist it.

Although disturbances can be beneficial, if their frequency and intensity exceed the forest's threshold, they can also be harmful. More often than not, disturbances created or potentiated by climate change surpass these thresholds lead to severe alterations on the trees such as reducing leaf function, deforming tree structure and destroying seed banks and physical environment, through soil erosion, nutrient loss and landscape heterogeneity (Dale *et al.*, 2000).

Among the environmental climate change related disturbances, wildfires are especially harmful. They can significantly change the habiting wildlife and potentially cripple the ecology of the burnt area. As a succession event starts, previous inhabitant species might be replaced by others more apt to the new conditions (Donato, Harvey and Turner, 2016). But while on the one hand these disruptions can have beneficial effects, on other occasions, wildfires can leave long-lasting marks. For example, Stevens-Rumann et al. (2018) found out that post-wildfire forests showed a lower tree regeneration and more tree regeneration failures – longer regeneration periods or shifts to lower density forests – than in the last century.

Mediterranean forests are under the Mediterranean climate, which is characterized by dry hot summers and cold wet winters. These characteristics make wildfires very frequent in the summer. The occurrence of wildfires on the Mediterranean basin and regions under a Mediterranean climate is not new, so that Mediterranean biomes such as Chaparral were moulded by natural wildfires and their flora and fauna was constituted of species adapted to these disturbances. However, this balance has been interrupted with the increasing severity and duration of wildfires mainly due to climate change and plantation of highly flammable species, which in combination with land abandonment resulted in an increase in fuel load (Lloret, 2004; Moreira *et al.*, 2009; Carmo *et al.*, 2011; Shakesby, 2011).

According to the Jesus et al. (2019) report, on European countries, most of 2018-2019's burnt area was on the ones with Mediterranean climates such as Portugal, Spain and Italy. Although many of these fires were not naturally occurring but intentionally lit, the increasing duration and severity is due to climate change.

Portugal is a great example of a Mediterranean country that is going through a wildfire-related environmental crisis. Portugal has an interesting history regarding its forest. Before the glaciations, Portugal was covered by an evergreen flora (Laurissilva). Then, on

continental Portugal after the glaciations, Laurissilva has been replaced by a mixed forest (Fagossilva), known by the vast Oak forest, while on the Açores, Canárias and Madeira, Laurissilva remained after the glaciations due to the thermoregulatory properties of the surrounding ocean. With the arrival of mankind to Fagossilva, its degradation began. Agriculture and naval construction during the age of discovery were among the main causes for deforestation. In the last centuries, Oaks have been replaced by Pine tree (*Pinus pinaster*), and more recently, a vast area of Eucalypt (*Eucalyptus globulus*) has been introduced in Portugal, giving birth to Ignisilva (Paiva, 2017). These new plantations, especially the eucalypts are highly flammable and together with the increased heat and dryness that comes with climate change cause a serious environmental problem for Portuguese forests and interacting biomes, such as adjacent waterbodies. Figure 1 demonstrates the processes that lead to the implantation of Ignisilva in Portugal, and its consequences.

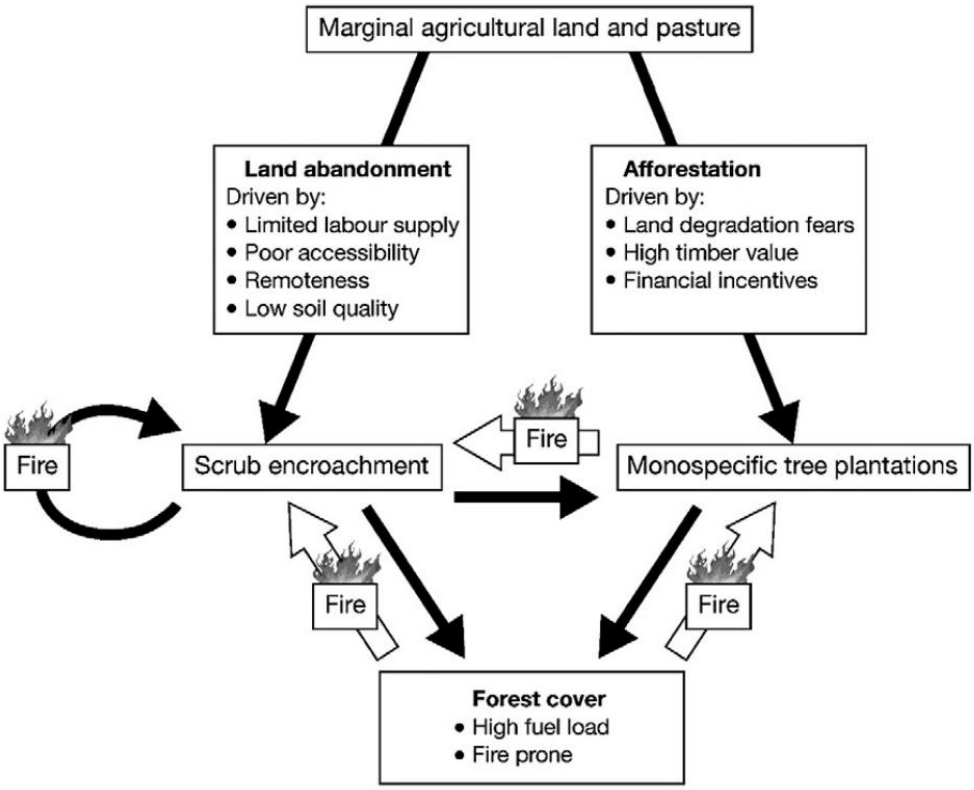

Figure 1 – Impact of agricultural abandonment, scrub encroachment and forestry activity in the Mediterranean (taken from Shakesby (2011), based on Moreira & Russo (2007)).

Extensive pine and eucalypt plantations facilitated not only the frequency but also the severity and duration of wildfires. So that between 2009 and 2018, of the EFFIS network, Portugal was the country that showed the highest number of fire occurrences (18345) and largest burnt area (138841 ha) (Jesus *et al.*, 2019). In 2020 though, there was a harsh decrease both in number of fires (48%) and burnt area (52%) (ICNF, 2020). Although this harsh decrease might lead to the conclusion that the impact of wildfires in Portugal is decreasing, that may not be the case. Although there seems to have been an improvement on the forest management, and firefighter action, the influence of the climate is still present and one of the limiting factors for the occurrence of wildfires may still be the biofuel availability – Forests and plantations – so it is understandable that while the flora recovers and a new generation of trees grows, the frequency, severity and length of wildfires may be reduced. This is clearly showed by (ICNF, 2020), where, for instance, in 2012-2013 there was a vast

burnt area (more than 100 000 ha) while on the following two years the burnt area drastically decreased, only to increase again in 2016-2017. This means that there is a tendency for wildfire impacts to be cyclical and dependant on the available biofuel and that the low 2020 values may be that way because the forests are still recovering from previous years' wildfires, more specifically, 2017, which was the worst year of the last decade, with 537 143 ha of burnt area.

Wildfires have three levels of impacts to soil-dwelling organisms (Fig. 2; Abrantes, 2016; Engstrom, 2010; Whelan et al., 2002). (1) Direct impacts such as mortality or injury to organisms through the direct action of the wildfire (heat, gases); (2) Indirect impacts such as the ecological conditioning that occurs to organisms of survivor populations (predation, starvation, competition, immigration); (3) Evolutionary effects to soil dwelling organisms, namely adaptation to post-fire forests.

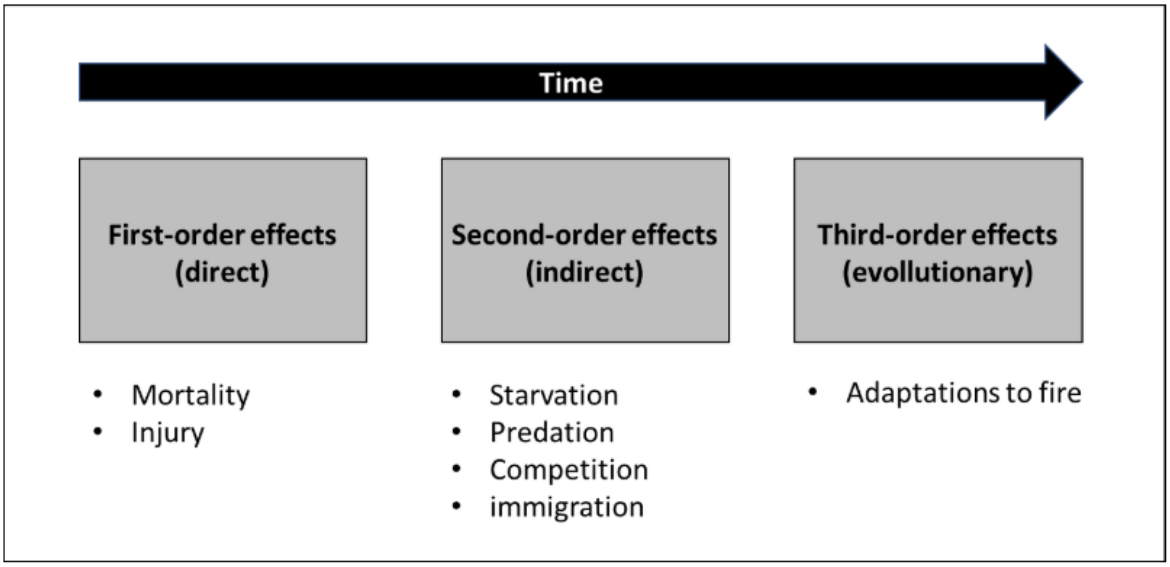

Figure 2 – Levels of wildfire effects on soil organisms. Taken from Abrantes (2016), based on Engstrom (2010).

Wildfires can affect chemical properties of the soil such as pH and osmotic potential, which can lead to a post fire germination inhibition (Henig-Sever, Eshel and Ne'eman, 1996) and even increase the amount of toxic compounds such as metals (Khanna, Raison and Falkiner, 1994; Campos *et al.*, 2015, 2016) and polycyclic aromatic hydrocarbons (Enell et al., 2008) in the soil. These can leach out through ash runoffs and become bioavailable, thus affecting the forest and waterbodies' biology (Barry, 2011; Sevcikova *et al.*, 2011;

Shuhaimi-Othman, Nur-Amalina and Nadzifah, 2012; Silva *et al.*, 2016). Wildfires can cause long lasting marks on trees that can even kill them, along with the remaining vegetation and organisms that depend upon the forest. Above-individual effects are also extremely relevant since they include changes in species, communities and overall ecology. For instance, in habitats where wildfires are frequent events, species tend to either resist the fire through, in case of trees, a thick bark, or find a way to recolonise their previous habitat after fire (resilience). As wildfire severity and duration is increasing, due to global temperature increase, these resistance and resilience strategies are increasingly less efficient (Stevens-Rumann *et al.*, 2018), which indicates that wildfires have an increasing impact on the habitats ecology, since they affect the capacity of species to either resist or recolonise the post-fire habitat, ultimately crippling the ecological succession that would take place. Fire transforms the organic matter (fuels) into materials with different physical and chemical properties. Bodí et al. (2014) define ashes as "the particulate residue remaining, or deposited on the ground, from the burning of wildland fuels and consisting of mineral materials and charred organic components" by following four components: (1) An organic fraction derived from the combustion of vegetation. Sometimes unburnt vegetation can be a part of it. (2) An inorganic fraction consisting of oxides, phosphates, carbonates, sulfates, and amorphous phases that either were already present or were transformed by the combustion (Ulery, Graham and Amrhein, 1993; Quintana *et al.*, 2007; Vassilev *et al.*, 2010), often called "mineral ash" (Smith, Eitel and Hudak, 2010). (3) Mineral soil particles that deposit on the vegetation before (Frandsen, 1987), or after the fire (Topoliantz, Ponge and Lavelle, 2006; Woods and Balfour, 2010; Pérez-Cabello *et al.*, 2012). (4) Exogenous chemicals deposited from the atmosphere unto the vegetation canopy. Although not directly derived from the original organic fraction, they can affect meaningfully the chemical component of the ash (Smith, Eitel and Hudak, 2010).

The process of ash generation depends on the completeness of the combustion. Wildfires have an initial phase of heating when volatile gases are released through pyrolysis. On this phase, temperatures can vary between 450 and 1400 °C (Ormeño *et al.*, 2009; Saura-Mas *et al.*, 2010), although, under most wildfire conditions, oxygen is the limiting factor, and a smouldering combustion takes place at 250 to 400 °C (Bodí *et al.*, 2014). Complete combustion leads to the formation of volatiles or gases (CO_2, CO, CH_4 or NO_2) leaving only the mineral fraction (DeBano, Neary and Ffolliott, 1998). Although, as wildfires tend to have

limited oxygen, they favour the formation of pyrogenic organic compounds, through incomplete combustion (Bodí *et al.*, 2014). The amount of ash depends on the total fuel available, the type of fuel and the completeness of combustion. These parameters vary spatially due to outside phenomena such as wind and precipitation, which sweep and wash the ashes away, respectively (Bodí *et al.*, 2014).

The chemical composition of ashes varies mainly with the completeness of combustion. It is possible to ascertain the amount of organic content on ashes through its colour. High organic C ashes tend to be dark-coloured, while low organic C ashes tend to be light-coloured. Incomplete combustion produces ashes with high organic C content that are for that reason darker, whereas complete combustion produces ashes with low organic C content (most of the C volatilizes) and a lighter colour (Kuhlbusch and Crutzen, 1995; Pereira, Úbeda and Martin, 2012). The main organic element of ashes is C, but other elements such as N can also be present in lower amounts (Bodí *et al.*, 2014). During combustion, organic N is the first element to volatilize, followed by oxygen and hydrogen, and lastly, C (Almendros, Knicker and González-Vila, 2003). Although Organic components of ash tend to have a low water-solubility (Quill *et al.*, 2010; Zhao *et al.*, 2010), polycyclic aromatic hydrocarbons (PAHs) can be a source of important water pollutants (Smith *et al.*, 2011).

As the combustion temperature increases, ashes' chemical properties change and organic components convert to inorganic ones and volatize (Bodí *et al.*, 2014). From 350 °C to 450-500 °C the ash colour becomes lighter (Lentile *et al.*, 2009; Hogue and Inglett, 2012; Pereira, Úbeda and Martin, 2012). At this stage, the main components, which depend on the plant species and affected ecosystem (Bodí *et al.*, 2014), are Ca, Mg, K, Si, and in lower proportions P, Na, S and trace metals such as Al, Fe, Mn and Zn (Qian *et al.*, 2009; Pereira, 2010; Gabet and Bookter, 2011). Between 500 and 1400°C, the differences between which plant species were used as fuel is reduced, with the main differences being the relative proportions of minerals in the ash (Úbeda *et al.*, 2009). The main components produced at 500 °C are carbonate compounds such as $CaCO_3$, $MgCO_3$ and $K2CO_3$ (Bodí *et al.*, 2014). At 580 up to 1100 °C, carbonate compounds dissociate to oxides, depending on the compound (Quill *et al.*, 2010; Pereira, Úbeda and Martin, 2012).

The Physical properties of ashes also change with combustion completeness, which is in turn related to the temperature. The most obvious physical parameter is colour, which is, as previously mentioned, lighter the higher the combustion completeness is. This parameter also influences the mass of the fuel, with higher temperatures associated with a reduced ash mass. At lower combustion temperatures (350 °C), the main mass loss is due to the volatilization of water and elements (Úbeda *et al.*, 2009), and occurs at different rate depending on the plant species and flammability (Dimitrakopoulos and Panov, 2001). The particle size of ash is also affected by temperature (Balfour and Woods, 2013), with particle size becoming finer with increased temperatures (Bodí *et al.*, 2014). The influence of temperature on particle size is overshadowed by the influence of the species of plant that forms the ash (Bodí *et al.*, 2014).

Ash deposition forms a two-layer system, and the hydrologic process associated with it is not straightforward. Some authors indicate that the ash layers store water, hence delaying and reducing overland flow rates (Leighton-Boyce *et al.*, 2007). On the other hand, Woods & Balfour (2010) demonstrated that ashes can also increase overland flow. Bodí et al. (2014) attribute this variability to three main factors: (1) ash depth and type; (2) soil type; and (3) rainfall characteristics (timing, duration and intensity). Ash layers have high porosity and are generally more permeable than soil, which expectedly leads to a delayed overland flow and runoff, proportionally to their thickness (Cerdà and Doerr, 2008; Woods and Balfour, 2008; Larsen *et al.*, 2009; Zavala *et al.*, 2009). When the ash layer becomes saturated with water, an overland flow occurs, as well as a subsurface flow between the ash and the soil (Bodí *et al.*, 2012; Ebel, Moody and Martin, 2012). This process leads to a runoff that incorporates additional downhill ash and other fine particles (Burns, 2007; Gabet and Sternberg, 2008).

Ash runoffs can reach streams, reservoirs or the sea (Shin, Sidharthan and Shin Young, 2002; Spencer, Gabel and Hauer, 2003; Ranalli, 2004), as they do, these runoffs carry the substances present on ashes and can lead to changes in the water chemistry (Earl and Blinn, 2003). Spencer et al. (2003) reported a substantial increase in nutrients on streams near wildfires in Montana. Furthermore, other substances such as PAHs and other trace elements are carried in the runoffs and find their way to waterbodies (Campos et al., 2012; Carvalho et al., 2019). Olivella et al. (2006) found out that after one month of the wildfire occurrence, the sum of PAHs in riverine waters increased by a factor of three and decreased in the

following months. They attributed this decrease to the degradation of PAHs by atmospheric agents. Ash overland runoffs also mobilize and transport trace elements into waterbodies (Campos *et al.*, 2015, 2016) that have shown to have an ecological impact (Atli *et al.*, 2006; Barry, 2011; Shuhaimi-Othman, Nur-Amalina and Nadzifah, 2012; Araújo *et al.*, 2014).

Changes in water chemistry, caused by ash runoffs, along with the introduction of contaminants like toxic trace elements and PAHs can have a deep impact on aquatic biota on many levels. Silva et al. (2015) assessed the effect of aqueous extracts of ashes (AEA) on two primary aquatic producers - *Raphidocelis subcapitata* and *Lemna minor*. The authors found out that AEA inhibited these species' growth. These results were corroborated by the *in-situ* assays performed by Ré, Campos, Saraiva, et al. (2020) on the same species. Carvalho et al. (2019) also observed an adverse effect of ash runoffs on microbial communities and invertebrate shredders, leading to a decreased leaf litter decomposition with consequences for the normal functioning of riverine systems. The effect of ash runoff is not restricted to lower trophic levels. *Corbicula fluminea* showed to be capable of filtering contaminants from the AEA at the expense of their lives (Silva *et al.*, 2016). *Daphnia magna*, *Atyaephyra desmarestii* and *Gambusia holbrooki* showed a feeding inhibition when exposed to *in-situ* ash contaminated streams (Ré, Campos, Puga, *et al.*, 2020). Thus, ash runoffs can be seriously detrimental to freshwater life, since they can impair several trophic levels and functions, affecting not only pelagic organisms but benthic ones as well (Ré *et al.*, 2021).

Although the effects of post-fire contamination on aquatic organisms have been recently investigated, there is a lack of fundamental understanding of their impacts on amphibians. Over the last years, amphibians have been facing a global decline (Stuart *et al.*, 2004). Some of the main causes for this decline are the infection by the globally emerging chytrid fungus (Lötters *et al.*, 2009), global climate change (Rohr and Raffel, 2010) and pollution (Wagner, Lötters and Naturschutz, 2013). The increasing occurrence of extreme events such as wildfires affects amphibians at a community level. Literature describes a fast increase in amphibian occupancy after the occurrence of a wildfire followed by a steady decrease over time (Hossack and Corn, 2007; Hossack and Pilliod, 2011; Hossack, Lowe and Corn, 2013). The introduction of chemical pollutants into amphibians' habitats has affected amphibians on a large scale (Taylor *et al.*, 2005; Polo-Cavia, Burraco and Gomez-Mestre, 2016). One of the main sources of chemical pollution is agriculture through the use of fertilizers and pesticides (Rouse, Bishop and Struger, 1999; Watt, Oldham and de Wijer,

2003) and toxic metal leaching from metal extraction mines (Adlassnig *et al.*, 2013; Marques *et al.*, 2013).

Amphibians are a group of organisms that plays an important role in the ecosystems they inhabit. Throughout their development they occupy different trophic levels. Most anuran tadpoles are filter feeders that use suction to produce a flow of water to trap suspended particles, while most adults are typically carnivores (Pough *et al.*, 2015). Furthermore, these organisms are excellent as environmental bioindicators. Most stream-dwelling amphibians have a set of ecological traits that allow them to be reliable as indicators of the stream condition. They are highly philopatric, long-lived and exist in stable populations (Welsh and Ollivier, 1998). These traits make them potentially better indicators than fishes and macroinvertebrates (Welsh and Ollivier, 1998). Amphibians are also extremely exposed to changes in their habitat, especially larval stages and adults that live in waterbodies. Their naked bodies make them highly susceptible to changes in the water chemistry and contaminants such as pesticides, metals, among others. Tadpoles are especially exposed due to their feeding method. Since most of them are filter feeders, they tend to absorb chemical contaminants in the water. One of the most used species as a model for ecotoxicology assays is *Xenopus laevis*. This South African frog is used as a model because it is known to be relatively sensitive, is fully aquatic throughout their entire development and are easy to handle and maintain in the laboratory.

The main goal of this work is to assess the toxic effects of wildfire ashes from to distinct proveniences (eucalypt and pine plantations) in larval stages of amphibians. For this purpose, aqueous extracts of ashes (AEA) were prepared to mimic the post-fire runoff and two types of assays, an acute (96 hours) teratogenicity assay on embryos and a chronical (14 days) ecotoxicity assay on tadpoles were performed in two species of amphibians, *Xenopus laevis* and *Pelophylax perezi*. The effects were evaluated in several endpoints – mortality, development, growth (total length, snout-to-vent length, tail length and weight) and biochemical response, which was divided in oxidative stress biochemical marker response (catalase, acetylcholinesterase, glutathione-S-transferase, total glutathione, lipidic peroxidation) and energy metabolism response (carbohydrates, lipids, proteins and oxygen consumption).

Materials and Methods

2.1. Sampling of ashes and preparation of aqueous extracts

In order to perform the assays, ashes were gathered from a wildfire that occurred in July 2013 in the region of Talhadas, Aveiro district (N 40° 39′ 54″, W8° 21′ 47″), which affected 815 ha, mainly covered by eucalypt (*Eucaliptus globulus*) and maritime pine (*Pinus pinaster*) plantations. The ashes were collected in July, immediately after the wildfire. For that, two hillslopes burnt at low severity but differing in the main vegetation cover were selected: one site was predominantly covered by eucalypt plantations (ELS) and the other one by the maritime pine plantations (PLS). In order to have representative ash-samples of each site (ELS and PLS), a transect was laid out across each slope, and five equidistant sampling points were selected. At each sampling point, a 50 x 60 cm plot was selected to firstly collect, with a small spoon and brush, the entire ash layer, and then the upper 2 cm of the mineral soil, removing the gravel and stones. After collection, ashes from the 5 points of each site were mixed to get a composite sample. The ashes collected at both sites exhibited a dark-grey colour, though PLS ashes were darker than ELS ones. The ashes were then transported on 5L plastic water bottles to the laboratory, where they were thoroughly mixed and stored at -18°C in the dark.

The aqueous extracts of ashes (AEAs), used to perform the ecotoxicity assays, were obtained by weighing 40 g of ashes into 4000 mL of the test medium FETAX (Dawson and Bantle, 1987), which is used in ecotoxicity assays with aquatic life stages of amphibians. This aqueous extract was mixed vigorously before being further diluted with FETAX medium to obtain the tested dilutions. AEAs were prepared freshly at the beginning of the assays and each time the test media was changed throughout the duration of the assays.

2.2. Chemical characterization of the ash extracts

The AEAs were analysed in order to measure total nitrogen (TN), ammonia (NH_3-N) nitrite (NO_2-N), nitrate (NO_3-N), phosphate (PO_4^{3-}), total hardness, calcium hardness and levels of metals. The TN was measured through an oxidative digestion followed by quantification by molecular absorption spectrometry (MAS) (APHA, method 4500-N C, 2017). Nitrate and nitrate contents were measured through chromotropic acid method (West

and Lyles, 1960) and and N-(1-Naphthyl)-ethylendiamine method (ISO 15923-1, 2013), respectively. Ammonia and phosphate were measured through the phenate method (APHA, method NH_3 F, 2017) and by the ascorbic acid method (ISO 15923-1, 2013). Total hardness and calcium were measuded through metallphathaleine and murexide method, respectively (Aqualytic Handbook of Methods, 2019). The levels of metals were measured following nitric-perchloric acid digestion protocols developed by APHA, method 3030 H (2017). The concentrations of vanadium (V), chromium (Cr), manganese (Mn), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd) and lead (Pb) were measured for both the ashes and AEA, determined by inductively coupled plasma mass spectrometry (ICP-MS).

2.3. Study species

Two species of anurans were selected as model species for this study: (i) *Xenopus laevis* (Daudin, 1802), which is a standard species recommended by most standard guidelines to perform ecotoxicity assays with amphibians; and (ii) *Pelophylax perezi* (López-Seoane, 1885), a species autochthonous to the Iberian Peninsula and South France, which inhabits an area considered to be a wildfire hotspot. Furthermore, this species is well distributed throughout the Iberian Peninsula, is abundant (which helps obtaining embryos from refence populations) and holds the status of "Least Concern", according to the "IUCN Red List of Threatened Species" (2012).

For *X. laevis*, test organisms were obtained by inducing breeding in adults from a husbandry maintained at the Department of Biology of the University of Aveiro (Portugal). Sexually mature males and females were injected, into the dorsal lymph sac, with 100 and 400 International Units of the human chorionic gonadotropin hormone (hCG 5000 IU, UK), (Sigma, Aldrich), respectively. Afterwards, the male and female were paired together in a breeding container and left overnight to allow amplexus and reproduction to occur. The obtained eggs were harvested, and their viability checked under a stereomicroscope (Zeiss Stemi 508). Part of the embryos were immediately used to perform the embryo teratogenicity assay and the remaining embryos were reared until reaching NF45-46 (at this stage tadpoles initiate independent feeding; Nieuwkoop and Faber, 1994), when they were used to perform the tadpole's toxicity assays. During this period, the organisms were maintained in an

aquarium filled with FETAX with continuous aeration, at 23 ± 1 °C and with a photoperiod of 14:10 h light:dark.

For *P. perezi*, eggs were collected at the freshwater pond Lagoa da Boavista (40°36´16´´N, 8°41´48´´W; Gafanha de Áquem, Aveiro). In the laboratory, the viable eggs were separated into an aquarium filled with FETAX medium and reared, under the same conditions as described for *X. laevis*, until reaching developmental stage G25 (stage when the organisms open the mouth and start independent feeding; Gosner, 1960), when they were used to perform the tadpole toxicity assay.

2.4. Embryo teratogenicity assays

The embryo teratogenicity assays were performed according to the guideline ASTM (1998). Embryos of *X. laevis* at developmental stage NF 8-11 were exposed to a control (consisting of FETAX medium) and to a set of concentrations of ELS and PLS AEA (26.9%, 35%, 45.5%, 59.2%, 76.9% and 100%). The assay design consisted of four replicates per control and dilution, with 10 organisms per replicate. Each replicate consisted of a covered Petri dish filled with 10mL of either FETAX medium or concentrations of the each AEA. The organisms were exposed through 96 hours at 23 ± 1 °C with a photoperiod of 14:10 h light: dark. Mortality was checked daily, and the dead organisms were removed in order to avoid the proliferation of microorganisms, which could impair the viability of the surviving organisms. After 48 hours of exposure, the medium was replaced in all replicates, and the water physical-chemical parameters measured (dissolved oxygen, pH and conductivity) using WTW 3410 meter (Multi 3410 SET C). At the end of the assay, water physical-chemical parameters were measured and the following parameters were monitored: mortality, hatching, malformations, developmental stage, and body length. In order to measure the organisms without inducing much stress and allow its fast deep freezing for ulterior analysis of biochemical markers, all the surviving larvae of each replicate were photographed under a graph paper by using a camera. The body measurements were made ulteriorly in each photography by using the software ImageJ.

2.5. Tadpole ecotoxicity assays

Tadpoles of *X. laevis* and of *P. perezi*, at developmental stages NF45 and G25, respectively, were exposed to a control (consisting of FETAX medium) and to several concentrations of the AEAs obtained from the ELS and PLS ashes (26.9%, 35%, 45.5%, 59.2%, 76.9% and 100%), following the protocol of ASTM (2002) with some modifications, specifically the duration of the assay was extended for a period of 14 days. Five replicates were used per dilution and for the control, with four organisms per sample. Exposure occurred with continuous aeration at 23 ± 1 °C and a photoperiod of 14:10 h light:dark.

During the assay period, test solutions were changed every 48 h and food was added at a quantity of 0.06 g of Tetramin™ per replicate. In order to do so, 3.9 g of Tetramin™ were mixed with 32.5 mL of FETAX medium, afterwards 0.5 mL (containing 0.06 g of Tetramin™) were pipetted to each replicate. Whenever the medium was changed, water parameters (pH, conductivity and dissolved oxygen) were measured at the old medium. Mortality was checked every 48h, with dead organisms removed from the test vessel to prevent the growth of microorganisms that could compromise the survival of the remaining tadpoles. At the end of the assay, the following endpoints were monitored: mortality, malformations, developmental stage, body length and weight. As well, to avoid stress in the organisms and enable its rapid deep freezing for ulterior analysis of biochemical markers, all surviving tadpoles were photographed under a graph paper by using a camera. Measurements were made in the photographs by using the software ImageJ. After photographing and weighing the tadpoles, they were introduced in 2 mL eppendorfs, with all the medium removed, and immediately frozen in liquid nitrogen, to avoid stress. The samples were then stored at -80 °C to be used later in biochemical marker analysis.

2.6. Biochemical markers and cellular energy allocation analysis

A battery of biochemical markers was analysed to assess the potential sub-individual effects of AEA on embryos and tadpoles. The analysed markers were related with oxidative stress (total glutathione TG, catalase-CAT, lipid peroxidation-LPO), biotransformation (glutathione S-transferase-GST), neurotransmission (acetylcholinesterase-AChE), and energy reserve parameters (electron transport system-ETS, total lipids, total carbohydrates and, total proteins). To proceed with these analyses, the frozen samples of embryos and tadpoles were gradually unfrozen on ice. The organism weight was highly variable,

therefore, in order to attempt to standardize this difference, the samples were diluted according to their sum weight, with the heavier samples diluted with more water, all to a concentration of 160 mg/ml of ultrapure water. Then, the samples were homogenized on an ultra-sonic homogenizer for 10-12s until all the organic material was homogeneous. The homogenization was performed on ice to prevent the denaturation of the organic material.

The diluted samples were then separated into different test tubes for different analysis. A volume of 300µL was used for ETS, 300µL for lipids, 300µL for carbohydrates and reserve proteins, 200µL for LPO – with 4µL Butylated hydroxytoluene (BHT) to stop lipid oxidation, and 500µL for the other biochemical markers – diluted before freezing with 500 µL K-phosphate buffer (0.2M; pH = 7.4). The ETS fraction needed a short preparation procedure before freezing the samples, where 150µL of homogenization buffer were added to the samples, following a centrifugation at 1000G for 10 minutes at 10 °C and removal of the supernatant.

The oxygen consumed (ETS) and the energy reserves available (carbohydrates, lipids and proteins) were determined following protocol of De Coen & Janssen (1997) with adaptations from Rodrigues et al. (2015). ETS was measured at 490nm every 20s for three minutes, blanks removed. Proteins were measured following the Bradford (1976) method at 592nm and compared with a bovine serum albumin standard. Total lipid content was measured at 375nm and compared with a tripalmitin standard curve. Carbohydrate content was measured at 462nm and compared with a glucose standard curve.

Analyses of TG, CAT, GST, and AChE were performed in one lab session. In this session, the tubes were divided into aliquots for each enzyme. For TG, GST and AChE were required 220µL of sample each, while CAT and protein only required 60µL each. Protein was measured here in order to normalize the data between samples.

TG was determined with the post-mitochondrial substrate (PMS) measured at 412 nm every 20s for three minute, resulting of the recycling reaction between reduced glutathione (GSH) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase (GR) (Tietze, 1969; Baker, Cerniglia and Zaman, 1990). GST was determined with the PMS fraction by mixing GSH with 1-chloro-2,4-dinitrobenzene (DTNB) and measured at 340 nm every 20s for five minutes (Habig, Pabst and Jakoby, 1974). CAT was determined with the PMS measured at 240 nm every 20s for two minutes through the degradation of

H_2O_2, using ultraviolet microplates (Clairborne and Greenwald, 1985). AChE was measured at 414 nm every 20s for five minutes using acetylthiocholine as a substrate (Ellman *et al.*, 1961). Protein was determined by adapting the Bradford method (Bradford, 1976), and was measured at 600 nm and compared with the standard concentrations. LPO was determined by measuring tiobarbituric acid reactive substances (TBARS) at 535 nm with the blanks subtracted (Bird and Draper, 1984).

The method of filling the microplates was similar for every analysis. The first column was left empty to facilitate statistical analysis. Four technical replicates were used vertically for each sample. The blanks – if necessary – fully occupied the second column. The standard curves – when used – occupied the space of the first samples, with the samples starting after the standard curves.

2.7. Data analysis

The existence of significant differences, for the monitored endpoints, between the AEA concentrations and respective controls were evaluated by one-way analysis of variance followed by the pairwise comparison Dunnett's post-hoc test. Assumptions of ANOVA were checked by using Shapiro-Wilks test for normality of data and Bartlett's test for the homoscedasticity of variances. Whenever any assumption was not met, the data was transformed with sqrt(x), log(x) or 1/x, to guarantee a normal distribution of the data. Selection of the transformation was based on skewness. The less skewed transformation would be chosen. If after transforming the data, assumptions could still not be met, then a non-parametric Kruskal-Wallis test followed by a pairwise comparison Dunn's test were performed. The significance level was set at $p < 0.05$.

Results

3.1. Chemical characterization of the ash extracts

In table 1 are showed the concentrations of the elements and chemical compounds quantified in the two types of ashes and respective AEAs. The ashes collected both at the eucalypt and pines forest, showed a much higher concentration (at least 197-fold higher) of the analysed elements than the respective AEAs (Table 1). The most abundant metals in the AEA were Mn > Zn > Cu > V > Pb > Cr = Ni > As > Co > Cd for ELS, and Mn > Zn > Cu > V > Pb > Ni > Cr > As > Co > Cd for PLS. When comparing the types of ashes, it was observed that the levels of Cd, Co, Cr, Mn, and Zn were higher in ELS; while those of As, Pb and V were higher in PLS. As for the AEAs, a slightly different pattern was observed: the ELS AEA exhibited higher values of nitrate, As, Cd, Co, Cr, Pb and V; and the PLS AEA higher values of Cu, Mn, Ni and Zn.

Table 1 – Concentrations of the chemical elements measured in the two types of ashes and corresponding aqueous extracts of ashes (AEA); eucalypt forest (ELS) and pine forest (PLS).

Chemical elements	Ashes (mg Kg^{-1})		AEA (mg L^{-1})	
	ELS	**PLS**	**ELS**	**PLS**
NO_3 (1-30 mg $L^{-1)}$	-	-	10.6	10.4
NO_2 (0.01-0.5 mg L^{-1})	-	-	<0.01	<0.01
N (0.5-25 mg L^{-1})	-	-	<0.50	<0.50
NH_3 (0.02-1 mg L^{-1})	-	-	0.30	<0.02
PO_4 (0.05-4 mg L^{-1})	-	-	<0.05	<0.05
Total hardness (2-50 mg L^{-1})	-	-	<2	<2
As	24±0.7	28±0.1	0.077±0.0193	0.023±0.0058
Cd	0.62±0.030	0.17±0.009	0.002±0.0005	<0.001
Co	8.6±0.05	2.5±0.09	0.026±0.0065	0.013±0.0033
Cr	22±0.7	19±0.3	0.083±0.0208	0.055±0.0138
Cu	84±0.8	83±1.9	0.228±0.0570	0.314±0.0785
Mn	413±30.6	373±19.9	1.201±0.3003	1.297±0.3243
Ni	20±0.04	20±0.3	0.083±0.0208	0.086±0.0215
Pb	50±0.1	53±0.3	0.128±0.0256	0.118±0.0236
V	29±0.5	42±0.6	0.147±0.0368	0.141±0.0353
Zn	270±0.7	244±2.9	0.601±0.1202	0.699±0.1398

3.2. Embryo teratogenicity assays with *Xenopus laevis*

In table 2 are described the values of the physical and chemical parameters of the test solutions, measured at the beginning and at the end (96 h) of the assay. As expected, the conductivity values increased with increasing tested concentrations of the AEAs. The pH did not change much with the AEA concentrations, rounding from 7.8 to 8.2. The dissolved oxygen never went below 7.0. The values of pH and dissolved oxygen were similar at the beginning and at the end of the assay. However, the values of conductivity showed a decrease at the end of the assay, both for ELS and PLS (Table 1).

Table 2 – Physical and chemical parameters measured at the beginning (New) and at the end (Old) of the assay performed with embryos of *Xenopus laevis*.

Ashes		pH		Conductivity		Dissolved oxygen (mg/L)
		New	Old	New	Old	
Control		7.8	8.2	684	684	7.3
ELS	26.9	8.0	8.2	826	692	7.9
	35.0	8.0	8.2	839	700	7.6
	45.5	8.1	8.2	866	727	7.1
	59.2	8.0	8.2	824	765	7.8
	76.9	7.9	8.2	937	801	7.6
	100	8.0	8.1	1087	856	7.2
PLS	26.9	-	8.2	-	630	-
	35.0	8.0	8.2	828	650	7.0
	45.5	8.1	8.1	819	655	7.1
	59.2	8.1	8.1	893	673	7.2
	76.9	8.1	8.1	956	694	7.2
	100	8.0	8.1	926	733	7.4

Mortality of embryos of *X. laevis* was slightly higher than the control (10% mortality) on 100% ELS (25% mortality) and PLS (27.5% mortality). Although, no significant differences were registered comparatively to the respective control ($p \geq 0.504$; Figure 3).

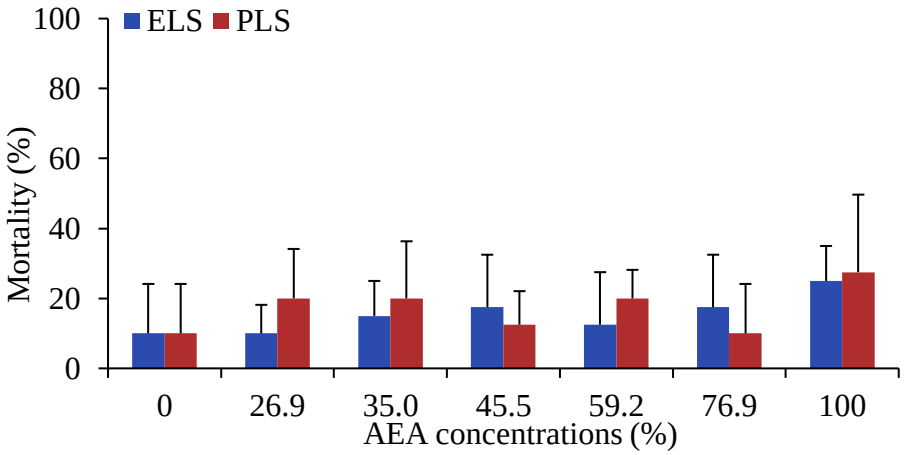

Figure 3 – Average mortality of embryos of *Xenopus laevis*, exposed for 96 hours to concentrations of aqueous extracts of ashes (AEA) of eucalypt (ELS) and pine (PLS) forests. Error bars represent standard deviation.

Developmental stage was only significantly affected on ELS ($p < 10^{-2}$), where embryos from the 26.9% concentration (NF44.3) were, on average, significantly less developed than the control ones (NF46.0; $p < 10^{-2}$; Figure 4).

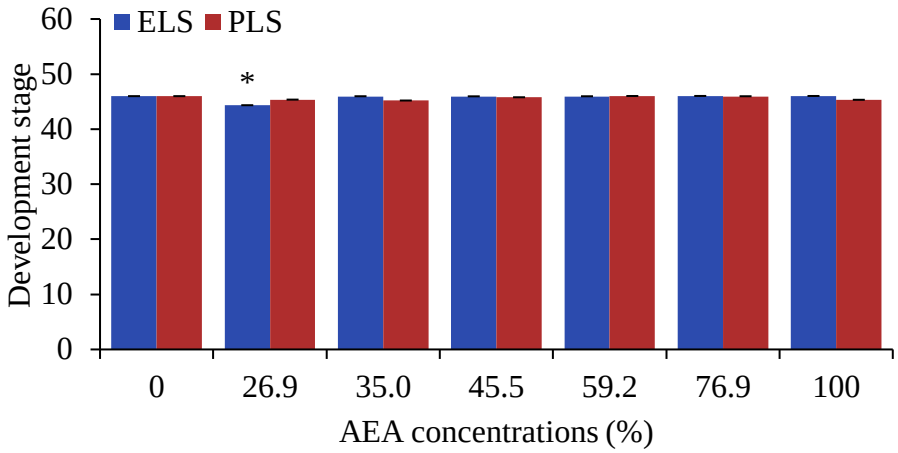

Figure 4 – Average development stage of embryos of *Xenopus laevis*, exposed for 96 hours to concentrations of aqueous extracts of ashes (AEA) of eucalypt (ELS) and pine (PLS) forests. Error bars represent standard deviation. * represents statistical difference from the control.

The surviving organisms at the end of the teratogenicity assay showed no significant differences in SVL ($p \geq 0.0793$; Figure 5A) and tail length ($p \geq 0.0631$; Figure 5B), for both AEA. For total length, the ANOVA showed a significant difference on organisms exposed to PLS ($p = 0.0476$), however, this difference was not shown in the Dunnett' pairwise comparisons between the AEA concentrations and the control ($p \geq 0.0510$; Figure 5C).

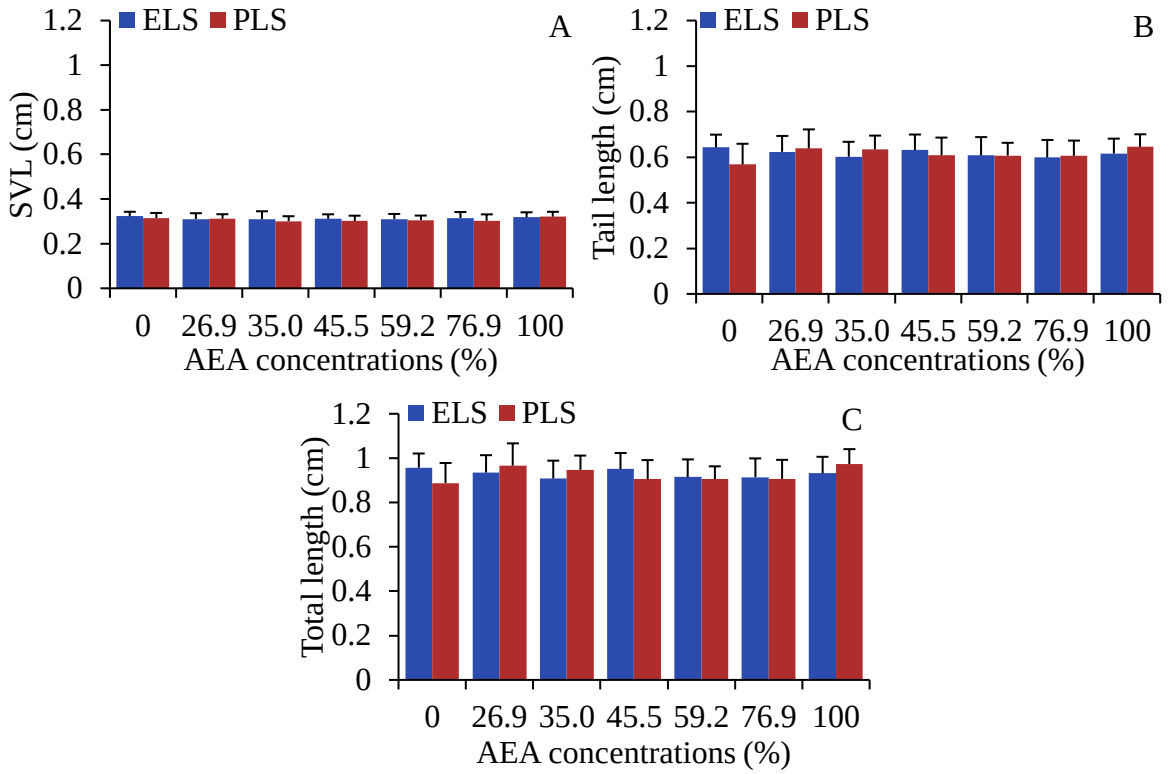

Figure 5 – Average of morphological lengths (A – snout-to-vent; B – tail; and C – total length) in larvae of *Xenopus laevis* after an exposure of 96 hours to concentrations of aqueous extracts of ashes (AEA) from eucalypt (ELS) and pine (PLS) forest. Error bars represent standard deviation.

The different AEA had little to no effect on biochemical markers activity as well as on LPO damage on *X. laevis* embryos. For CAT, AChE and TG (only for ELS), though some alterations on their activities were observed, were not significantly different from the control ($p \geq 0.188$; Figure 6A, B and D). However, GST activity showed a significant reduction, comparatively to the control, in organisms exposed to 26.9% of PLS ($p = 0.0140$; Figure 6C). There was no significant effect of any of the tested AEA on LPO damage ($p \geq 0.102$; Figure 6E).

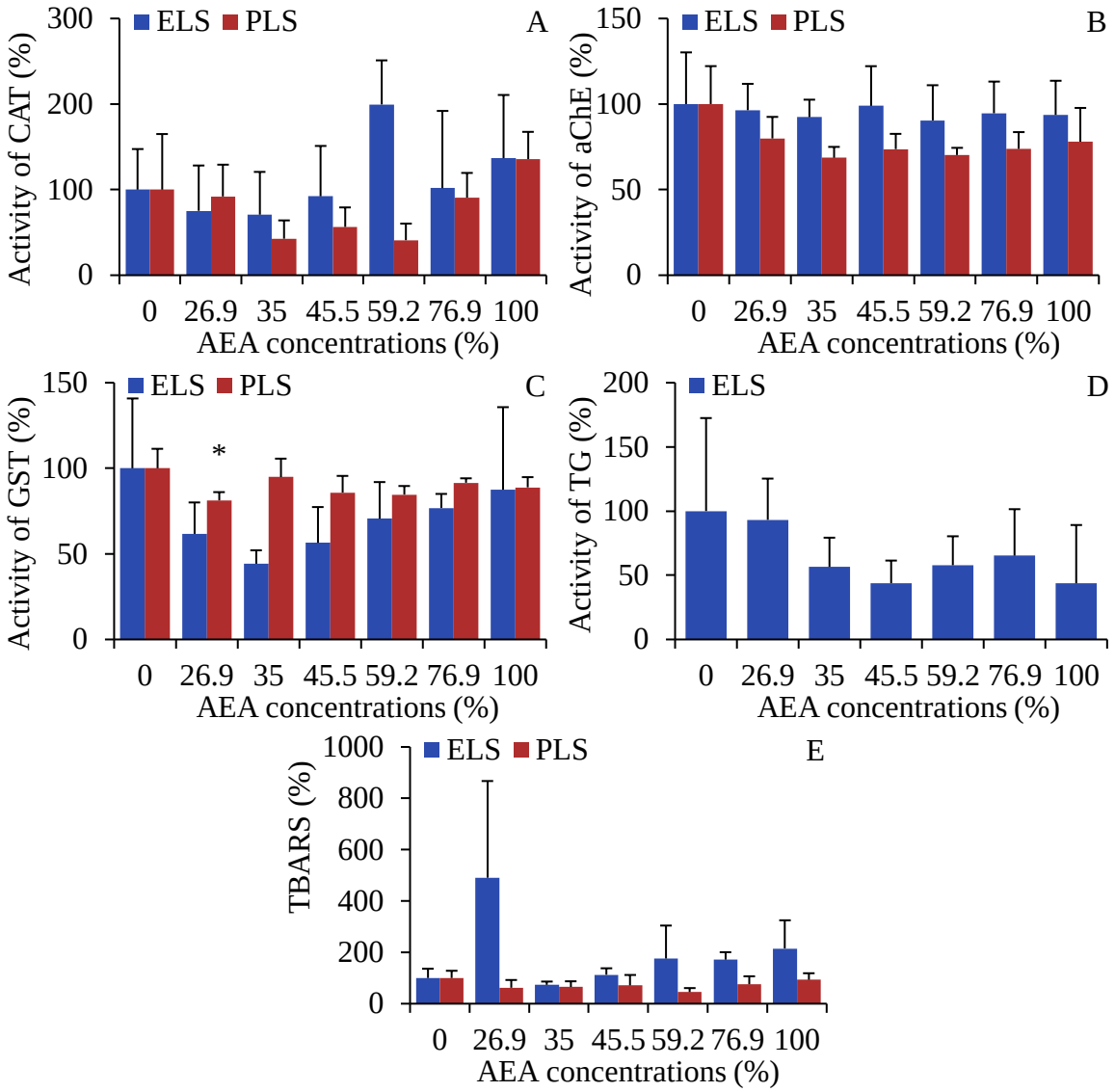

Figure 6 – Average of enzymatic activity (A – catalase; B – acetylcholinesterase; C – glutathione-S-transferase; D – total glutathione) and lipid peroxidation (LPO) damage (E – thiobarbituric acid reactive substances) in embryos of *Xenopus laevis* exposed, for 96 hours, to concentrations of aqueous extracts of ashes (AEA) from eucalypt (ELS) and pine (PLS) forest. Error bars represent standard deviation. * indicates significant differences from the respective control ($p < 0.05$).

As for energy reserves, no significant alterations were registered in organisms exposed to ELS ($p \geq 0.182$; Figure 7). However, in organisms exposed to concentrations equal or higher than 59.2% of PLS a reduction of up to 29.9% on carbohydrate content was observed ($p \leq 0.0273$; Figure 7A). Concerning oxygen consumption, significant changes were also only observed in organisms exposed to AEA of PLS. When compared to the control, organisms exposed to 35.0%, 45.5%, 76.9% and 100% of PLS showed a significant increase in oxygen consumption ($p \leq 0.0245$; Figure 7D).

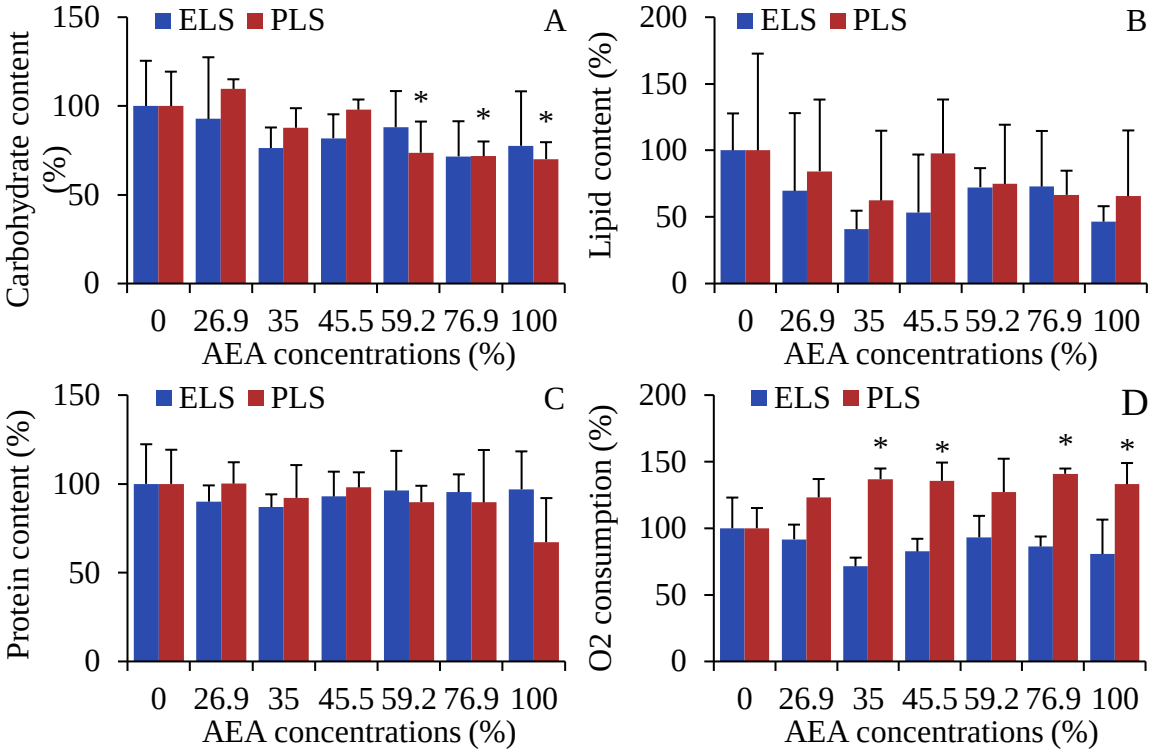

Figure 7 – Average of energy reserves (A – Sugar content; B – Lipid content; C – Protein content; D – Oxygen consumption in larvae of *Xenopus laevis* exposed, for 96 hours, to concentrations of aqueous extracts of ashes (AEA) from a pine (PLS) and eucalypt (ELS) forest. Error bars represent standard deviation. * indicates significant differences from the respective control ($p < 0.05$).

1.3. Tadpole ecotoxicity assays

3.3.1. *Xenopus laevis*

The pH values of the two AEA varied within the range of 7.8 and 8.4, while the conductivity increased with increasing concentrations (Table 3). Levels of dissolved oxygen were always above 7.0 mg/L (Table 2 – Physical and chemical parameters measured before and after changing the test solutions in the assay performed with tadpoles of *Xenopus laevis*.). Similarly, to what was observed with the aqueous extracts prepared for the assays with the embryos of *X. laevis*, the values of pH and dissolved oxygen were similar in the new and old solutions, while the values of conductivity decreased after the 48 h period (i.e. within the period for medium changes).

Table 2 – Physical and chemical parameters measured before and after changing the test solutions in the assay performed with tadpoles of *Xenopus laevis*.

Ashes		pH		Conductivity		Dissolved oxygen (mg/L)
		New	Old	New	Old	
Control		7.8	8.1	684	537	7.3
ELS	26.9	8.0	8.1	826	636	7.9
	35.0	8.0	8.2	839	625	7.6
	45.5	8.1	8.2	866	649	7.1
	59.2	8.0	8.2	824	684	7.8
	76.9	7.9	8.2	937	726	7.6
	100	8.0	8.4	1087	780	7.2
PLS	26.9	-	8.2	-	670	-
	35.0	8.0	8.2	828	597	7.0
	45.5	8.1	8.2	819	619	7.1
	59.2	8.1	8.2	893	633	7.2
	76.9	8.1	8.2	956	663	7.2
	100	8.0	8.2	926	709	7.4

Although an increase in mortality seems to occur in organisms exposed to ELS and PLS (≥20%), there were no significant differences comparatively to the respective control ($p \geq 0.0747$; Figure 8).

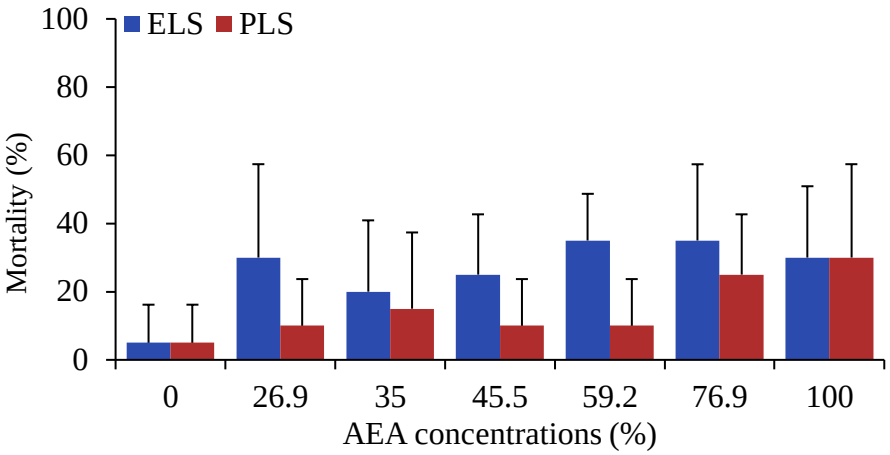

Figure 8 – Average of mortality of tadpoles of *Xenopus laevis* exposed, for 14 days, to concentrations of aqueous extracts of ashes (AEA) of eucalypt (ELS) and pine (PLS) forests. Error bars represent standard deviation.

Both AEAs significantly affected the development of the tadpoles ($p < 10^{-2}$). On ELS, the organisms on all the concentrations were significantly less developed than the ones on the control ($p \leq 0.0271$; Figure 9). On PLS, only the organisms exposed to concentrations of 35.0% and higher were significantly less developed than the ones on the control ($p \leq 0.0398$; Figure 9).

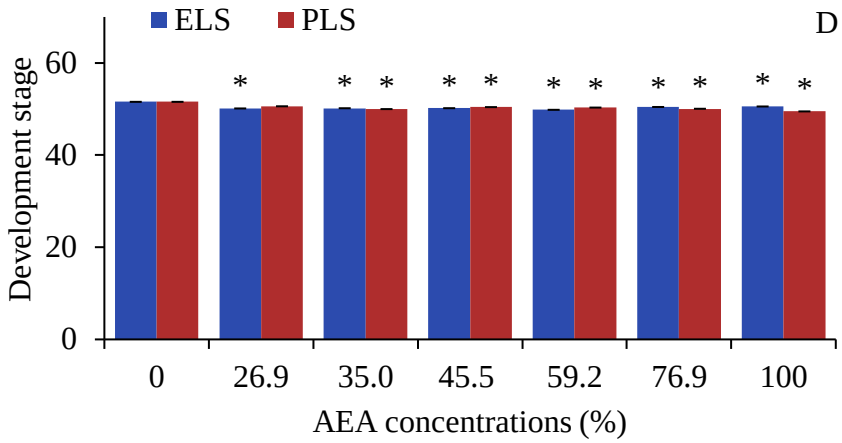

Figure 9 – Average of development stage of tadpoles of *Xenopus laevis* exposed, for 14 days, to concentrations of aqueous extracts of ashes (AEA) of eucalypt (ELS) and pine (PLS) forests. Error bars represent standard deviation. * represents statistical difference to the respective control.

Exposure to the two types of AEA adversely affected SVL, tail length and total length ($p \leq 0.0179$). On ELS, SVL was significantly different from the control at concentrations 59.2% to 100% ($p \leq 0.0348$; Figure 10A), tail length differed significantly from the control at 35.0% and from 59.2% to 100% ($p \leq 0.0381$; Figure 10B) and total length was different

from the control in all AEA concentrations ($p < 10^{-3}$; Figure 10C). For PLS, SVL ($p < 10^{-4}$), tail length ($p < 10^{-4}$) and total length ($p < 10^{-3}$) were significantly lower in the AEA concentrations comparatively to the control (Figure 10). The LOEC and NOEC values, represented in Table 3 show that, regarding SVL and tail length, PLS starts to induce significant effects at lower concentrations than ELS. Similarly, exposure to both AEA significantly altered the weight of tadpoles ($p < 10^{-6}$). All AEA concentrations significantly reduced the weight of tadpoles comparatively to their respective control group, for both ELS ($p \leq 0.0002.6$; Figure 10D) and PLS ($p \leq 0.00023$; Figure 10D). The tadpoles exposed to 100% of ELS weighed, on average, approximately 58% less than the control ones and those exposed to 100% of PLS weighed 47% less. As shown in Table 3, LOEC and NOEC values were 26.9% and < 26.9%, respectively, for both AEA.

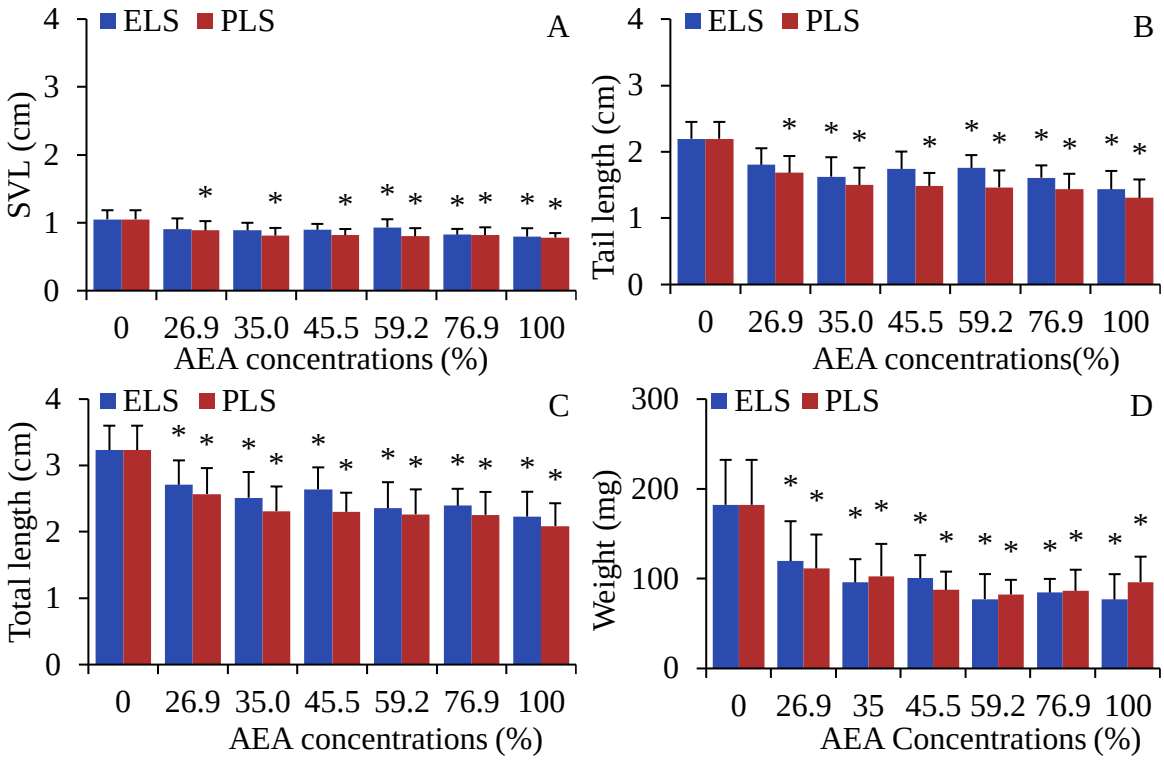

Figure 10 – Average of body lengths (A – snout-to-vent; B – tail; and C – total length) and weight (D) in tadpoles of *Xenopus laevis* exposed, for 14 days, to concentrations of aqueous extracts of ashes (AEA) from a pine (PLS) and eucalypt (ELS) forest. Error bars represent standard deviation. * indicates significant differences from the respective control ($p < 0.05$).

Regarding the endpoints monitored at sub-individual level, no significant effects were observed for AChE and TG activity ($p \geq 0.160$; Figure 11B and D). CAT activity altered

significantly with both AEA ($p \leq 0.00821$). For ELS, although there was an apparent decrease in CAT activity, only the concentration of 35% showed to be significantly different from the control ($p = 0.00693$; Figure 11A). In PLS a significant decrease in the activity of this enzyme was observed at all the concentrations ($p < 10^{-3}$; Figure 11A) comparing to the control. The activity of GST was also significantly altered on both AEA types. On ELS, the GST activity on concentrations from 59.2% to 100% was significantly lower than the control one ($p \leq 0.00150$; Figure 11C). On PLS, GST activity was significantly lower than the control in all concentrations ($p < 10^{-7}$; Figure 11C). On the 100% concentration, GST activity was 42.6% and 70.3% lower than in the control for ELS and PLS, respectively. LPO only differed significantly for ELS ($p = 0.0349$), where there was a signigicant increase in TBARS on the 35% concentration ($p = 0.0379$; Figure 11E).

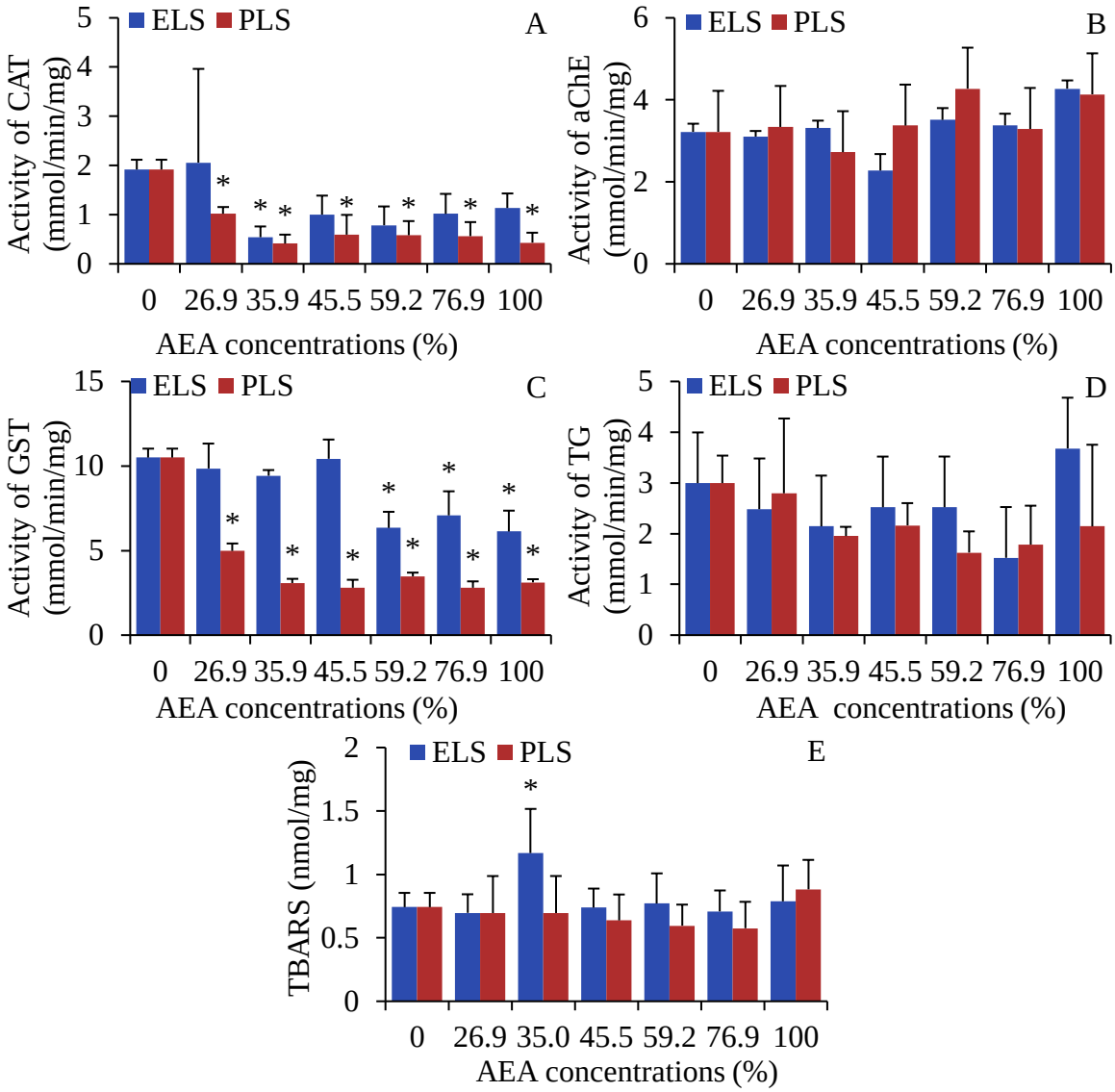

Figure 11 – Average of enzymatic activity (A – catalase; B – acetylcholinesterase; C – glutathione-S-transferase; D – total glutathione) and lipid peroxidation (LPO) damage (E – thiobarbituric acid reactive substances) in tadpoles of *Xenopus laevis* exposed, for 14 days, to concentrations of aqueous extracts of ashes (AEA) from a pine (PLS) and eucalypt (ELS) forest. Error bars represent standard deviation. * indicates significant differences from the respective control ($p < 0.05$).

Carbohydrate content of *X. laevis* tadpoles was significantly altered with the exposure to both AEAs ($p \leq 10^{-5}$), with decreases relatively to the control of up to 65% on 100% ELS (Figure 12A), and 83% in 76.9% PLS (Figure 12A). Lipid content significantly decreased on samples also exposed to both types of AEA at concentrations equal and above 45.5% ($p \leq 0.0151$; Figure 12B). On the other hand, protein content suffered no significant alteration on organisms exposed to any AEA ($p \geq 0.194$; Figure 12C). Oxygen consumption significantly increased in organisms exposed to both AEA ($p < 10^{-2}$), although, for ELS, the pairwise comparison showed no significant difference between any concentration and the control ($p \geq 0.161$; Figure 12D). On PLS, the oxygen content increased significantly in the 26.9%, 35.0%, 76.9% and 100% concentrations ($p \leq 0.0323$; Figure 12D).

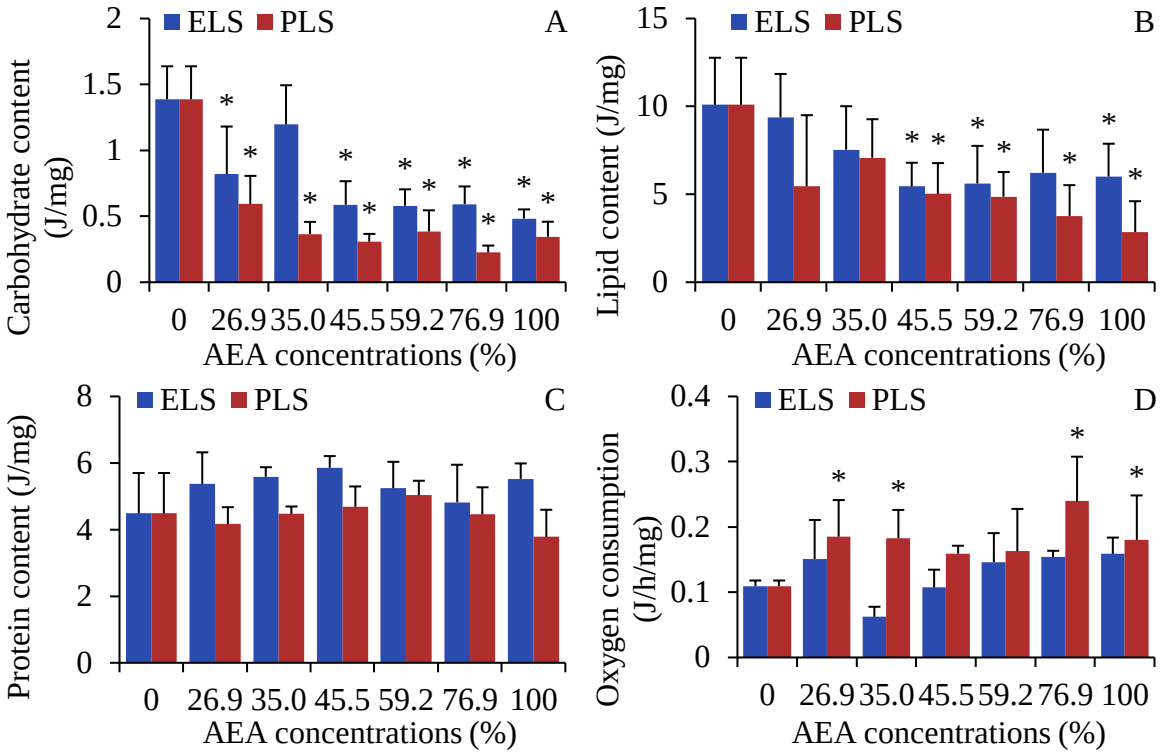

Figure 12 – Average of energy reserves (A – carbohydrate content; B – Lipid content; C – Protein content; D – Oxygen content in tadpoles of *Xenopus laevis* exposed, for 14 days, to concentrations of aqueous extracts of ashes (AEA) from a pine (PLS) and eucalypt (ELS) forest. Error bars represent standard deviation. * indicates significant differences from the respective control ($p < 0.05$).

Table 3 – Values of the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) for various biological and biochemical parameters in tadpoles of *X. laevis* exposed, for 14 days, to concentrations of AEA of an eucalypt (ELS) and pine forest (PLS).

	ELS		PLS	
	LOEC	NOEC	LOEC	NOEC
Mortality	-	100%	-	100%
Development	26.9%	< 26.9%	35.0%	26.9%
SVL	59.2%	45.5%	26.9%	< 26.9%
Tail length	35.0%	45.5%[a]	26.9%	< 26.9%
Total length	35.0%	45.5%[a]	26.9%	< 26.9%
Weight	26.9%	< 26.9%	26.9%	< 26.9%
CAT	35.0%	100%	26.9%	< 26.9%
aChE	-	100%	-	100%
GST	59.2%	45.5%	26.9%	< 26.9%
TG	-	100%	-	100%
LPO	35.0%	100%[a]	-	100%
Carbohydrates	26.9%	35.0% [a]	26.9%	< 26.9%
Lipids	45.5%	76.9% [a]	45.5%	35.0%
Proteins	-	100%	-	100%
ETS	-	100%	26.9%	59.2%[a]

a-This concentration was considered as the NOEC since no significant effects were here registered. Though it must be highlighted that at a lower concentration significant effects were observed relatively to the control.

3.3.2. *Pelophylax perezi*

The pH varied from 7.8 and 8.1 throughout the 14 days of the assay. Conductivity was increased in higher concentrations of AEA, ranging from 684 to 1087. Oxygen levels kept within the acceptable range and were on all occasions 7.0 or higher.

Table 4 – Physical and chemical parameters measured before and after changing the test solutions in the assay performed with tadpoles of *Pelophylax perezi*.

Ashes		pH	Conductivity	Oxygen
Control		7.8	684	7.3
ELS	26.9	8.0	826	7.9
	35.0	8.0	839	7.6
	45.5	8.1	866	7.1
	59.2	8.0	824	7.8
	76.9	7.9	937	7.6
	100	8.0	1087	7.2
PLS	26.9	-	-	-
	35.0	8.0	828	7.0
	45.5	8.1	819	7.1
	59.2	8.1	893	7.2
	76.9	8.1	956	7.2
	100	8.0	926	7.4

A significant mortality of *P. perezi* tadpoles exposed to ELS was observed at concentration 76.9% ($p = 0.0138$; Figure 13). For organisms exposed to PLS, though a mortality above 20% was registered at the two highest tested concentrations, it was not significantly different from the control, which could be due to the high variability in the responses registered at these concentrations (Figure 13).

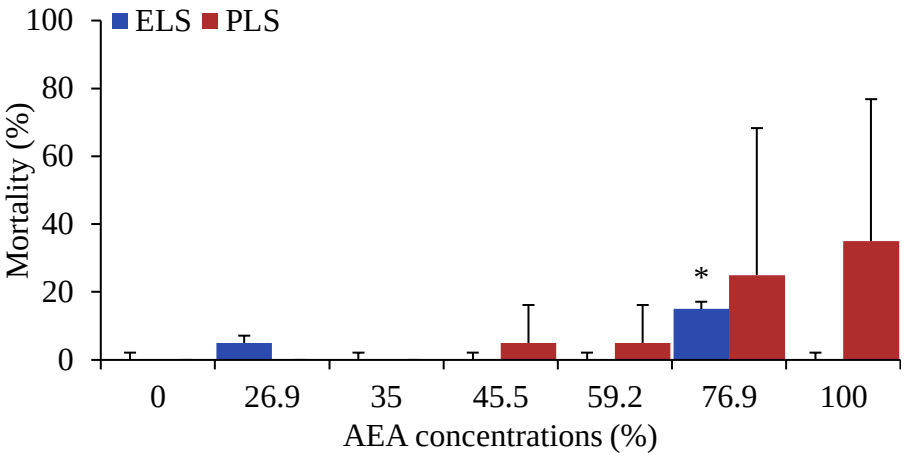

Figure 13 – Average of mortality of tadpoles of *Pelophylax perezi* exposed, for 14 days, to concentrations of aqueous extracts of ashes (AEA) of eucalypt (ELS) and pine (PLS) forests. Error bars represent standard deviation. * indicates significant differences from the respective control ($p < 0.05$).

As for development stages, this endpoint was not significantly affected by the exposure to ELS or PLS AEA ($p \geq 0.199$; Figure 14).

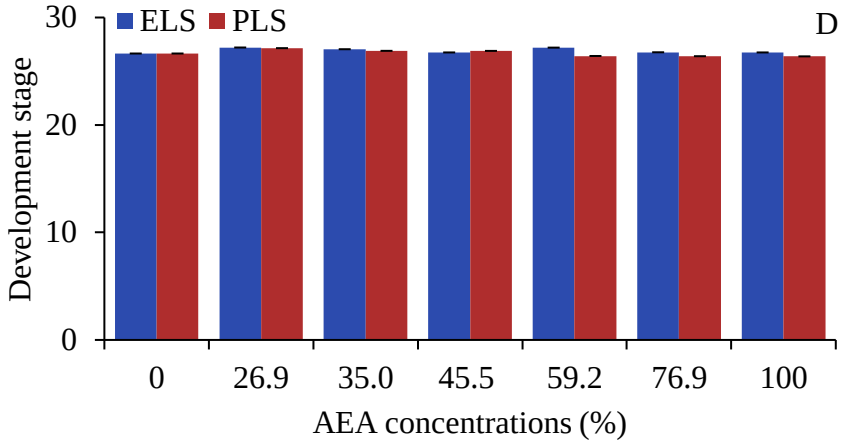

Figure 14 – Average of development of tadpoles of *Pelophylax perezi* exposed, for 14 days, to concentrations of aqueous extracts of ashes (AEA) of eucalypt (ELS) and pine (PLS) forests. Error bars represent standard deviation. * indicates significant differences from the respective control ($p < 0.05$).

ELS exposure did not affect the SVL, tail or total length of *P. perezi* tadpoles ($p \geq 0.0980$; Figure 15). However, PLS caused a significant decrease in SVL ($p \leq 0.0439$; Figure 15A) at concentrations from 35% to 100%, and in tail and total length at concentrations from 45.5% to 100% ($p \leq 0.0170$; and $p \leq 0.0343$, respectively; Figure 15B and C). Concerning

the weight, no significant changes were observed between tadpoles exposed to ELS and the respective control ($p \geq 0.116$). Though, as observed for length, tadpoles of *P. perezi*, exposed to concentrations of 35% to 100% of PLS, weighted less than tadpoles from the control group ($p \leq 7.48*10^{-4}$; Figure 15D).

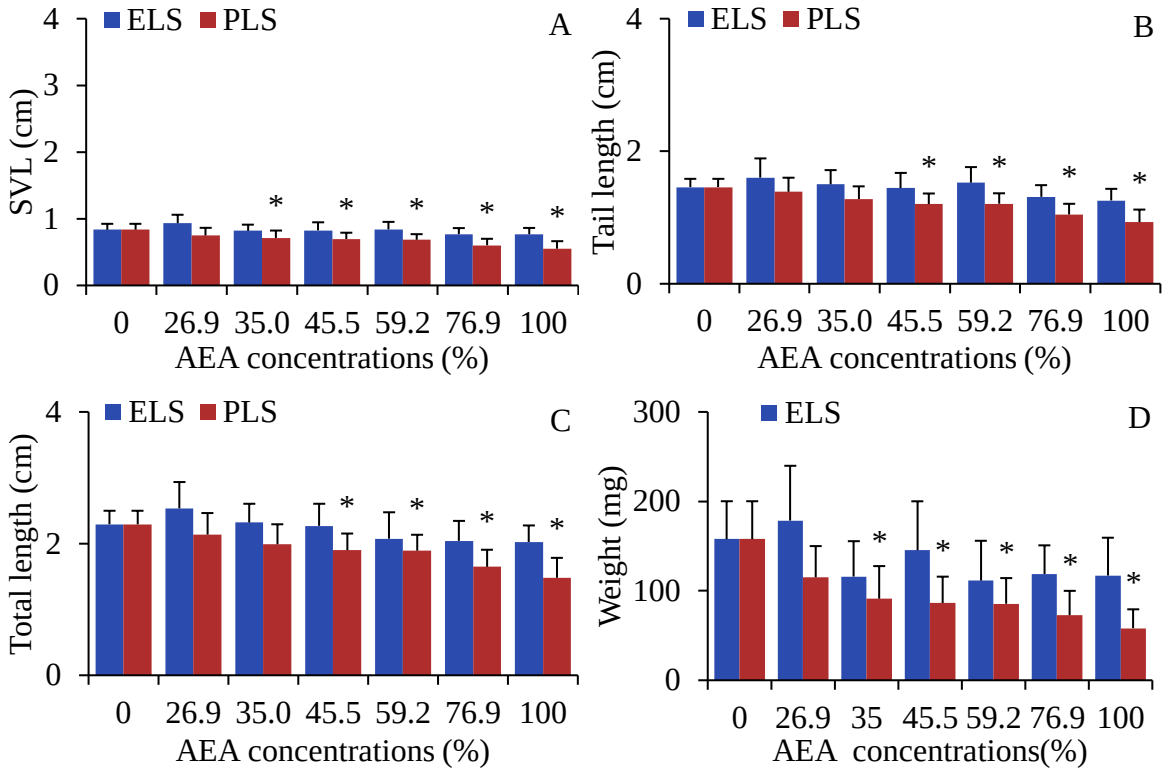

Figure 15 – Average of body lengths (A – snout-to-vent; B – tail; and C – total length) and weight (D) in tadpoles of *Pelophylax perezi* exposed, for 14 days, to concentrations of aqueous extracts of ashes (AEA) from a pine (PLS) and eucalypt (ELS) forest. Error bars represent standard deviation. * indicates significant differences from the respective control ($p < 0.05$).

Pelophylax perezi tadpoles exposed to ELS showed no significant alterations in the activity of the analysed enzymes (CAT, AChE and GST) and on lipid peroxidation, comparatively to the control group ($p \geq 0.1943$; Figure 16). As for organisms exposed to PLS, the activity of CAT was similar among organisms exposed to the AEA concentrations and to the control, also no significant lipid peroxidation was observed at any exposed group of organisms ($p \geq 0.0708$; Figure 16D). However, significant alterations were observed in the activities of AChE and GST: tadpoles exposed to 100% of PLS showed a significant

increase in the actvity of AChE ($p < 10^{-4}$; Figure 16B), while a reduction in GST activity was observed in tadpoles exposed to all concentrations of PLS ($p < 0.0401$; Figure 16C).

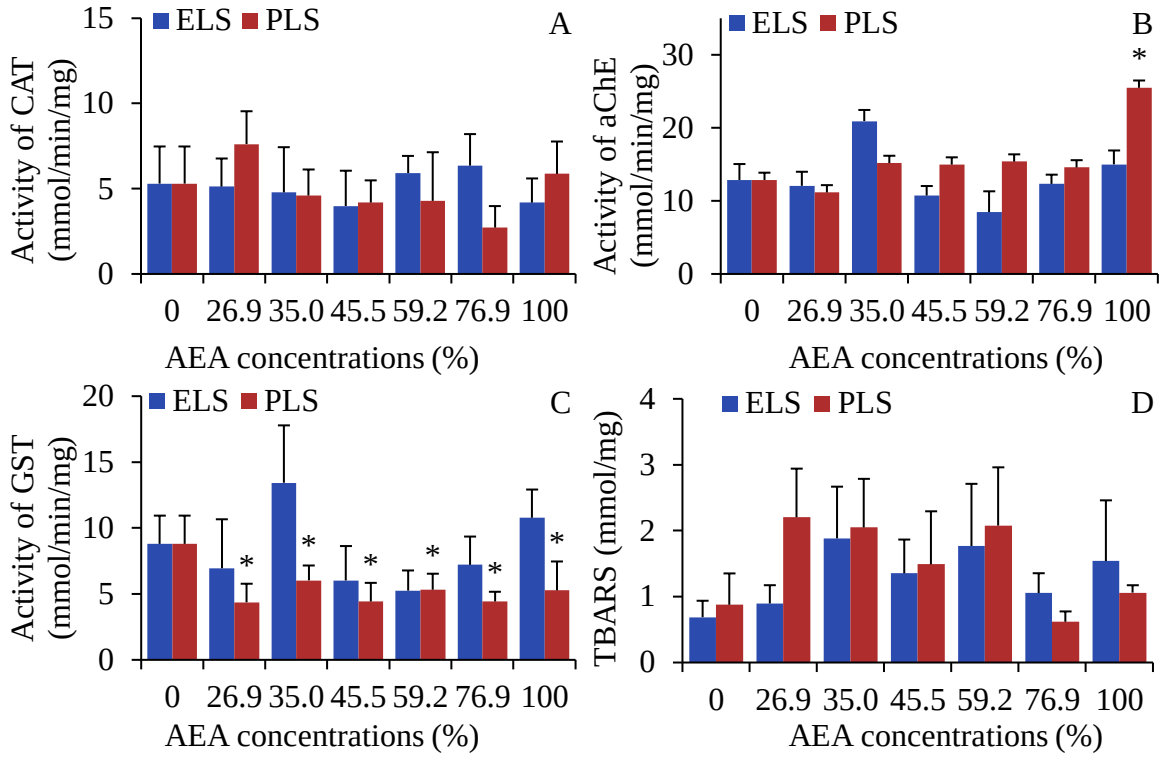

Figure 16 – Average of enzymatic activity (A – catalase; B – acetylcholinesterase; C – glutathione-S-transferase) and lipid peroxidation (LPO) damage (D – thiobarbituric acid reactive substances) in tadpoles of *Pelophylax perezi* exposed, for 14 days, to concentrations of aqueous extracts of ashes (AEA) from a pine (PLS) and eucalypt (ELS) forest. Error bars represent standard deviation. * indicates significant differences from the respective control ($p < 0.05$).

Carbohydrate, lipid and protein content were not significantly altered with the exposure to any of the tested concentrations of ELS or PLS ($p \geq 0.0544$; Figure 17A, B, C). Regarding oxygen consumption, only in organisms exposed to PLS a significant increase in consumption was observed at 26.9%, 59.2%, 76.9% and 100% concentrations, when comparing to the control ($p \leq 0.0446$; Figure 17D).

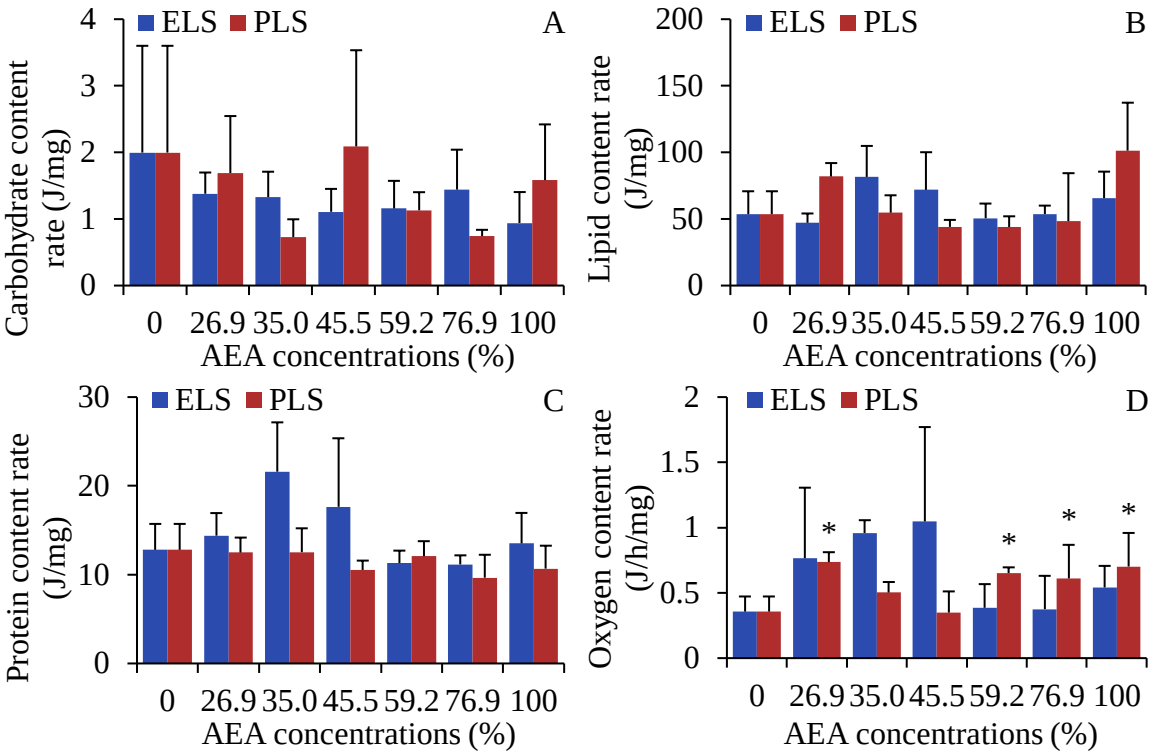

Figure 17 – Average of energy reserves (A – carbohydrate content; B – Lipid content; C – Protein content; D – Oxygen content in tadpoles of *Pelophylax perezi* exposed, for 14 days, to concentrations of aqueous extracts of ashes (AEA) from a pine (PLS) and eucalypt (ELS) forest. Error bars represent standard deviation. * indicates significant differences from the respective control ($p < 0.05$).

The LOEC and NOEC values for all the analysed endpoints are shown in

Table **5**. For ELS, no significant effects were observed for any of the monitored responses, so a NOEC of 100% was established for all the endpoints. For PLS, NOEC and LOEC values varied according to the assessed endpoint: for mortality, CAT, LPO, carbohydrates, lipids and proteins the determined NOEC was 100%, for GST was less than 26.9%, for SVL and weight was 26.9%, for tail and total lengths was 35.0%, for ETS was 45.5% and for aChE was 76.9%.

Table 5 – Values of the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) for various biological and biochemical parameters in tadpoles of *P. perezi* exposed for 14 days to concentrations of AEA.

	ELS		PLS	
	LOEC	NOEC	LOEC	NOEC
Mortality	-	100%	-	100%
Development	-	100%	-	100%
SVL	-	100%	35.0%	26.9%
Tail length	-	100%	45.5%	35.0%
Total length	-	100%	45.5%	35.0%
Weight	-	100%	35.0%	26.9%
CAT	-	100%	-	100%
aChE	-	100%	100%	76.9%
GST	-	100%	26.9%	< 26.9%
LPO	-	100%	-	100%
Carbohydrates	-	100%	-	100%
Lipids	-	100%	-	100%
Proteins	-	100%	-	100%
ETS	-	100%	26.9%	45.5%[a]

a-This concentration was considered as the NOEC since no significant effects were here registered. Though it must be highlighted that at a lower concentration significant effects were observed relatively to the control.

Discussion

The elements present at higher concentrations in the ashes collected from pine and eucalypt plantations were Mn, Zn and Cu. Silva et al. (2015) found similar results, except their study showed a higher concentration of V than Cu, while on this study, Cu showed a higher concentration than V for ELS and PLS ashes and the two AEA. The ashes characterized by Silva et al. (2015) are however a representation of a mixed stand of eucalypt (mainly) and pine. All metals (As, Cd, Co, Cu, Mn, Ni, Pb, Zn) quantified by Silva et al. (2015) except V were in the same order of magnitude as the two types of ashes here studied, although those authors reported a concentration of Mn 96% higher than that measured in the present work. The metals present at higher concentrations on the ashes (Mn, Zn and Cu) were also the ones present at the highest concentrations on the AEA, showing that the studied ashes have the same relative metal representation than the AEA, except for Pb and As, that were represented at higher levels on the original ashes. Most of the concentrations of metals in our findings are in agreement with other studies (Plumlee *et al.*, 2007), although the authors results referred to the collected runoff, whereas ours referred to an elutriate mixed in the lab to mimic an ash runoff. Plumlee et al. (2007) also found concentrations <1 mg/L for As, Cr, Cu, V and Zn and <0.05 mg/L for Cd in postfire runoffs. On our study, Co, Ni and Pb concentrations were 0.05 – 1 mg/L of AEA and Mn concentration was higher than the 1 mg/L of AEA, whereas Plumlee et al. (2007) found a <0.05 mg/L Co, Ni and Pb concentration and a 0.05 – 1 mg/L Mn concentration. Although there are similarities in terms of magnitude and abundance matrix, the here tested AEA presented some differences in terms of composition. PLS revealed a higher concentration of the three most abundant metals (Mn, Zn and Cu) in the AEA. Some of these, have been shown to be highly associated with toxicity to biota (Shuhaimi-Othman, Nur-Amalina and Nadzifah, 2012; Araújo *et al.*, 2014).

The teratogenicity assay showed that exposure of *X. laevis* embryos to ELS and PLS mainly induced effects at the biochemical level. Mortality, development, length and oxidative stress responses were not affected by the AEA. These results suggest a low sensitivity of embryos to the ashes and chemicals present in the two AEA, which may be related to the fact that they possess a jelly coat and the embryonic membranes that may serve as a physical barrier for contaminants in the medium.

Though the absence of significant mortality relatively to the control (most probably, due to a high variability registered in these responses), it must be highlighted that at 100% of ELS and PLS a mortality above 20% (considered the threshold for significant lethal effects by several guidelines) was reported. Actually, the concentrations of some metals in the AEA are more than enough to justify the occurrence of some mortality. Herkovits & Alejandra Helguero, (1998) showed that Cu concentrations of 0.085 mg/l can cause 50% mortality on *Rhinella arenarum* embryos. The concentrations of this very same metal on our study were more than double, which would predict the occurrence of a mortality higher than the one that was observed (~20%) (Table 1). This might be explained by the AEA's high pH value (Table 2). It is known that lower pH tend to favour metal solubility and consequently bioavailability (Buchwalter, Linder and Curtis, 1996; Wilde *et al.*, 2006; Marques *et al.*, 2013; de Paiva Magalhães *et al.*, 2015), hence, alkaline AEA may have a lower metal bioavailability, potentially leading to lower mortality despite high metal concentrations. Nonetheless, this may not be enough reason to justify absence of mortality. Furthermore, Herkovits & Alejandra-Helguero (1998) point out that Zn can have an antagonistic interaction with Cu acting as a protection for Cu toxicity and in cases where Zn itself has a toxic effect Cu can also nullify that effect. In our study, Zn was one of the most concentrated metals present in the AEA (Table 1) and therefore may have acted as a protection from other metals, specifically Cu.

Energy reserves on the other hand showed a slight response to PLS exposure. With increasing PLS concentrations, there was a decrease in carbohydrate content and an increase in oxygen consumption. Other authors also found exposure to stressors such as metals (Cd, Cu or Zn) or herbicides to induce alterations in the energy reserves and oxygen consumption in amphibians (Durou, Mouneyrac and Amiard-Triquet, 2005; Freitas *et al.*, 2019). These changes were likely caused by an increase in the metabolism in order to cope with the exposure to PLS and the metals present in it. This resulted in an increase in respiration rate, and consequently in a decrease in the energy reserves, namely carbohydrate content (Rabasa and Dickson, 2016). The first reserves to respond to an increase in metabolism are usually carbohydrates, as they are easily available. Though, if carbohydrate molecules are not enough to accommodate the metabolic increase, glycogen is broken into glycose to be consumed. If these are not enough to respond to the metabolic stress, lipids are also integrated in the glucose catabolism pathways. As a last resort, proteins can also be

consumed in order to obtain energy. On *X. laevis* embryos, PLS only caused a decrease in carbohydrates, indicating that the energetic demands did not induce an increase in metabolism enough to require lipid or protein breakdown. The fact that such metabolic changes were only observed for embryos exposed to PLS and not ELS may be an early indication of a higher toxicity of the former. Higher concentrations of Mn, Zn and Cu, which are the most abundant metals on PLS might be an explanation for this differential toxicity.

Tadpoles of *X. laevis* were the tested organisms that showed the highest sensitivity to both ELS and PLS AEA. Though a statistically significant effect was not observed, mortality above 25% occurred in tadpoles exposed to the two AEA. Also, a delay in development and reduction in body lengths and weight were observed for tadpoles exposed to the two AEAs. Furthermore, there was an oxidative stress response of CAT and GST, as well as an unbalance of the energy metabolism, demonstrated by a general decrease in energy reserves and an increase in oxygen consumption. The higher sensitivity of tadpoles, comparatively to embryos of the same species may be explained by the fact that tadpoles do not hold a jelly coat or embryonic membranes to serve as a physical barrier to the contaminants, while exhibit other organs that may potentiate exposure such as the gills, a highly vascularized and permeable skin, and a developed mouth that promoted the uptake of ashes and contaminants through ingestion. This means that, besides the dissolved component of the AEA, the deposited ashes may also have a relevant impact on these organisms. Figure 18 depicts the accumulation of ashes on the digestive tract ant throughout the body (Annex 1).

Although there is an earlier effect on ELS, higher concentrations show that on average, PLS seems to affect development in a more pronounced way than ELS. This is an indicative that although ELS requires lower concentrations to affect development, at higher ones, PLS is more severe. Haywood et al. (2004) studied the effects of Zn, Cu, Pb and Cd on *X.laevis* embryos. They refer that at near lethal concentrations, metal exposure might lead to a halt in development, and sub-lethal concentrations negatively affect hatching success. The here studied AEA concentrations did not show significant lethal effects. Although, the slower development may be an indication that energy which would usually be used for development and growth is being relocated to metal detoxification mechanisms.

The results demonstrate that both AEA deeply affect growth with both snout-to-vent and tail lengths decreasing with increasing AEA concentrations. Tadpole weight follows the

same tendency but more intensely. The fact that the AEA affected growth on tadpoles but not on embryos might be explained due to the additional exposure pathways that may potentiate uptake of chemicals. The accumulated ashes in the tadpoles' gut might have prevented the tadpoles from feeding, leading to a potential malnutrition and ultimately less efficient growth. On snout-to-vent and tail lengths, there is a distinguishable difference between ELS and PLS. The latter has an earlier effect and appears to be harsher on higher concentrations. This difference might be inherent to the AEA composition, more specifically, the metal matrix. PLS had a higher concentration of Mn, Zn and Cu, the most abundant metals in both AEA. Of these, on the concentrations measured on the AEAs, only Cu seems to be at a concentration that may be toxic to larval stages of amphibians and other river-dwelling organisms (Herkovits and Alejandra Helguero, 1998; Shuhaimi-Othman, Nur-Amalina and Nadzifah, 2012). The previously mentioned antagonism between Cu and Zn seems particularly relevant here. The proportions of Cu/Zn are 0.38 on ELS and 0.45 on PLS. This difference might be the cause for PLS being more toxic and inducing more changes, since there is relatively less Zn in ELS than PLS, so copper toxicity may not be as antagonized in PLS ad it is in ELS.

On the one hand, results from the oxidative stress biochemical markers agreed with the previous results, with PLS having an earlier and harsher effect than ELS, but were on the other hand unexpected, with only an effect on CAT and GST and no lipidic damage. CAT is an important enzyme in the ROS metabolism, catalysing hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) molecules. This enzyme's activity decreased in both AEA concentrations, although only significantly in PLS. Some metals present in the ash matrix and respective AEA have been shown to induce an increase in CAT activity (Radwan, El-Gendy and Gad, 2010) while others such as Cd, Cu and Zn can cause an inhibition (Ahmad *et al.*, 2005; Hansen *et al.*, 2007; Loro *et al.*, 2012). Furthermore, Atli et al. (2006) showed that even in the same organism, CAT activity can vary significantly and even show opposite responses. This issue is escalated by the complex metal matrix present in our ashes, making effect predictions extremely difficult. Metals present in the ash matrix and AEA, such as Cu, V, Cr and Co are known to induce the formation of free radicals such as H_2O_2 (Annex 2; Figure 19). Although CAT inhibition is associated with an excessive oxidative stress, there was no lipidic damage. One reason for such is the interaction of Zn, which is also associated with the oxidative stress pathway, but in an opposed way. Zn is known to act as an

antioxidant by preventing free metal formation (Valko, Morris and Cronin, 2005). GST activity also decreased for both AEA. PLS showed an earlier and harsher effect than ELS. The complex metal matrix on the AEA make it hard to predict a biomarker response. GST enzymes are deeply connected to reduced glutathione (GSH), and have the role of catalysing nucleophilic attacks by GSH on nonpolar compounds, preventing their interaction with crucial cellular proteins and nuclear acids (Hayes, Flanagan and Jowsey, 2005). There is also a wide variety of responses to metals by this enzyme. Loro et al. (2012) demonstrated that an exposure of *Fundulus heteroclitus* to zinc has significantly increased GST activity. Contrastingly, Radwan et al. (2010) showed that Cu causes an inhibition of this enzyme. GST activity decreases found in this study may also be a consequence of the complex ash matrix. Interestingly, although there was an effect on the oxidative stress enzymatic response, there was no damage on the lipids (LPO), which might be an indicative that although there was an inhibition of some enzymes, it was still enough to negate lipidic damage.

Energy reserves also showed some interesting results in this assay. There was a decrease in both carbohydrate and lipid contents of organism exposed to ELS and PLS AEA. Although, PLS decreases were overall harsher and started at lower concentrations. There is a typical sequence for stress induced energy reserve consumption (Wang, Hung and Randall, 2006) and these results are no exception. There is a clear sequence of effects starting with carbohydrates, following to lipids and ending on proteins. Even lower AEA concentrations induced a decline in carbohydrate content on *X. laevis* tadpoles, whereas lipid content was only negatively affected by higher AEA concentrations, and no effect at all was found in protein content for the tested concentrations. This sequence was also previously associated with an increase in oxygen consumption. However, on this experiment, only organisms exposed to PLS showed an increase in oxygen consumption, indicating that for tadpoles the response may be more complex than for embryos. On the teratogenicity assay, only carbohydrates were affected by the AEA, suggesting that *X. laevis* tadpoles are more sensitive than embryos. These differences might occur due to the previously mentioned additional exposure pathways. Physical ash accumulation might also explain the oxygen consumption response on tadpoles. On the one hand, Comoglio et al. (2005) found out that false southern king crabs (*Paralomis granulosa*) under starvation suffer a decrease in lipid content and an increase in oxygen consumption. On the other hand, it is known that in

mudflat fiddler crabs (*Uca rapax*), metal exposure leads to a decrease in oxygen consumption (Capparelli, Abessa and McNamara, 2016). Since there may exist a starvation effect (*sensu* decreased food ingestion and decreased nutrient adsorption in the gut) due to the accumulated ashes and a metal exposure effect, there might an antagonism between the two factors, and since oxygen consumption increased in *X. laevis* tadpole exposure, the starvation effect seems to be more relevant than the metal exposure itself. This starvation hypothesis is also supported by the overall decrease in size and weight as well as slower development found in organisms exposed to the AEA. Furthermore, the difference between the AEA might be a consequence of how efficient the ash deposits are as acting as a physical blocking barrier, thus indicating that deposits form PLS might form a sturdier barrier preventing the tadpoles from feeding.

P. perezi tadpoles showed similar responses to that of the *X. laevis* tadpoles, but on a smaller scale. Organisms exposed to PLS were smaller, weighed less, showed a biochemical marker response and consumed more oxygen than the reference. Although ELS showed the same tendencies as the PLS, it induced a significantly higher mortality at 76.9%. Although PLS did not show any significant differences, there was also a tendency for higher concentrations leading to increased mortality. Development was not affected by either AEA and that was expected because *P. perezi* is a species that is capable of inhabiting contaminated waterbodies (Egea-Serrano, Tejedo and Torralva, 2009; Egea-Serrano and Tejedo, 2014).

Length and weight of *P.perezi* tadpoles followed the tendency of *X. laevis*, with general decreases in snout-to-vent, tail and total lengths, and a decreased weight. The scale of the effect, however, was totally different. Contrarily to what was observed with *X. laevis* tadpoles, on *P. perezi* only PLS exposure decreased tadpole length and weight, and even then, the difference was only significant on moderate concentrations (35.0% - 45.5%). This means that the higher toxicity of PLS was consistent across both studied species' tadpoles and that the tested AEA have a harsher impact in terms of biometric measurements on *X. laevis* than on *P. perezi*. Barry (2011) found out that, on *Sclerophrys arabica*, Cu negatively affects growth, and, with the tested concentrations, Zn has no effect, thus validating our results. However, even though the concentrations of these metals are higher in our AEA, the decrease was not as pronounced. That is because of the antagonistic interaction between Cu

and Z, besides, other metals might interfere as well. Furthermore, the alkalinity of our AEA does not favour metal solubility and therefore decreases bioavailability.

Biochemical marker response was poor on *P. perezi* tadpoles, with only an aChE activity increase in 100% PLS and a decrease in GST activity also in PLS. Although there was a significant change in these two enzymes' activities, no lipidic damage was found. A lower response when comparing *P. perezi* to *X. laevis* in terms of oxidative stress markers was already expected, since, while the latter is known to be sensitive to environment stimuli and stressors, the former is relatively more resistant and therefore able to cope better with the AEA exposures (Marques *et al.*, 2013). Nonetheless, aChE activity increased on PLS exposure. Frasco et al. (2005) found out that metals such as Ni, Cu, Zn and Cd inhibit the activity of this enzyme. Although, the authors also defend that the different metals have different affinities with the used procedure, thus, although there was an effect, this effect may be the result of over and under-representations of some metals due to their affinities. GST activity seems to be an exception for the increased *P. perezi* tadpole resistance. This enzyme's activity decreased in the same scale that the *X. laevis* one for PLS. Although, for ELS, this enzyme's activity was not affected. While on *X. laevis* the highest ELS concentrations also lead to a decrease in GST activity, this was not the case with *P. perezi*. There are therefore more accentuated differences between both AEA's effect on GST activity in *P.perezi* tadpoles.

On *P. perezi* tadpoles, energy reserves were not affected, except for oxygen consumption, by ELS or PLS AEA. Similarly to *X. laevis*, oxygen consumption suffered a general increase on PLS only, with an attenuation in mid-range concentrations. The fact that only oxygen consumption decreased and none of the other reserves suffered a change might indicate that there was no starvation effect present and the increased oxygen consumption was a result of a metabolic acceleration in order to cope with the stress from the AEA.

Final remarks and future research

Over the three assays, several endpoints were tested in order to demonstrate the impact of AEA on larval stages of amphibians, which allowed us to reach the following conclusions:

At the individual level, the sublethal impacts are constant throughout the assays. Exposure to the compounds present in the two AEA affected the studied organisms in a consistent manner. At the individual level, length and weight showed to be sensitive endpoints to evaluate the toxicity of AEA as they were significantly affected in tadpoles of the two species exposed to the AEA (except for ELS in *P. perezi*). As for biochemical parameters, those related with energetic metabolism showed the highest sensitivity, with oxygen consumption being the endpoint affected for embryos and tadpoles of *X. laevis* and tapoles of *P. perezi*.

The toxicity induced by the two AEA was developmental stage dependent. The embryos of *X. laevis* were more tolerant to the two AEA than the tadpoles, which could be related to the presence of the jelly coat and embryonic membranes that may act as a physical barrier to the uptake of chemicals and to the fact that tadpoles have gills and an open mouth that may contribute to a higher ash and associated chemicals uptake. These results suggest tadpoles to be an adequate model organism to assess the impact of ashes in aquatic life stages of anurans.

Likewise, the toxicity of the AEA was species dependent, with tadpoles of *X. laevis* being much more sensitive to the two AEA that the tadpoles of *P. perezi*, thus suggesting the former to be adequate to be used in the risk assessment of wildfire originated ashes for amphibians.

Finally, plant coverage proved to affect the chemical composition and toxicity of the ashes, and subsequently the AEA. In this study, ashes originated from Pine Forest, in general, induced a higher toxicity to all the tested organisms.

Future research is still needed on this topic. The ash matrix is complex, and in order to attribute its effect, further studies on their compounds are required. Metallothionein proteins are an interesting way of measure metal toxicity and therefore should be addressed in the future. It is also known that water pH can also influence the bioavailability of the toxic compounds on the ashes, so it might be interesting to replicate the waterbodies' pH on the

burned site in order to assess pH's influence on ash toxicity. Assays have been performed with embryos and tadpoles, but to better replicate what happens naturally, long-term assays comprising both larval stages of amphibians can be performed in order to evaluate the chronical effects of AEA exposure. Another interesting study would be the link between ash toxicity and tadpole behaviour, to be assessed through an avoidance assay. This assay would provide an ecological response of the tadpoles to the AEA exposure. In order add ecological relevance, in-situ assays would also be relevant, because the organisms would be exposed to the exact post-fire conditions.

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Annex 1

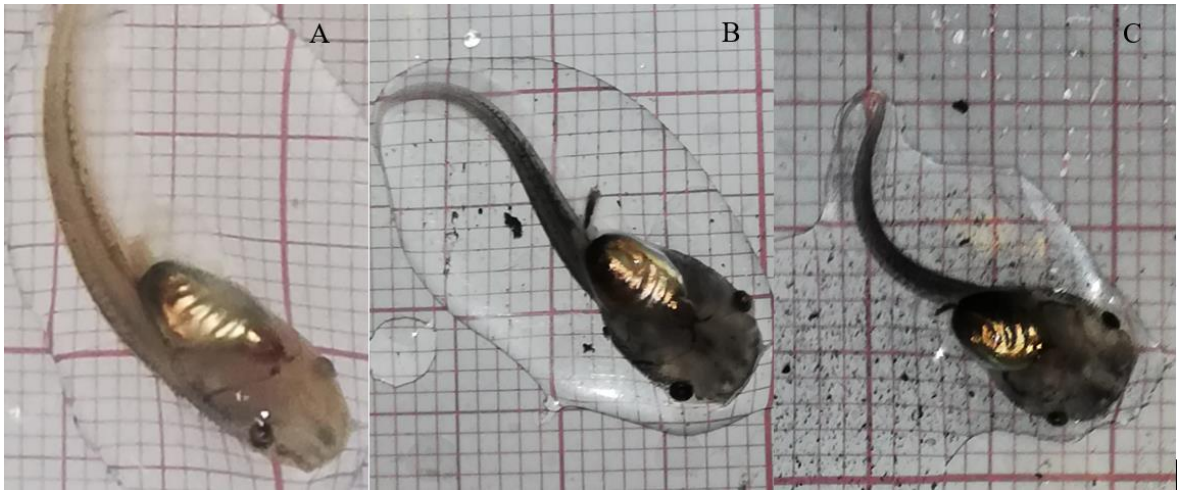

Figure 18 – Photographs of *X. laevis* exposed for 14 days to (A) control FETAX medium, (B) 100% ELS and (C) 100% PLS.

Annex 2

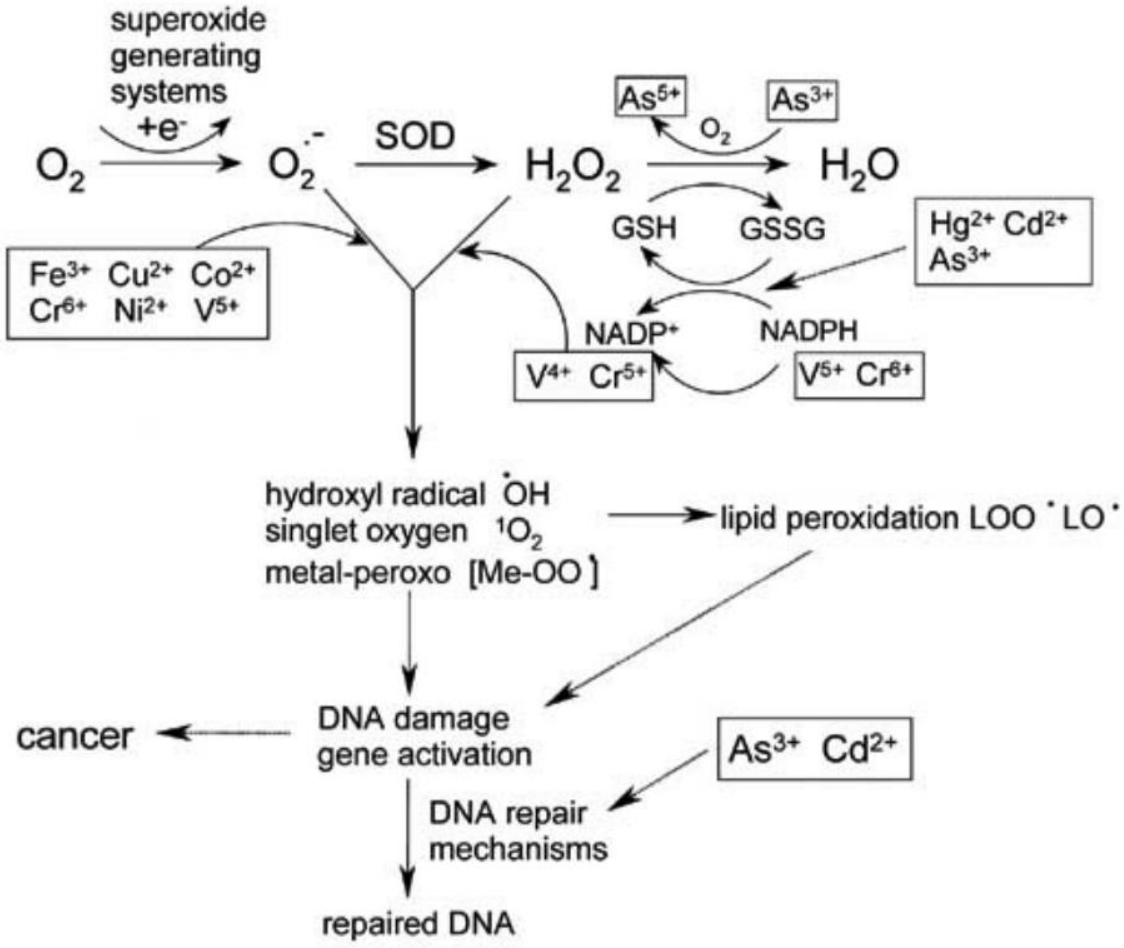

Figure 19 – Pathway of metal-induced oxidative stress. Taken from Valko et al. (2005).