Universidade de
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2020

INÊS BEATRIZ DA SILVA FERREIRA

INFLUÊNCIA DA SEVERIDADE DE INCÊNDIOS FLORESTAIS NA TOXICIDADE DE CINZAS EM ESTÁDIOS DE DESENVOLVIMENTO INICIAIS DE ANFÍBIOS

INFLUENCE OF WILDFIRES SEVERITY ON ASH TOXICITY TO EARLY LIFE STAGES OF AMPHIBIANS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Isabel Maria Cunha Antunes Lopes, investigadora principal do CESAM (Centro de Estudos do Ambiente e Mar) e do Departamento de Biologia da Universidade de Aveiro e do Doutor Nelson José Cabaços Abrantes, investigador auxiliar do CESAM (Centro de Estudos do Ambiente e Mar) e do Departamento de Ambiente e Ordenamento da Universidade de Aveiro.

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o júri

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palavras-chave

Incêndios florestais, severidade, extratos aquosos de cinzas, ecotoxicidade, *Xenopus laevis*, *Pelophylax perezi*.

resumo

A severidade dos incêndios florestais depende de vários fatores e das suas interações, nomeadamente, da temperatura, geomorfologia, características do combustível, condições meteorológicas, entre outras, que por sua vez influenciam a quantidade de cinzas produzidas durante o incêndio e a sua composição química. As cinzas produzidas durante incêndios florestais constituem uma matriz complexa composta por vários compostos orgânicos e inorgânicos, os quais constituem uma grande preocupação ambiental devido à sua elevada toxicidade, persistência no ambiente e tendência para bioacumulação. De facto, estudos recentes têm confirmado que os incêndios florestais atuam como fonte difusa de contaminação nos ecossistemas aquáticos, provocando alterações na sua estrutura e função. No contexto da contaminação causada por cinzas de incêndios florestais, os estádios de vida iniciais (embriões e girinos) de anfíbios, devido à sua ecologia e vulnerabilidade a perturbações ambientais, podem apresentar elevada suscetibilidade a este tipo de contaminação, por estarem mais expostos às substâncias químicas libertadas pelas cinzas, através da coluna de água e dos sedimentos. Este trabalho teve como objetivo avaliar a influência da severidade de incêndios florestais em povoamentos de pinheiro na ecotoxicidade de extratos aquosos de cinzas (AEA) em estádios iniciais de vida de duas espécies de anuros: *Pelophylax perezi* e *Xenopus laevis*. Para atingir esse objetivo, embriões de *X. laevis* e girinos das duas espécies foram expostos por 96 horas e 14 dias, respetivamente, a uma série de concentrações (26.9 a 100 %) de AEA resultantes de um incêndio de moderada severidade (MS) e alta severidade (HS). No final dos ensaios, foram avaliadas as seguintes respostas: mortalidade, malformações, estádio de desenvolvimento, comprimento corporal (cauda, rostro-cloaca e corpo total) e massa corporal (apenas para girinos). Para além destes critérios de avaliação apicais, os efeitos ao nível sub-individual também foram monitorizados para stress oxidativo (peroxidação lipídica, catalase, glutationa total, glutationa S-transferase - também uma enzima de transformação de xenobióticos), neurotoxicidade (acetilcolinesterase) e metabolismo energético (sistema transporte de eletrões, lípidos totais, carbohidratos e proteínas).

Foram ainda realizadas análises químicas aos dois tipos de cinzas e AEA. Ao comparar MS e HS-AEA, verificamos que o primeiro apresentou uma dureza inferior. Além disso, cinco (As, Co, Mn, Ni, V) dos dez elementos analisados estavam presentes em maiores concentrações no HS. Os ensaios realizados com embriões e girinos, das duas espécies de anuros, revelaram influência do tipo de AEA, espécie e estádio de vida, nos efeitos ecotoxicológicos observados. Quanto à toxicidade letal, observou-se uma redução semelhante e significativa na sobrevivência de embriões de *X. laevis* e girinos de *P. perezi* expostos a 100 % de MS e HS. Porém, os girinos de *X. laevis* foram mais sensíveis a MS do que a HS. Em relação ao efeitos sub-letais, HS foi mais tóxico para embriões de *X. laevis* do que MS, pois o primeiro tipo de AEA reduziu significativamente o CC, CRC e o CTot em todas as concentrações testadas, enquanto MS apenas afetou o CC. Pelo contrário, os girinos de *X. laevis* mostraram-se mais sensíveis ao MS; os comprimentos e peso corporais diminuíram significativamente para todas as concentrações testadas de MS, embora apenas as duas concentrações mais altas de HS afetassem esses parâmetros. Os girinos de *P. perezi* revelaram maior sensibilidade ao HS, tendo, em geral, o comprimento e peso corporais sido significativamente reduzidos em concentrações ≥ 26,9 % e 59,2 % de MS, respetivamente. No geral, os marcadores bioquímicos não foram significativamente afetados pela exposição aos dois tipos de AEA. Porém, é de salientar que a atividade da enzima catalase aumentou em embriões de *X. laevis* e girinos de *P. perezi* expostos a AEA, indicando a ocorrência de stress oxidativo. Este é o primeiro estudo sobre os efeitos ecotoxicológicos de AEA em anfíbios, e mostrou que a entrada de cinzas, geradas durante incêndios florestais, e de compostos químicos que lhes estão absorvidos, podem constituir um risco para as fases de vida aquáticas deste grupo de organismos.

keywords

Wildfires, severity, aqueous extracts of ashes, ecotoxicity, *Xenopus laevis*, *Pelophylax perezi*.

abstract

Wildfire severity depends on many factors and on their interactions, namely on temperature, geomorphology, fuel characteristics, meteorological conditions, among others. This, in turn, influences the amount of ashes produced during the fire and their chemical composition. Ashes produced by forest fires are a complex matrix composed of organic and inorganic compounds, which are of great environmental concern due to their high toxicity, environmental persistence and tendency to bioaccumulate. Actually, recent studies have confirmed that wildfires act as diffuse source of contamination for the aquatic ecosystems, leading to impairments in their structure and functions. Within the context of contamination driven by wildfire ashes, aquatic early life stages (embryos and tadpoles) of amphibians, due their ecology and vulnerability to environmental disturbances, may be highly susceptible to this type of contamination as they will be exposed to chemical substances released by the ashes the water column and the sediment. This work intended to assess the influence of wildfire severity in pine stands on the ecotoxicity of aqueous extracts of the generated ashes (AEA) on aquatic early life stages of two anuran species: *Pelophylax perezi* and *Xenopus laevis*. To attain this goal, embryos of *X. laevis* and tadpoles of the two species were exposed for 96 hours and 14 days, respectively, to serial concentrations (26.9 to 100 %) of AEA of a medium severity (MS) and a high severity (HS) wildfire. At the end of the exposure periods, the following parameters were evaluated: mortality, malformations, developmental stages, body length (tail-TL, snout-to-vent-SVL and total body-TotL) and body mass (only for tadpoles). Adding to these apical endpoints, effects at the sub-individual level were also monitored for oxidative stress (lipid peroxidation, catalase, total glutathione, glutathione S-transferase-also a xenobiotic transformation enzyme), neurotoxicity (acetylcholinesterase), and energetic metabolism (electron transport system, total lipids, carbohydrate and proteins).

Chemical analyses were performed to the two types of ashes and AEA. When comparing MS and HS AEA, the former showed a lower hardness. Furthermore, five (As, Co, Mn, Ni, V) of the ten analysed elements were present at higher concentrations in MS, while only Cd and Pb were at higher concentrations in HS. The assays performed with embryos and tadpoles of the two anuran species, revealed influence of AEA type, species and life stage in the observed ecotoxicological effects. As for lethal toxicity, a similar and significant reduction in survival of embryos of *X. laevis* and tadpoles of *P. perezi* was observed at 100 % of MS and HS. Though, tadpoles of *X. laevis* were more sensitive to MS (survival impaired at concentrations ≥ 26.9 %) than to HS (survival impaired at concentrations ≥ 76.9 %). Regarding sublethal effects, HS was more toxic to embryos of *X. laevis* than MS, as the former type of AEA significantly reduced TL, SVL and TotL at all tested concentrations, while MS only affected TL. On the contrary, tadpoles of *X. laevis* were more sensitive to MS; their body lengths and weight were significantly reduced at all tested concentration of MS, though only the two highest concentrations of HS affected these parameters. Tadpoles of *P. perezi* were more sensitive to HS, having, in general, their body lengths and weight significantly reduced at concentrations ≥ 26.9 % and 59.2 % of MS, respectively. Overall, biochemical markers were not significantly affected by exposure to the two types of AEA. Though it must be emphasized that catalase increase in embryos of *X. laevis* and tadpoles of *P. perezi* after exposure to AEA, indicating the occurrence of oxidative stress. This is the first study on the ecotoxicological effects of AEA to amphibians, and showed that the input of wildfire generated ashes, and adsorbed chemicals, may constitute a risk to aquatic life stages of amphibians.

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Index of Acronyms

AChE- Acetylcholinesterase

AEA- Aqueous extracts of ashes

AEA-HS- Aqueous extracts of ashes from high severity

AEA-MS- Aqueous extracts of ashes from moderate severity

AOS- Antioxidant defense system

BHT- Butylated hydroxytoluene

CAT- Catalase

CDNB- 1-chloro-2,4-dinitribenzene

CMC- Criterium maximum concentrations

CRM- Certified reference materials

DGAV- Portuguese Institution responsible for authorizing animal experimentation research

DTNB- 5-5'-dithiobis (2-nitrobenzoic acid)

DTPA- Diethylenetriaminepentaacetic acid

EQS- Environmental quality standards

ETS- Electron transport system

FETAX- Frog embryo teratogenesis

G- Gosner developmental stage

GR- Glutathione reductase

GSH- Reductase glutathione

GST- Glutathione S-transferase

HS- High severity

ICP-MS- Inductively coupled plasma mass spectrometry

INT- Iodonitrotetrazolium

IU- International units

LOEC- Lowest observed effect concentration

LPO- Lipid peroxidation

MAS- Molecular absorption spectrometry

MS- Moderate severity

NADPH- β-Nicotinamide adenine dinucleotide 2'-phosphate

NF- Nieuwkoop and Faber

NOEC- Non- observed effect concentration

PAHs- Polycyclic aromatic hydrocarbns

PMS- Post-mitochondrial supernatant

ROS- Reactive oxygen species

SVL-Snout-to-vent length

TBA-2-triochlotoacetic acid

TCA- Trichloroacetic acid

TG- Total glutathione

TL- Tail length

TN- Total nitrogen

TotL- Total length

UV-B- Ultraviolet- B

Chapter I

Literature Review

1. Introduction

1.1 Climate Changes

Over the past few decades, natural and anthropogenic process have contributed with greater strength, weight, and speed to current climate changes (ENGASP- Engenharia e Técnicas Afins Lda, 2014). However, in the balance of these events, since the 20th century, Human action has been contributing at a greater weight in global warming of the Planet's surface (about 0.85 °C) (IPCC, 2019a). The issue of global warming began to gain more emphasis from the period of the Industrial Revolution (IPCC, 2019b; NASA's Jet Propulsion Laboratory, 2020), mainly due to the increase in human activity, such as the burning of fossil fuels, therefore leading to increased levels of greenhouse gases in the atmosphere (NASA's Jet Propulsion Laboratory, 2020; Secretary-General, 2019). The main causes of rapid temperature rise and consequent climate change are human activities, such as industrialization, agricultural development, deforestation, and, as before, the burning of fossil fuels, such as oil, gas and coal (ACCIONA, 2019; IPCC, 2019b).

In the long run, the gradual increase in temperature leads to more changes in the climate of Planet Earth (NASA's Jet Propulsion Laboratory, 2020), making events of a more extreme nature felt with greater intensity and occurrence in terms of severity and frequency, such as storms, hurricanes, floods and forest fires.

According to the report of the Intergovernmental Panel on Climate Change (2019a), with the gradual increase in the temperature of the Planet Earth's surface, it is likely that between 2030 and 2052 global warming will reach 1.5 °C, which, in addition to favouring occurrence of extreme climatic phenomena, there is also the risk of loss of certain species, though, for example, the spread of invasive species, pests, diseases. Thus, a reduction of species in percentages of less than 10 % is pointed out: six for insects, eight for plants and only four for vertebrates (IPCC, 2019b).

1.2 Environmental Impacts of Wildfires, case of Portugal

Since times before human occupation, fire has always been an integral part of the Mediterranean Region, and in this way, it was shaping the surrounding ecosystem and landscapes, thus contributing in an important way to the ecology of the region. However, the discovery of fire by Man led to new causes of ignition of fires, potentiating a greater periodicity of fire occurrences (Pausas & Fernández-Muñoz, 2011; Fernandes, 2009), transforming this natural phenomenon into a worrying social and environmental problem.

Over time, with special focus in recent years and in conjunction with projections of climate change verified today, the problem of forest fires has been attracting special attention. Not only, due to its increase in the number of occurrences, but also in the severity and intensity that it presents (Marlon *et al.,* 2008; Pausas & Keeley, 2009; Fernandes, 2009). Thus, the Mediterranean Basin has been the focus of this same situation, registering great changes in the temperature level (IPCC, 2019a). As in other countries in the Mediterranean region, namely in Portugal, during the first decade of the 21st century, the number of forest fires has been increasing, and consumed an average of 140,000 ha per year (ICNF, 2011; Pausas & Fernández-Muñoz, 2011).

Among the main causes that contribute to the increase in forest fires are natural and human hand. Among the natural causes, the climate changes associated with the Mediterranean climate are noteworthy. Characterized by mild, rainy winters, and hot, dry summers, it provides rapid growth of biomass, thus favouring a high fuel load for the propensity of easy occurrence and rapid spread of forest fires in the driest season (Campos *et al.,* 2012; Leite, 2011; Nogueira, 2013; Fernandes, 2009). Anthropogenic causes, on the other hand, are mainly related to the planting of highly flammable and resinous plant species, such as eucalyptus (*Eucalyptus globulus*) or pine (*Pinus pinaster*), or rural abandonment, fuelled by changes in the population's economy (Moreira *et al.,* 2009). This whole set of factors results in huge losses both at the environmental and socio-economic levels.

In Portugal between the period of January 1 and October 15, 2020, it was registered a 52% reduction in the average burnt area in the last 10 years, with a reduction in the number of wildfires that occurred in the same period of time. With the exception of the

catastrophic year of 2017 in which the ICNF reports as “the most severe of the last 15 years, with values similar to that of 2005, so far the most severe”, with about 65.887 ha burned, unfortunately exceeding a hundred lost lives (ICNF, 2020; República Portuguesa, 2020; Silva, 2017 *in* Público).

Taking into account the high number of forest fires in our country, and the severe economic and ecological implications and consequences associated with them, it is essential to apply fire risk assessment methodologies. Climate studies, from the European Union's Joint Research Center, reveal an alarming trend towards Europe, more precisely for southern countries, especially the Iberian Peninsula whose temperature levels (especially in summer) will be higher and precipitation levels lower than average (Beighley & Hyde, 2018).

Events such as wildfires are one of the phenomena with the greatest environmental impact, mainly due to the further accentuation of hydrological and erosive processes, since they increase the mineralization of organic matter in the soil, making it easily exportable by erosive and hydrological processes, thus increasing forms the repellence of the soil to water (Francos *et al.,* 2018; Guthrie, 1979; Leite, 2011; Malvar *et al.,* 2016). The extent of these effects depends particularly on the intensity and severity of the fire.

Among the main direct effects caused by forest fires, the consumption of organic matter, heat production, release of nutrients and sub-combustion products stand out (Gandara & Kageyama, 1998). As a consequence, the aquatic and soil systems are affected in several ways, changing its physical, chemical and biological properties (Andreu *et al.,* 2001; Leite, 2011), such as structure, porosity and infiltration as for the soil ecosystem (Certini, 2005; González-Pérez *et al.,* 2004; Fernandes, 2009).

1.3 Effect of Forest Fires on Soils and Ash Formation

Forest fires are a very important factor in the dynamics of many terrestrial ecosystems, exerting a predominant modifying effect in the short, medium and long term on the environment (Abrantes *et al.,* 2017; Alauzis *et al.,* 2004; Leite, 2011).

Depending on its intensity and severity (a term that refers to the description of how fire intensity affects the surrounding ecosystems), wildfires can cause irreversible changes in the chemical, physical and biological properties of soils (Andreu *et al.,* 2001; Keeley, 2009; Ryan & Noste, 1985). Organic matter begins to change when the soil is heated to 200 °C and consumed when it reaches 450 °C. In the case of certain chemical elements present in the biomass, they start to volatilize at temperatures from 100 °C to 180 °C, following changes to pH values (Fernandes, 2009; Pereira & Úbeda, 2010).

The formation and deposition of an ash layer on the topsoil is a direct effect of the occurrence of forest fires. Its complex physical and chemical composition depends on factors such as the temperature of the combustion of the fire, the type of soil, the type of surrounding vegetation, the part of the burnt plant species (root, stem or leaves) and the complete / partial combustion of the vegetation (Abrantes et al., 2017; Balfour & Woods, 2013; Bodí *et al.,* 2014; Goforth *et al.,* 2005).

In wildfires considered to be of low-moderate severity (below 450 ° C), the incomplete combustion of vegetation provides the formation of ashes with a darker colour (with lower density) and its composition is essentially composed of organic compounds (Bodí *et al.,* 2014; Forbes *et al.,* 2006; Goforth *et al.,* 2005). In high severity wildfires (above 450 ° C), due to the complete combustion, oxidation is more powerful which results in ash with a lighter shade (grey and white), with high density and essentially composed of inorganic compounds (Balfour & Woods, 2013; Goforth *et al.,* 2005; Úbeda *et al.,* 2009). Thus, the severity of wildfires plays an important role in terms of the formation of the main elements of the ashes composition.

The ashes resulting from wildfires are a complex matrix. They are composed of oxides, hydroxides, carbonates and organic and inorganic compounds. Among the main inorganic components that compose the ashes are the magnesium (Mg), silicon (Si), potassium (K) and calcium (Ca). At lower concentrations is sodium (Na), phosphorous (P) and sulphur (S), as well as certain major and trace elements as aluminium (Al), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni) cooper (Cu), zinc (Zn), cadmium (Cd), mercury (Hg) and lead (Pb) (Bodí *et al.,* 2014; Burton *et al.,* 2016; Campos *et al.,* 2015; Cerrato *et al.,* 2016; Pereira & Úbeda 2010; Plumlee *et al.,* 2007). As for the

main organic components, polycyclic aromatic hydrocarbons (PAHs) stand out (Kim *et al.,* 2003; Silva *et al.,* 2015; Simon *et al.,* 2016; Vergnoux *et al.,* 2011).

Studies carried out in burnt stands of Eucalyptus and Pine Maritime in the northern region of Portugal, revealed the presence of several metals in the ashes collected immediately after fire, namely: V, with a concentration of 47 μg g^{-1} and 45 μg g^{-1}, respectively; Mn, with 590 μg g^{-1} and 270 μg g^{-1}, respectively; Co, with 2.4 μg g^{-1} and 5.0 μg g^{-1}, respectively; Ni, with 23 μg g^{-1} and 22 μg g^{-1}, respectively; Cu, with 55 μg g^{-1} and 35 μg g^{-1}, respectively; Cd, with 0.50 μg g^{-1} and 0.30 μg g^{-1}, respectively and Pb, with concentrations of 60 μg g^{-1} and 110 μg g^{-1}, respectively (Campos *et al.,* 2015).

1.4 Wildfires as an Aquatic Diffuse Source of Pollution

Due to wildfires, the removal of vegetation, litter and organic matter increases the potential for soil erosion, failing to provide protection to the soil surface(Campo *et al.,* 2006; Cerdà & Lasanta, 2005; Chafer, 2008; Leite, 2011; Moench & Fusaro, 2012). This significant reduction in the content of organic matter, increases water repellence, inducing a reduction in infiltration rates (Bodí *et al.,* 2012; Keizer *et al.,* 2005; Shakesby & Doerr, 2006), with increased overland flow and surface transport (Balfour & Woods, 2013; Bodí *et al.,* 2014; Francos *et al.,* 2017; Guthrie, 1979; Leite, 2011).

Through surface runoff after post-fire rain events, ashes and top soil from the burnt areas can reach downstream aquatic systems. Thus, linked to ashes and soil particles, occurs the input of metals and PAHs, among others, to watercourses causing a loss in water quality. In fact, wildfires have been identified as a diffuse source of contamination to water bodies (Campos *et al.,* 2012a; Costa *et al.,* 2014; Mansilha *et al.,* 2014; Nunes *et al.,* 2017; Smith *et al.,* 2011; Vila-Escalé *et al.,* 2007).

Besides the input of toxic compounds, such as metals and PAHs, wildfires can also cause changes in pH, temperature and conductivity levels, with a consequent decrease in the oxygen available (Smith *et al.,* 2011; Tsai *et al.,* 2017).

As above mentioned, fires are responsible for the mobilization of metallic compounds, which in certain concentrations can become toxic to living beings. Certain

components of ash are considered harmful contaminants and thus considered priority pollutants according to the United States Environmental Protection Agency (US EPA) and the European Community (EC) (EC, 2008- DIRECTIVE 2008/105/EC; USEPA, 2017). Among the several metals that enter the aquatic systems due to wildfires, elements such as Cu, Fe and Zn are considered essential elements for the survival of organisms, participating in the reactions of the electron transport chain and providing stability in the protein structure. Notwithstanding their importance, at certain concentrations they can become toxic to the organisms. In the case of Cd, Hg and Pb they are able to displace and replace essential metals by interfering with enzyme activity, and therefore they can cause toxic and harmful effects to aquatic organisms (Gifford *et al.,* 2004). Moreover, elements such as As, Cd, Cr, Cu, Pb, Hg, Ni and Zn can bioaccumulate through food chain impairing all ecosystem (Noll, 2003).

In fact, several recent studies have pointed out the toxicity of runoff from burnt areas and ashes extracts to aquatic organisms, including effects on the growth rate of producers, as the microalgae *Raphidocelis subcapitata* and the macrophyte *Lemna minor*; effects on the bioluminescent bacteria *Vibrio fischeri* (Campos *et al.,* 2012a; Ré *et al.* 2020a; Silva *et al.,* 2015); feeding inhibition and biochemical effects on the fish *Gambusia holbrooki* (Nunes *et al.,* 2017; Ré *et al.,* 2020b)*;* effects on the feeding inhibition of the crustacean *Daphnia magna* and the shrimp *Atyaephyra desmarestii* (Ré *et al.,* 2020b); and effects in microbial decomposer communities (fungi and bacteria), as well as in the invertebrate shredder *Allogamus ligonifer* (Pradhan *et al.,* 2020). Pilliod *et al.,* (2003) also reported a negative impact of wildfires on amphibians.

1.5 Relevance of Wildfire Ash Contamination to Amphibians

Changes in water quality can thus affect the structure of the communities of organisms that coexist in it (Dangles *et al.,* 2004; Niyogi & Townsend, 2003; Nogueira, 2013). The trend towards bioaccumulation and bioamplification, through the food chain, of metallic contaminants with high environmental toxicity (Noll, 2003), such as As, Cd, Cu,

Pb, Hg, Ni and Zn, has been playing an increasingly important role of great environmental concern for certain aquatic species.

Thus, in freshwater biota it is considered relevant to assess the effects of ash in aquatic life stages. Among the species most threatened, the amphibian group stands out, due to its widespread decline in recent times (Stuart *et al.,* 2004). This group deserves a special attention, since the majority of species require both terrestrial and aquatic environments to complete their complex life cycle (reproduction, embryonic and larval development, metamorphosis) (Katzenberger *et al.,* 2012). In this way, amphibians are considered organisms sensitive to environmental disturbances, due to the fact that many species have geographical restrictions occurring only in a certain microhabitat (Pilliod *et al.,* 2003) due to their ectotherm, permeable and bare skin, irrigated and devoid of protective structures, such as hair or scales. Thus, processes such as surface runoff from ash and other compounds resulting from forest fires may affect the development of these organisms, due to the easy gas exchange of contaminants between the environment and the dermis, thus leading to the occurrence of malformations or even unviability of the eggs, right in the early stages of their development (Toledo, 2016).

The use of amphibians as model species to assess the effects of pollutants dates back to 1970's (Power *et al.,* 1989). Essentially due to the continuous intense global decline in amphibian populations, an increasing concern arose regarding the protection and conservation of this group of organisms (Alford, 2010; Stuart *et al.,* 2004). Being chemical contamination one of the major causes of such decline, it is important to understand the effects chemical stressors may exert in the different life stages of amphibians (Gendron, 2013). Of the laboratory amphibian models most used in ecotoxicological tests, the species *Xenopus laevis*, also known as African clawed frog, is the most commonly used. Of the main reasons that stand out for their use, is due to the fact that they are totally aquatic, with easy handling and maintenance in the laboratory; reproduces easily through the induction by hormones; without major associated maintenance costs (Xenbase, 2020).

1.6 Aim of Study

The present work intended to evaluate the effects caused by aqueous extracts of ashes (AEA) from wildfires of different severities on the embryonic and larval development of two species of anurans, *Xenopus laevis* and *Pelophylax perezi*. For this purpose, embryonic teratogenicity and tadpole toxicity assays were carried out, lasting 96 hours and 14 days, respectively.

The effects of the AEA were evaluated for several endpoints, including the survival, the success rate of hatching, the occurrence of morphological changes in the embryos and larvae, the stage of development of the eggs and tadpoles. Effects at the sub-individual level were also monitored using distinct biochemical parameters.

To address the goals of this work, the present thesis is structured into four chapters, as follows:

- Chapter I- A contextualization of the research topic is provided in this chapter. Namely, it provides an overview on the changes in climatic conditions associated with the problematic of wildfires, with a consequent increase in its intensity and severity. It also pointed the associated contamination of adjacent aquatic systems due to surface runoff process of the ashes formed during wildfires and its possible impacts on biota, namely aquatic life stages of amphibians.
- Chapter II- This chapter focuses the lethal and sublethal effects that aqueous extracts of ashes, originated from moderate and high severity wildfire, may cause in embryos and tadpoles of the standard amphibian species *Xenopus laevis*.
- Chapter III- In this chapter are studied the lethal and sublethal effects that aqueous extracts of ashes, originated from moderate and high severity, induce in an amphibian species autochthonous, *Pelophylax perezi*, of the Iberian Peninsula, a region highly affected by wildfires.
- Chapter IV- This chapter provides a general and integrated discussion on the obtained results. Also, it identifies additional research questions to be tackled in future works.

Chapter II

Influence of fire severity on the ecotoxicity of the released ashes to early life stages of *Xenopus laevis*

Chapter II- Influence of fire severity on the ecotoxicity of the released ashes to early life stages of *Xenopus laevis*

2.1 Introduction

On a global scale, there is evidence that climate is changing. The associated increase in hot and dry conditions during the summer and westward movement in winter climate makes the Mediterranean one of the most vulnerable regions to climate changes (Giorgi & Lionello, 2008; Lionello *et al.,* 2006). Increasing temperatures (Hansen *et al.,* 2006) and drier conditions may increase the frequency in the occurrence of extreme events in the Mediterranean region, such as forest fires (Vélez, 2002). Among the several environmental impacts of wildfires, they can affect the biota of aquatic ecosystems. In fact, through the ash overland flow, promoted by post-fire rain events, several noxious compounds associated to ashes (e.g. metals and polycyclic aromatic hydrocarbons) can reach the surface water and sediment and altering its ecological quality, releasing toxic or noxious compounds for the aquatic systems (Campos *et al.,* 2012; Nunes *et al.,* 2017; Pradhan *et al.*, 2020; Ré *et al.,* 2020b; Silva *et al.,* 2015). Among the several taxonomic groups, amphibians can be particularly affected by post-fire contamination (Kerby *et al.,* 2010; Pilliod *et al.,* 2003; Todd *et al.,* 2011). In fact, their dependency on the aquatic environment in the early stages of development, the restrictive habitat of certain species (Moreira *et al.,* 2010), their inability to regulate temperature (ectotherm animals), their thin permeable skin and their complex life cycle (Katzenberger *et al.,* 2012), make them particularly susceptible to the exposure of these chemical compounds. Besides that, their extremely irrigated dermis, allowing an easy gas exchange of chemical compounds with the environment, reinforces and underlines the need to include amphibian species in ecotoxicological assessment (Antunes *et al.,* 2010; Matozzo *et al.,* 2013). Several studies revealed that the amphibian epidermis in advanced larval stages, can accumulates greater amounts of cadmium, copper, lead and zinc (Prokić *et al.,* 2016). Thus, disturbances in aquatic systems can alter the life cycle of amphibians, causing adverse effects, among others, in the process of metamorphosis (Pilliod *et al.,* 2003). On the other hand, it is important to consider that the gelatinous layer that surrounds the embryo, in its early

stages of the life cycle, can function as a protective barrier against contaminants (Edginton *et al.,* 2007; Marques *et al.,* 2008). Hence and considering that wildfires are a major environmental disturbance in Mediterranean Countries of Southern Europe, it is of crucial importance to understand the effects of post-fire contamination in this vulnerable group. In this sense, in this chapter, the present work intends to evaluate the effects of aqueous extracts of ash, originating from wildfires of different severities, in the first stages of the life cycle development of the species *Xenopus laevis*.

2.2 Material and Methods

2.2.1 Collection of ashes and preparation of aqueous extracts of ash (AEA)

Ashes from a burnt forest- located in north-central Portugal (Nespereira de Cima, Oliveira de Azeméis, Aveiro) (Figure 1), were collected in March 2019, immediately after the wildfire occurrence. The wildfire burnt an area of approximately 320.39 ha that was mainly covered by stands of maritime pines (*Pinus pinaster*). The severity was assessed according to the methodology described in Shakesby & Doerr (2006) and Keeley (2009). Two hillslopes, one burnt at moderate severity (MS) and the other burnt at high severity (HS), were selected in the burnt area for the ash sampling. On each slope, one transect was laid out across the full length of the slope section and five equidistant points were established from the top to the bottom, to take into account ash spatial heterogeneity. At each of the five sampling points, a grid was laid out and a plot of 50 x 60 cm was sampled for ash. At each sampling plot the entire ash layer was collected with a brush and a spoon, in order to avoid mixture with soil, and after that, the ash samples were sieved separately through a 2 mm mesh and transported to the laboratory in plastic bags, under dark conditions. In the laboratory, the sieved ash samples were air-dried and then mixed in a container to produce a single composite sample, which was stored at – 20 °C in dark plastic bags (to reduce microbial activity) until the preparation of the aqueous extracts of ashes (AEA) (Campos *et al.,* 2016; Silva *et al.,* 2015).

Figure 1: *Geographical localization of the ash collection study area: Nespereira de Cima village, Oliveira de Azeméis municipally, Aveiro district (scale 200 m). Retrieved form GoogleMaps (2020a, 2020b) in 28 December 2020.*

In the laboratory, the AEA from the moderate and high severities were prepared for posterior chemical analysis and to perform the ecotoxicological assays.

2.2.2 Chemical analytical procedures of AEA samples

The AEA samples were evaluated for total nitrogen (TN), ammonia (NH_3- N), nitrite (NO_2-N), nitrate (NO_3- -N), phosphate (PO_4^{3-}), total hardness and calcium hardness and metal concentrations. To quantify the levels of TN the samples were subjected to oxidative digestion followed by quantification by molecular absorption spectrometry (MAS) (APHA, method 4500-N C, 2005). As for the levels of nitrite and nitrate, the samples were analysed

using the chromotropic acid method (West & Lyles, 1960) and the N- (1-Naphthyl) -ethylendiamine method (ISO 15923-1, 2013), respectively. Ammonia and phosphate levels were determined by the phenate method (APHA, method 4500-NH_3, 2017) and by the ascorbic acid method (ISO 15923-1, 2013). The total hardness and the calcium hardness were quantified by the metallapataleine and murexide method, respectively (Aqualytic Method Manual) (USEPA, 1983). The concentration of TSS (total suspended solids) in AEA was quantified gravimetrically through the filtration of 50-150 mL of water through a 1.2 µm glass fibre filter, followed by drying at a constant weight at 105 °C (APHA, method 2540 D, 2017).

The levels of metals in the AEA samples were following nitric-perchloric acid (APHA, method 3030 H, 2017). Briefly, 50 ml of each water sample was digested with 3 ml of HNO_3 (65 %) in teflon beakers at 90 °C and evaporated to 15-20 ml. After cooling, 10 ml of HNO_3 (65 %) and 10 ml of $HClO_4$ (70 %) were added to the teflon cup and heated on a hot plate at 90 °C until dense white vapours of $HClO_4$ appear. After the teflon beakers cooled to room temperature, their walls were washed with Milli-Q® water and the solutions were filtered using a 0.45 µm Whatman® NucleporeTM membrane. The filtrates were transferred to volumetric polypropylene tubes and subsequently diluted to 100 ml with Millipore water. To quantify the levels of metals in ash, approximately 500 mg of dry ash (40 °C) was digested with HNO_3 (65 %) (95°C for 2 h 45 min) in a DigiPrep HotBlock, redissolved with H_2O_2 (30 %) and Milli-Q water, and heated for 2 h at 95 °C. After cooling down, the solution was filtered through 0.45 µm Whatman® NucleporeTM and was diluted to 50 ml with Milli-Q water (USEPA, method 3050B, 1996). In ash and AEA, concentrations of metals such as vanadium (V), chromium (Cr), manganese (Mn), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd) and lead (Pb) were determined by inductively coupled plasma mass spectrometry (ICP-MS). Through the analysis of certified reference materials (CRM) and by testing every 10 samples in duplicate, the quality control of the analytical procedures was guaranteed. Blanks were prepared following the same analytical procedure and performed in parallel with the CRM and the samples.

2.2.3 Test Organisms

The amphibian species X*enopus laevis* (Daudin 1802) was selected as the model species to assess the effects of ashes in early life stages of amphibians. This species is widely used as a model species in ecotoxicological studies to assess the impacts that chemicals may pose to amphibians. Within the context of the present work, the selection of this species gains further relevance since it exhibits a life cycle totally aquatic, thus, being expected that all developmental stages may, at some point, be exposed to ashes released by wildfires.

The embryos and tadpoles used to perform the ecotoxicological assays were obtained from in-house sexually mature adults, kept at a facility of the Department of Biology, University of Aveiro (Aveiro, Portugal). These organisms were maintained in glass aquaria containing dechlorinated tap water, under controlled conditions of temperature (23 ± 1 °C) and photoperiod (14:10 h light: dark), being fed every other day with a mixed diet of larvae of mealworms (*Tenebrio molitor*) and of pellets XE 40 (Mucedola srl). The spawning of adults of *X. laevis* was induced by one single injection of one female (with 400 International Units-IU) and one male (with 100 IU) with human chorionic gonadotropin (hCG 5000 IU, UK), (Sigma, Aldrich), into the dorsal lymph sac. The released eggs were retrieved from the breeding tank, being transferred to a recipient containing FETAX medium. Afterwards the viability of embryos was checked at a stereo microscope (Zeiss Stemi 508). A part of the viable embryos was immediately used to perform the embryonic teratogenesis assay. The other part was kept in an aquarium, with continuous aeration and at the same temperature and photoperiod as for the adult colonies, until reaching the Nieuwkoop and Faber (NF) developmental stage NF 45-46 (which corresponds to the stage where tadpoles open the mouth and start independent feeding; (Niewkoop & Faber, 1994). Once the tadpoles opened their mouth, were maintained for a few more days in the aquaria, being fed with fish food (TetraMin™ from Tetra™), until being used in the toxicity assays. During this whole period, the FETAX medium of the aquaria was changed every other day and dead animals were removed on a daily basis to avoid the degradation of the quality of the medium.

2.2.4 Embryo teratogenicity assay

Embryos of *X. laevis*, at developmental life stage NF 7-8, were exposed to a set of concentrations of the AEA (10g of ashes per litre, corresponding to 100%) according to the Frog Embryo Teratogenesis Assay with minor changes (ASTM, 2012). The embryos were exposed to a control (consisting of FETAX medium) and to six concentrations of the two eluates: 26.9 %, 35 %, 45.5 %, 59.2 %, 76.9% and 100 % (obtained by diluting the ash eluate with FETAX medium). Four replicates were performed for the control and for each dilution. Each replicate, consisted of a Petri dish (with a diameter of 6 cm) filled with 10 mL of the test solution and 10 embryos of *X. laevis* (totalising 40 embryos per treatment). Exposure occurred for a period of 96 h at a constant temperature (23 ± 1 °C) and photoperiod (14:10 h light: dark) conditions. After 48 h of exposure, the test solutions were renewed. Mortality was checked every 24 h and dead animals removed from the test recipient to avoid the proliferation of microorganisms, which could compromise the viability of the remaining alive organisms. Along with mortality, hatching was also checked every 24 h. At the end of the exposure period, the following endpoints were monitored: mortality, malformations, and developmental stage (according to the table of (Niewkoop & Faber, 1994), and body length (tail, snout-to-vent and total body). After making these evaluations, larvae were immediately deep-frozen in liquid nitrogen and stored at -80 °C for ulterior analysis of biochemical responses. These responses were related with oxidative stress [lipid peroxidation (LPO), antioxidant responses (catalase (CAT), total glutathione (TG), glutathione S-transferase (GST), the latter is also a xenobiotic transformation enzyme], neurotoxicity [acetylcholinesterase (AChE)], and energetic metabolism [electron transport system (ETS), total lipids, carbohydrate and proteins] (please see below, in section 2.2.6, the methodologies used for the determination of these biochemical parameters).

At the end of assay, the following physical-chemical parameters were measured, by using a WTW 3410 meter (Multi 3410 SET C): temperature (°C), pH, dissolved oxygen (mg L^{-1}) and conductivity (µS cm^{-1}).

2.2.5 Tadpoles toxicity assay

The toxicity assay carried out with tadpoles followed the methodologies described in (ASTM, 2002) with some modifications, namely the duration the assay that was 14 days instead of 96 h. Tadpoles of *X. laevis* at developmental stages NF 46 were exposed to the same treatments as the embryos, i.e. a control of FETAX and six concentrations (26.9 %, 35 %, 45.5 %, 59.2 %, 76.9 % and 100 %) of the two types of AEA. For each treatment, four replicates were carried out, consisting of a high-density plastic recipient filled with 200 mL of the test solution and four tadpoles. Exposure occurred for a period of 14 days in a climatic chamber, under constant aeration, and controlled conditions of temperature (23 ± 1 °C) and photoperiod (14:10 h light: dark). Each 48 h the test medium as renewed, and organisms were fed with 0.06 g of TetraminTM (Tetrawerke, Melle, Germany) per replica. The mortality of tadpoles was checked on a daily basis, whenever an organism died it was removed from the test vessel to avoid the proliferation of microorganisms that could compromise the viability of the remaining alive animals. At the end of the assay, adding to mortality, developmental stage and malformation, the following biometric parameters were measured: weight and snout-to-vent, tail and total body length. After completing these measurements, tadpoles were deep-frozen in liquid nitrogen and stored at -80 °C, for ulterior analysis of biochemical responses. The biochemical parameters measured for the embryos (please see sub-section 2.2.3), were also here assessed for the tadpoles, and the methodologies used for their quantification are described in section 2.2.6).

During the assay, the following physical-chemical parameters were measured: temperature (°C), pH, dissolved oxygen (mg/L) and conductivity (µS/cm) values, measured ate the fresh and old medium by using a WTW 3410 meter (Multi 3410 SET C).

2.2.6 Methodologies for assessing biochemical endpoints

To *Xenopus laevis* embryos 1700 µL of ultrapure water was added and homogenization was performed by sonication (Brason Sonifer 250) for 10 seconds, maintaining samples always on ice. To *Xenopus laevis* tadpoles a volume proportional of ultrapure water to weight of the samples of organisms was calculated and posteriorly

homogenization was performed by sonication for 10 seconds, maintaining samples always on ice. From each sample, aliquots of 300 µL were taken for analysis of lipids, sugars and protein contents, and electron transport system (ETS) activity. One aliquot of 200 µL was used for determination of LPO. The remaining homogenate from each sample (~ 500 µL) was diluted with 500 µL of 0.2 M K-phosphate buffer (at pH 7.4), centrifuged for 20 min at 10,000 g (4 °C) to obtain the post-mitochondrial supernatant (PMS) and kept in -80 °C until further analyses of the other biochemical markers: AChE, CAT, GST activities, TG and protein content. All biomarkers determinations were performed out in 96-wells microplates at 25 °C and determined in a microplate reader spectrophotometer (Multiskan Spectrum, Thermo Fisher Scientific, Waltham, USA) by following the methodologies briefly described below.

Lipid peroxidation was determined on 200 µL of homogenate treated with 4 µL of 4% butylated hydroxytoluene (BHT) diluted in methanol, 100 µL of cold trichloroacetic acid 100% solution (TCA), 1000 µL of 0.73 % 2-thiobarbituric acid (TBA) in 60 mM Tris–HCl, with 0.1 mM diethylenetriaminepentaacetic acid (DTPA) at room temperature (25 ± 1°C), followed the protocol by Bird & Draper, (1984). The samples were vortexed and after 1 hour incubation period at 100 °C, centrifuged for 10 min at 6000 g. At 25 °C and in the dark conditions, the supernatant fraction (volume equally at 300 µL, make 3 replicates of 100 µL) was pipetted to a microplate and absorbance was read at 535 nm in microplate reader. LPO was expressed as nmol per mg. mL^{-1}.

In 10 µL of PMS embryos or tadpoles samples, Catalase activity was measured by the decomposition of the substrate Hydrogen peroxide (H_2O_2), (Claiborne, 1985), using reaction buffer contained 140 µL of 0.05 M K-phosphate (pH 7.0) and 150 µL of 30 % H_2O_2. Using microplates for UV light, the absorbance was read immediately at 240 nm, each 20 seconds, during 2 minutes. CAT activity was expressed as µmol per min per mg of protein.

Total glutathione content was determined in the PMS fraction according to Tietze, (1969) and Baker et al., (1990). In a microplate, 50 µL of PMS of embryos or tadpoles samples and 250 µL of buffer reaction containing 0.2 M Na-K phosphate buffer solution (pH 8.0), β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt solution (NADPH), 5-5'-dithiobis (2-nitrobenzoic acid) solution (DTNB) and Sodium

hydrogencarbonate ($NaHCO_3$), and Glutathione reductase (GR) solution were pipetted at room temperature (25 ± 1 °C) and protected from light. A solution of reduced glutathione (GSH) was attained by dissolving 0.0062 g of GSH (10 mM) in 2 mL of 0.2 M Na-K phosphate buffer solution (pH 8.0). For the standard curve GSH concentrations (100 mM; 1000 mM; 10 000 mM and 100 000 mM) ultrapure water was added. The absorbance was read at 412 nm during 3 min. TG levels was expressed as µM per min per mg of protein.

Glutathione S-Transferase activity was measured according to Habig *et al.,* (1974) using 50 µL of PMS embryos or tadpoles samples and 250 µL of the reaction solution that contains 0.2 M K-phosphate buffer (pH 6.5), GSH and 1-chloro-2,4-dinitrobenzene (CDNB) mixed. The absorbance was read at 340 nm, every 20 seconds, for 5 minutes. GST activity were expressed in nmol per min per mg of protein.

Acetylcholinesterase activity was measured on 50 µL of PMS embryos or tadpoles samples with 250 µL of reaction buffer following the colorimetric method of Ellman *et al.,* (1961), adapted by Guilhermino *et al.,* (1996). The reaction buffer contained a mixture of 0.1 M K- phosphate buffer solution (pH 7.2), 0.0075 M Acetylthiocholine iodide solution, and DTNB solution mixture, which contained 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) and Sodium hydrogencarbonate ($NaHCO_3$), was prepared at room temperature (25 ± 1 °C) and protected from light until needed. The absorbance was read at 414 nm, each 20 seconds, during 5 minutes. AChE activity was expressed in nmol per min per mg of protein.

The protein quantification in the PMS embryos and tadpoles samples were determined following Bradford method (Bradford, 1976), adapted from BioRad's Bradford microassay setup in a 96-multiwell plate, using bovine γ-globuline as a standard. For the reaction, 10 µL of PMS samples and 250 µL of the BioRad solution were pipetted and after 15 minutes in the dark and, agitated at 150 rev/min, the absorbance was read at 600 nm.

The quantification of energy available (carbohydrates, lipids and proteins) and ETS followed the method described by De Coen & Janssen (1997) with slight modifications described (Rodrigues *et al.,* 2015).

To determinate the ETS activity, 300 µL homogenate of embryos or tadpoles samples were mixed with 150 µL of homogenization buffer (0.3 M Tris base; 0.45 % (w/v) Poly Vinyl Pyrrolidone; 459 µM $MgSO_4$; 0.6 % (v/v) Triton X-100 at a pH of 8.5) and centrifuged at 1000 g for 10 minutes at 4 °C. Fifty µL of supernatant, 150 µL of the buffered substrate solution (containing Tris-(hidroximetil)-aminometane, Triton X-100, β—NADH; β –NADPH) and 100 µL of Iodonitrotetrazolium chloride solution (INT) were then pipetted to a microplate. The absorbance was measured kinetically over a 3 minutes period at 490 nm. ETS activity was expressed at J per h per mg of protein.

The total lipid content of each embryos and tadpoles was determined using 300 µL of homogenate sample adding 500 µL of chloroform (119.38 M), 500 µL of methanol (CH_3OH) (32.04 M) and 250 µL Mili-Q water. After centrifugation at 1000 g for 5 minutes, the organic phase (100 µL) of each sample was transferred to a clean glass tube and 500 µL of Sulphuric acid (H_2SO_4) were added and then incubated at 200 °C for 15 minutes. For the lipid standard curve, several tripalmitine calibration solutions were included and treated as samples. After cooling down to room temperature (25 ± 1 °C), 1500 µL of deionised water were added to each sample. The absorbance was read at 375 nm. Total lipid content was expressed at J lip per mg of protein.

The quantification of carbohydrate contents was performed by adding 100 µL of 15 % TCA to 300 µL of each homogenized sample, followed by incubation during 10 minutes at -20 °C. After centrifugation of samples at 1000 g during 10 minutes at 4 °C, 200 µL of 5 % phenol and 800 µL of H_2SO4 were added to the supernatant and vortexed. The samples and glucose solutions (that were used for the standard curve) were incubated at room temperature (25 ± 1 °C) during 30 minutes. Posteriorly, absorbance was measured at 492 nm in microplate Carbohydrates contents was expressed by J sugar per mg of protein.

The pellet from previous carbohydrates centrifugation was resuspended in 500 µL of NaOH, incubated during 30 minutes at 60 °C and added to 280 µL of HCl. Bradford's method (Bradford, 1976) was used for total protein content quantification in homogenate samples using bovine serum albumin as a standard. Absorbance was measured after 30 min incubation at room temperature (25 ± 1 °C) in the microplate at 592 nm.

This work was carried out according to the 3 R´s policy for animal experimentation and with the approval for research ethics from DGAV (Portuguese Institution responsible for authorizing animal experimentation research).

2.2.7 Statistical analyses

To identify differences in the monitored parameters, between the concentrations of each AEA and the respective controls, one-way analysis of variance was performed followed by the Dunnett's post-hoc test. This analysis allowed to determine the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) Normality of data was checked through the Shapiro-Wilk's test and the homoscedasticity of variances through the Bartlett's test. Significant differences were set at a p value 0.05. These analyses were carried out in the in Graphpad Prism 8 Software.

The dilution causing 20 % of mortality in the tadpoles was estimated by means of the Probit analysis (Lewis & Finney, 1972).

2.3 Results

2.3.1 Physical and chemical characterization of AEA

Table I present the levels of the chemical elements that were analysed in the ashes and in AEA originated from the MS and HS sampling sites.

In general, the concentration of metals was much higher in the ashes than in the corresponding extracts (AEA) samples, differing by more than one order of magnitude. In terms of concentrations, metals such as Mn and Zn stand out from the rest by the high concentration recorded. The elements of Co, Ni, Mn and V registered similar values for both ashes (MS and HS). The level of metals in AEA followed a similar pattern of the corresponding ashes with the following order of concentrations: Mn > Zn > Cu > V > Ni > Pb > As > Cr > Co > Cd for the AEA-MS and Mn > Zn > Pb > Cu > Cr > V > Ni > As > Cd > Co for the AEA-HS. Concerning the nutrients measured in both AEA, similar values were

registered, with exception of NO_2 N, that was found higher at AEA-MS. Total hardness also differed between AEA, with higher values in AEA-HS.

Table I: *Average (± standard deviation) concentrations of chemical elements in ashes (mg Kg^{-1}) and in aqueous extracts of ashes (AEA) (mg L^{-1}) from moderate severity (MS) and high severity (HS).*

Chemical elements	Ashes (mg Kg^{-1})		AEA (mg L^{-1})	
	MS	HS	MS	HS
Nitrate (NO_{3-} -N) (1-30 mg L^{-1})	-	-	9.7	9.25
Nitrite (NO_{2-} -N) (0.01-0.5 mg L^{-1})	-	-	0.17	0.07
Nitrogen (TN) (0.5-25 mg L^{-1})	-	-	14.6	18.4
Phosphate (PO_4^{3-}) (0.05-4 mg L^{-1})	-	-	<0.05	<0.05
Ammonia (NH3- N) (0.02-1 mg L^{-1})	-	-	<0.02	<0.02
Calcium Hardness ($CaCO_3$) (50-900 mg L^{-1})	-	-	<50	<50
Hardness Total (2-50 mg L^{-1})	-	-	27	41
Arsenic (As)	22.6 ± 1.3	8.2 ± 0.2	0.108 ± 0.0270	0.018 ± 0.0046
Cadmium (Cd)	0.3 ± 0.04	3.1 ± 0.5	0.002 ± 0.0005	0.016 ± 0.0032
Cobalt (Co)	6 ± 0.04	8 ± 0.08	0.042 ± 0.0105	0.015 ± 0.0038
Chromium (Cr)	23 ± 1.5	40 ± 5.6	0.097 ± 0.0243	0.092 ± 0.0230
Cooper (Cu)	56 ± 3.1	79 ± 2.6	0.294 ± 0.0735	0.259 ± 0.0648
Manganese (Mn)	2540 ± 78.6	2290 ± 84.6	16.7 ± 4.17	3.23 ± 0.808
Nickel (Ni)	30 ± 1.1	32 ± 1.3	0.151 ± 0.0378	0.05 ± 0.0125
Lead (Pb)	29 ± 2.1	64 ± 3.2	0.114 ± 0.0228	0.274 ± 0.0548
Vanadium (V)	35 ± 1.2	49 ± 0.9	0.19 ± 0.048	0.068 ± 0.0170
Zinc (Zn)	551 ± 20.3	322 ± 15.3	1.88 ± 0.376	1.32 ± 0.264

2.3.2 Embryo teratogenicity assays

The physical parameters of temperature (°C), pH, dissolved oxygen (mg L^{-1}) and conductivity (µS cm^{-1}), measured in the stock-solution (i.e. 100 % of AEA) and in the six AEA concentrations (0 %, 26.9 %, 35 %, 45.5 %, 59.2 % and 76.9 %), for both MS (Table II) and HS (Table III) were measured at the beginning and end of the embryos teratogenicity and tadpoles toxicity assays.

In general, the values of pH and conductivity were slightly lower in the assays with AEA-MS compared to AEA-HS. Furthermore, by comparing the values between the beginning and the end of both assays, it was observed a reduction in the pH values throughout the exposure and, by opposite, an increase in the conductivity values. Dissolved oxygen did not undertake major changes during the tests.

Table II: *Physico-chemical parameters measured in the samples of AEA from the moderate severity (MS) wildfire, at beginning (0 h) and end (96 h) of the embryo teratogenicity assay.*

Physico-chemical parameters (MS)							
AEA Concentrations (%)	**pH (0h)**	**pH (96h)**	**Conductivity (µS cm^{-1}) (0h)**	**Conductivity (µS cm^{-1}) (96h)**	**Dissolved oxygen (mg L^{-1}) (0h)**	**Dissolved oxygen (mg L^{-1}) (96h)**	**T (°C)**
0 %	7.9	7.6	569	880	8.	8.9	19
26.9 %	9.2	8.0	638	677	8.7	8.8	19
35 %	9.3	8.2	640	728	8.7	8.7	19
45.5 %	9.4	8.2	649	778	8.7	8.7	19
59.2 %	9.4	8.2	668	815	8.6	8.5	19
76.9 %	9.5	8.2	700	811	8.6	8.5	19
100 %	9.5	8.3	746	930	8.6	8.3	19

Table III: *Physico-chemical parameters measured in the samples of AEA from the high severity (HS) wildfire, at beginning (0 h) and end (96 h) of the embryo teratogenicity assay.*

Physico-chemical parameters (HS)							
AEA Concentrations (%)	**pH (0h)**	**pH (96h)**	**Conductivity (µS cm^{-1}) (0h)**	**Conductivity (µS cm^{-1}) (96h)**	**Dissolved oxygen (mg L^{-1}) (0h)**	**Dissolved oxygen (mg L^{-1}) (96h)**	**T (°C)**
0 %	7.9	8.0	566	545	8.6	8.6	18
26.9 %	9.8	8.2	715	813	8.6	8.6	18
35 %	9.9	8.3	736	861	8.6	8.3	18
45.5 %	9.9	8.4	770	973	8.6	8.3	18
59.2 %	10.0	8.4	804	999	8.6	8.2	18
76.9 %	10.1	8.4	903	1121	8.6	8.2	18
100 %	10.2	8.5	997	1283	8.5	8.2	18

The exposure to 100% of AEA of MS and HS, induced a significant mortality (27.5%) in embryos of *X. laevis* (F= 2.841; p< 0.05; F= 3.083; p< 0.05, respectively; Figure 2). The estimated 96 h LC_{20} (95% Cl) values were 83.1 % (59.9-282.4) and 92.4 % (66.3-374.7) for AEA-MS and AEA-HS, respectively.

At the end of the embryo exposure period the total average percentage of malformations were below to 10% for both types of AEA and most abnormalities verified were abdominal edemas, excess and lack of pigmentation (hyperpigmentation and hypopigmentation) and notochord curvature (Figure 3).

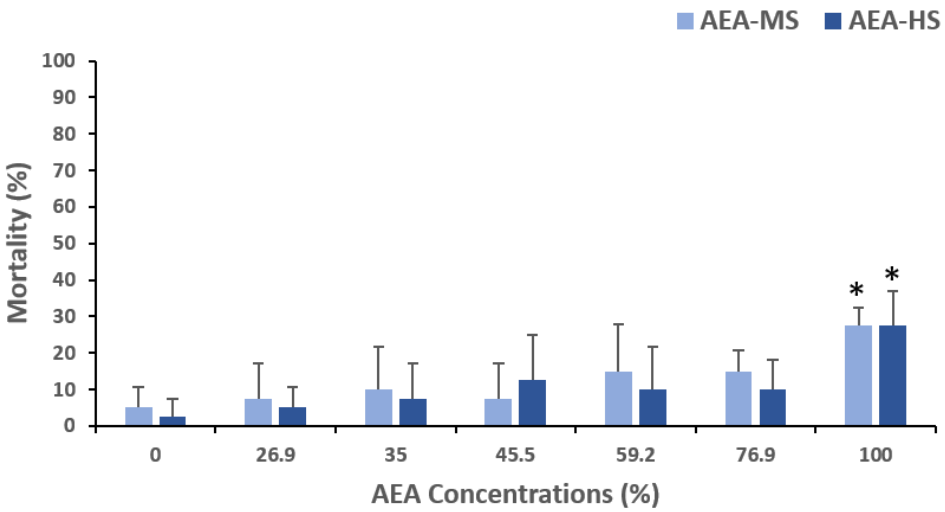

Figure 2: *Average mortality (%) of embryos of* Xenopus laevis*, after being exposed, for 96 h, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (MS) or high (HS) severity. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.05).*

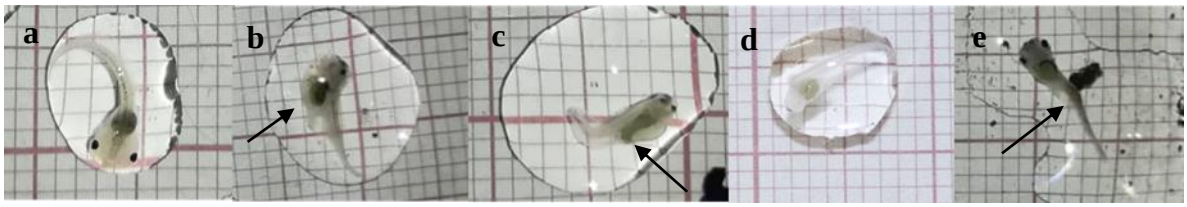

Figure 3: *Embryos of* Xenopus laevis *after being exposure, for 96 h, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (MS) or high (HS) severity.* ***a)*** *Control embryo without malformations;* ***b)*** *embryo exposed to 45.5 % AEA-MS concentration, showing an excess of pigmentation (hyperpigmentation) and with an abdominal edema;* ***c)*** *embryo exposed to 76.9 % AEA-MS concentration, showing an abdominal edema;* ***d)*** *embryo exposed to 59.2 % AEA-HS concentration, showing the lack of pigmentation (hypopigmentation);* ***e)*** *embryo exposed to 100 % AEA-HS concentration, showing a bent-notochord.*

Exposure to the AEA influenced the developmental stage of *X. laevis* embryos (Figure 4). Most of the organisms exposed to AEA of MS and HS where at the development stage NF 46 (> 80 %) after 96h of exposure. Moreover, embryos exposed to 76.9 % AEA-MS revealed a significant difference in development stages relatively to the control (F= 1.292; p< 0.0001). For the AEA-HS a significant delay in the development stage was observed for organisms exposed to 100 % (~75 % were at NF 46, while only ~25 % were at NF 45) after the 96 h period (F= 3.703; p< 0.0001).

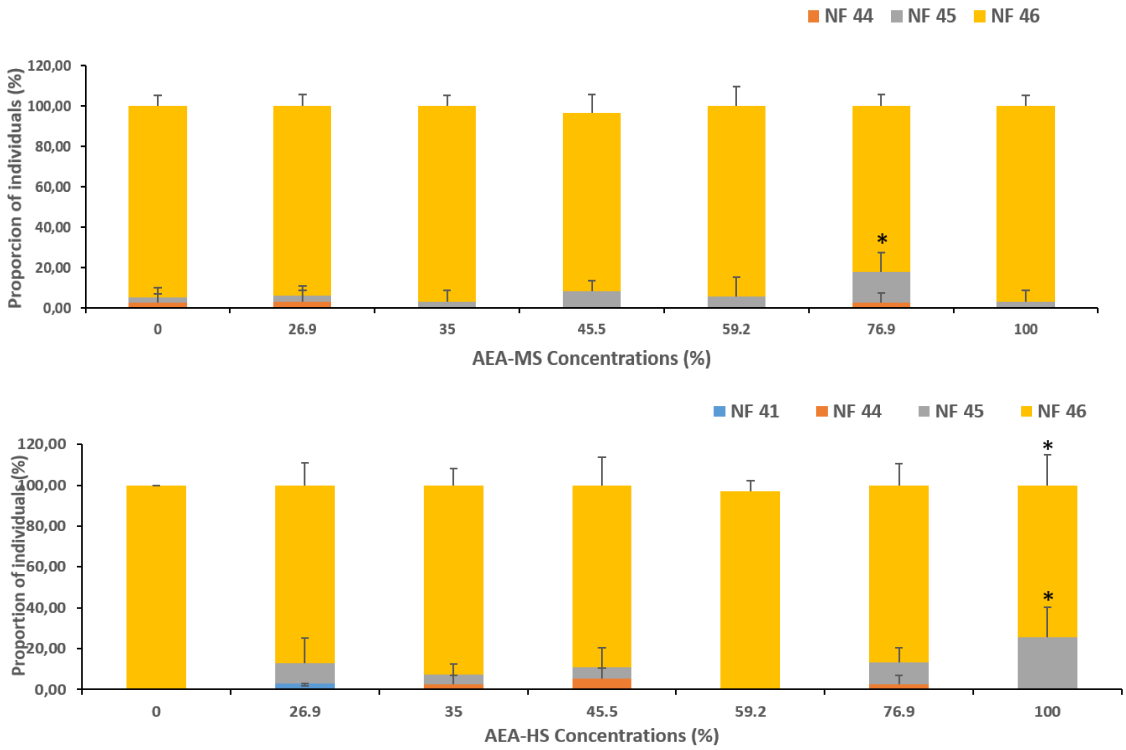

Figure 4: *Average proportion (%) of embryos of* Xenopus laevis *at NF developmental stages NF 41, NF 44, NF 45 and NF 46, after being exposed, for 96h, to several concentrations of aqueous extracts of ashes obtained from wildfires of moderate (AEA-MS; up) or high (AEA-HS; down) severity. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.05).*

The exposure of the embryos to AEA-MS caused a significant decreased in the tail length of larvae at all tested ash concentrations ($F= 7.811$; $p< 0.05$; Figure 5). In the case of AEA-HS, besides the significant reduction in the tail length, it, was also observed a significant reduction in the body and total length at all ash concentrations ($F= 11.09$; $F= 6.855$; $F= 13.18$; $p< 0.05$; Figure 5.

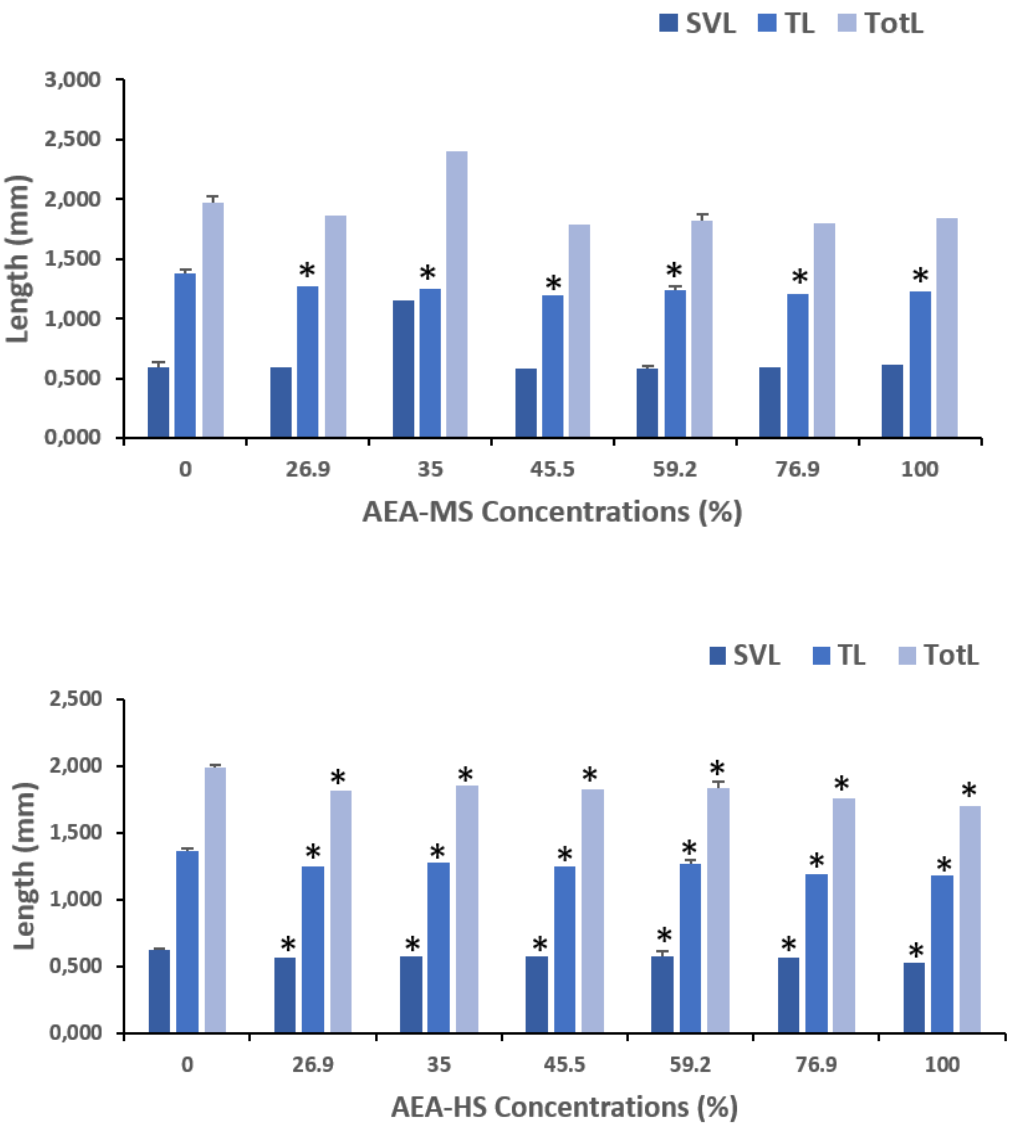

Figure 5: *Average length of snout-to-vent (SVL), tail (TL) and total (TotL) of embryos of* Xenopus laevis*, after being exposed, for 96 h, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (AEA-MS; up) or high (AEA-HS; down) severity. Error bars represents standard deviation. * indicates significant differences relatively to the control ($p<0.05$).*

2.3.2.1 Biochemical endpoint responses

The results in Figure 6a show that in lipid peroxidation there were significant effects in 45.5 % and 59.2 % concentrations for AEA-MS, and in 45.5 % for AEA-HS. For CAT activity (Figure 6b) was significantly stimulated at 26.9 and 35 % for AEA-MS and at 100 % for AEA-HS ($F= 10.71$; $p< 0.0001$; $F= 4.969$; $p= 0.0026$). At 45.5 % AEA-HS exposure, we found that the embryos experienced a significant increase in total glutathione activity (Figure 6c). For GST, no significant changes were observed during the exposure of the *X. laevis* embryos to ashes (Figure 6d). Results in Figure 6e show that AChE activity in the AEA-MS and AEA-HS was significantly inhibited in embryos exposed to 76.9 and to 100 % ash concentrations ($F= 4.193$; $p= 0.0063$). For ETS activity, a tendency for non-linear response pattern were observed for AEA-MS and AEA-HS (Figure 6f), with only significant differences in the concentration 35 % for AEA-MS. For lipid activity (Figure 6g) and carbohydrates (Figure 6h), no statistically significant differences were observed for both ashes. Concerning the total protein content, significant reduction was observed at 100 % AEA-MS ($F= 3.778$; $p= 0.0104$; Figure 6i).

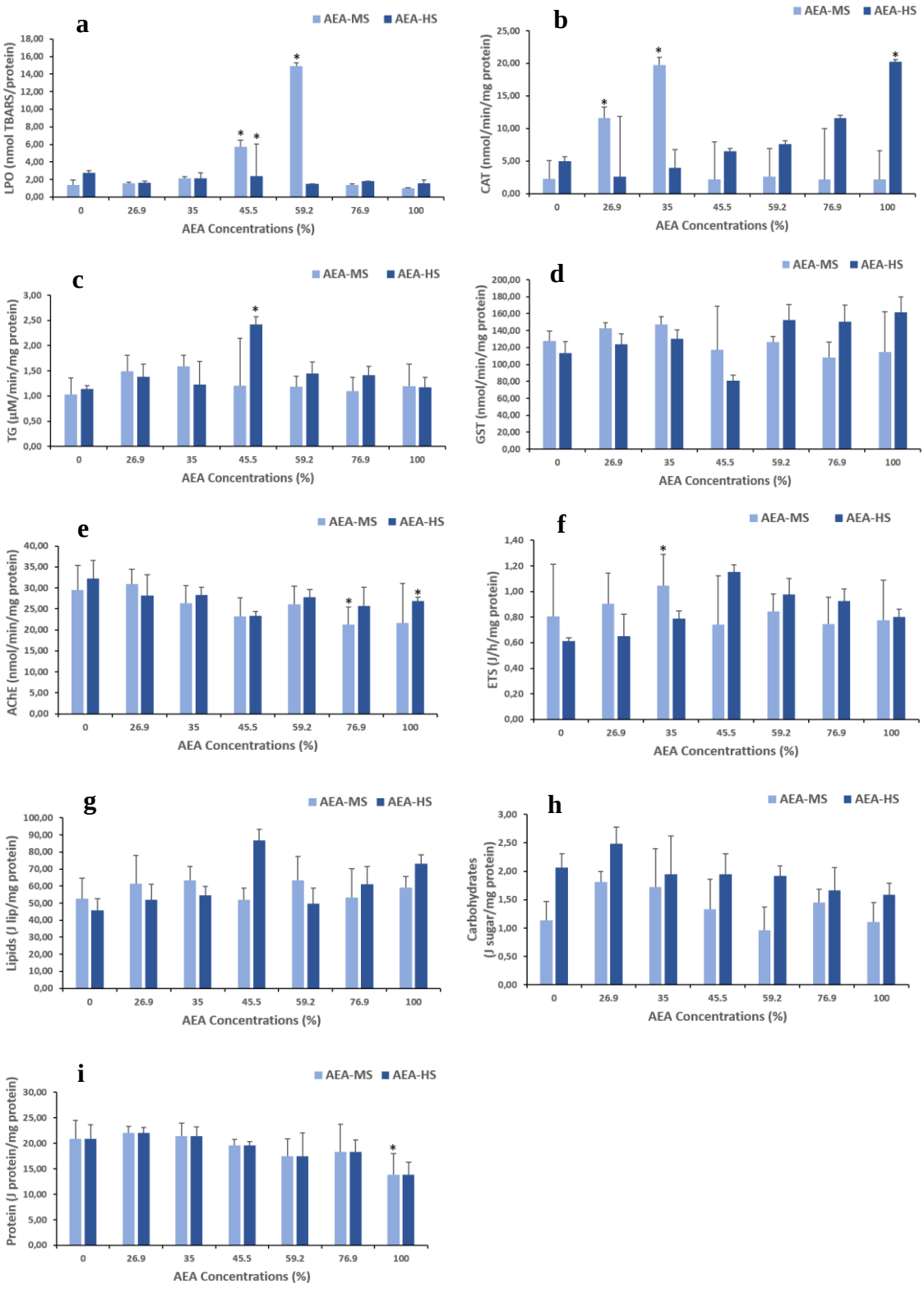

Figure 6: *Sub-individual effects in the embryos of* Xenopus laevis, *after being exposed, for 96 h, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (MS) or high (HS) severity.* ***a)*** *LPO= lipid peroxidation;* ***b)*** *CAT= catalase;* ***c)*** *TG= total glutathione;* ***d)*** *GST= glutathione S-transferase;* ***e)*** *AChE= acetylcholinesterase;* ***f)*** *ETS= electron transport system;* ***g)*** *Lipids;* ***h)*** *Carbohydrates;* ***i)*** *Protein activity. All values are presented as means ± SD. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.05).*

2.3.3 Tadpoles toxicity assay

The levels of pH, conductivity (µS cm^{-1}), dissolved oxygen (mg L^{-1}) and temperature (°C) for AEA-MS and AEA-HS *X. laevis* tadpoles toxicity assays are indicated in Table IV and Table V, respectively. Briefly, for the AEA-MS and AEA-HS, the pH values decreased during the assays, while an increase in the conductivity was registered for all ash concentrations. Dissolved oxygen values remained constant for both assays.

Table IV: *Physico-chemical chemical parameters measured in the samples of AEA from the moderate severity (MS) wildfire, at beginning (0 h) and after changing medium solution (48 h) of tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (µS cm^{-1}) (0h)**	**Conductivity (µS cm^{-1}) (48h)**	**Dissolved oxygen (mg L^{-1}) (0h)**	**Dissolved oxygen (mg L^{-1}) (48h)**	**T (°C)**
0 %	7.9	7.8	566	643	8.8	8.8	19
26.9 %	9.2	8.1	625	776	8.7	8.7	19
35 %	9.3	8.2	638	784	8.7	8.7	19
45.5 %	9.4	8.2	654	820	8.8	8.7	19
59.2 %	9.5	8.2	681	857	8.8	8.6	19
76.9 %	9.7	8.3	704	899	8.7	8.7	19
100 %	9.7	8.3	751	982	8.7	8.6	19

Table V: *Physico-chemical chemical parameters measured in the samples of AEA from the high severity (HS) wildfire, at beginning (0 h) and after changing medium solution (48 h) of tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (µS cm^{-1}) (0h)**	**Conductivity (µS cm^{-1}) (48h)**	**Dissolved oxygen (mg L^{-1}) (0h)**	**Dissolved oxygen (mg L^{-1}) (48h)**	**T (°C)**
0 %	7.9	7.6	598	619	8.9	8.8	18
26.9 %	9.6	8.3	701	811	8.8	8.7	18
35 %	9.8	8.6	736	884	8.8	8.7	18
45.5 %	9.9	8.7	778	909	8.8	8.7	18
59.2 %	10.0	8.7	818	974	8.8	8.7	18
76.9 %	10.1	8.8	896	998	8.9	8.7	18
100 %	10.2	8.9	989	1097	8.9	8.5	18

The exposure of *X. laevis* tadpoles to AEA-MS resulted in a significant mortality at all tested concentrations (F= 7.156; p< 0.05; Figure 7). In contrast, the exposure of tadpoles to the AEA-HS only induced a significant mortality at 76.9 %, with 40% of mortality (F= 3.480; p< 0.05; Figure 7). Concentrations causing 20 % of mortality (95 % Cl) were 25.0 % (0.1-39.9) and 67.3 % (49.7-109.0) for AEA-MS and AEA-HS, respectively. The percentage of malformations in tadpoles exposed to AEA were lower than 60 and 35 % for AEA-HS and AEA-MS, respectively. The most frequent malformation were bent notochord and abdominal edema (Figure 8).

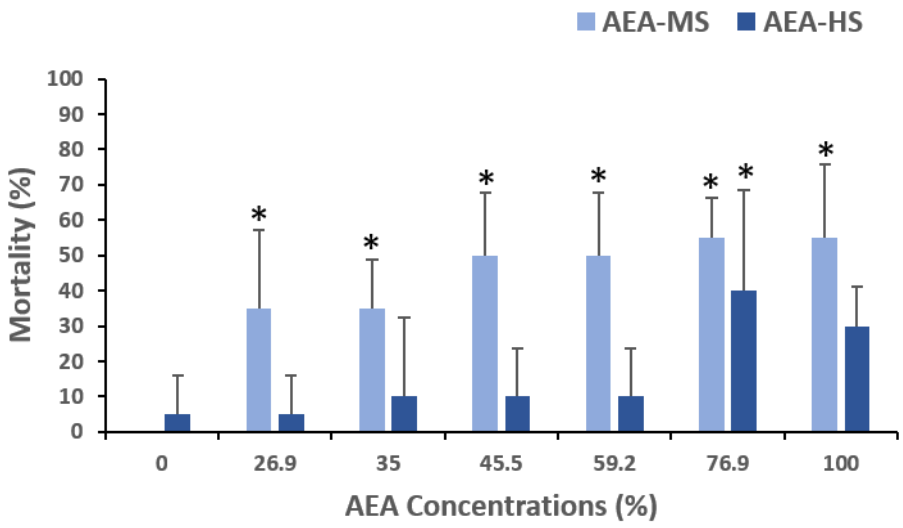

***Figure 7**: Average mortality of* Xenopus laevis*, after being exposed, for 14 days, to several concentrations of aqueous extracts (AEA) of ashes obtained from wildfires of moderate (MS) or high (HS) severity. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.05).*

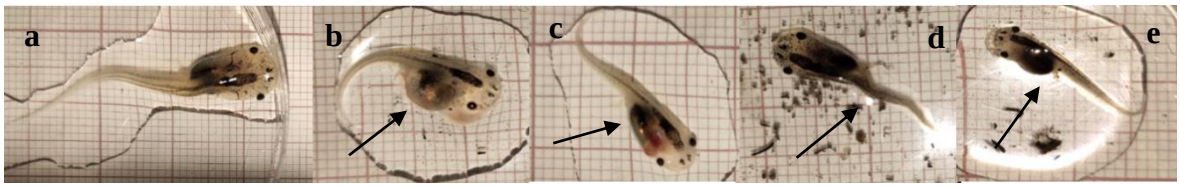

***Figure 8:** Tadpoles of* Xenopus laevis *after being exposure, for 14 days, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (MS) or high (HS) severity. **a)** Control tadpole without malformations; **b)** tadpole exposed to 35 % AEA-MS concentration, showing an abdominal edema; **c)** tadpole exposed to 45.5 % AEA-HS concentration, showing an abdominal edema; **d)** tadpole exposed to 76.9 % AEA-HS concentration, showing a bent notochord; **e)** tadpole exposed to 100 % AEA-HS concentration, showing an excess of pigmentation (hyperpigmentation) with an abdominal edema.*

Exposure to the AEA affected the development stage of the *X. laevis* tadpoles (Figure 9). In general, individuals exposed to 26.9, 45.5, 59.2, 76.9 and 100 % concentration of AEA-MS demonstrated a significant difference at the development stage NF 50 (F= 5.866; p< 0.0001), compared to the control, that the majority of the tadpoles were in NF 51 and 52, while at concentrations > 45.5 % tadpoles were mostly at development stage NF 50. For tadpoles exposed to AEA-HS, a significant effect was also observed in development stage (F= 6.431; p< 0.0001). Most tadpoles of the *X. laevis* exposed to all concentrations were in NF 50.

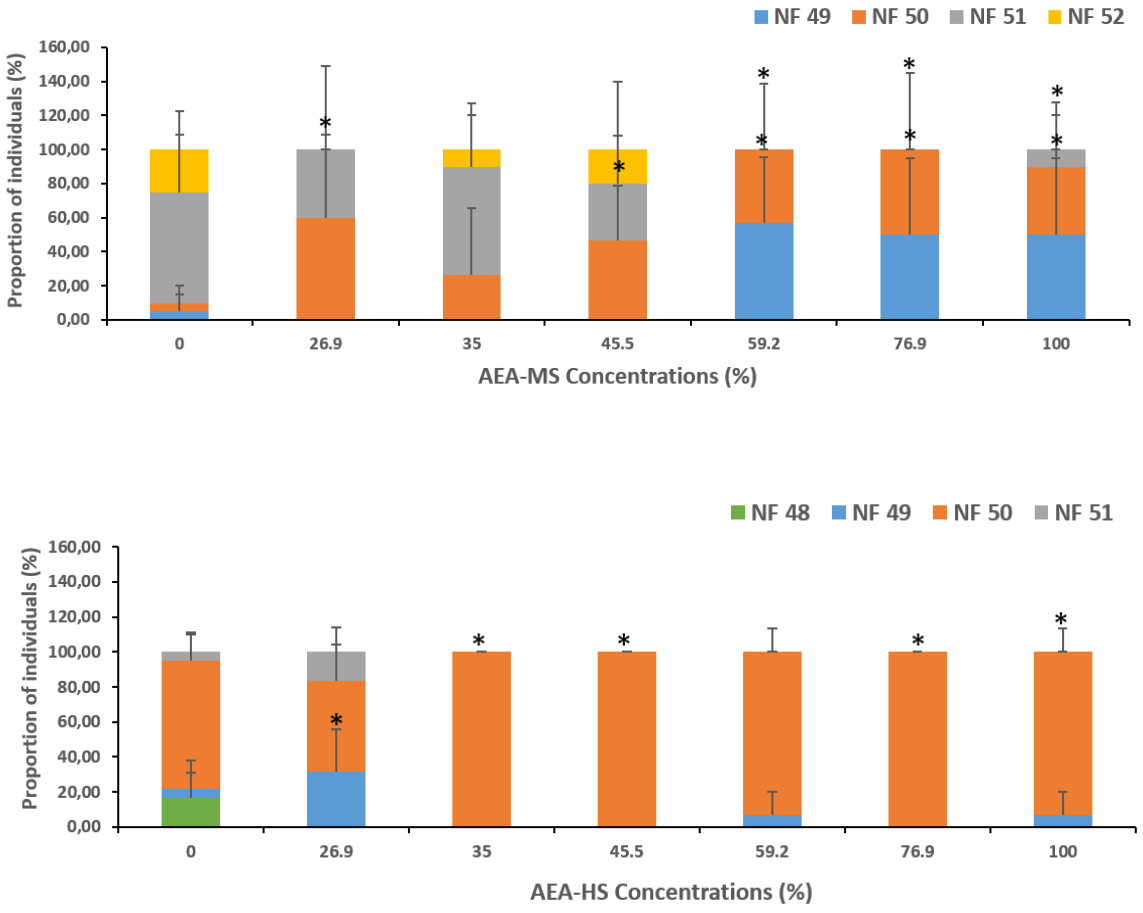

Figure 9: *Average proportion (%) of tadpoles of* Xenopus laevis *at NF developmental stages NF 48, NF 49, NF 50, NF 51 and NF 52, after being exposed, for 14 days, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (AEA-MS; up) or high (AEA- HS; down) severity. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.05).*

The snout-to-vent (SVL), tail (TL) and total (TotL) lengths of tadpoles exposed to all concentrations of AEA-MS were significantly reduced (F= 10.61; F= 11.42; F= 13.96; p< 0.05; respectively; Figure 10). For tadpoles exposed to AEA-HS a reduction in tail length, at 76.9 and 100 % (F= 8.422; p< 0.05), and of total length, at 100 % (F= 4.728: p< 0.05), was reported (Figure 10).

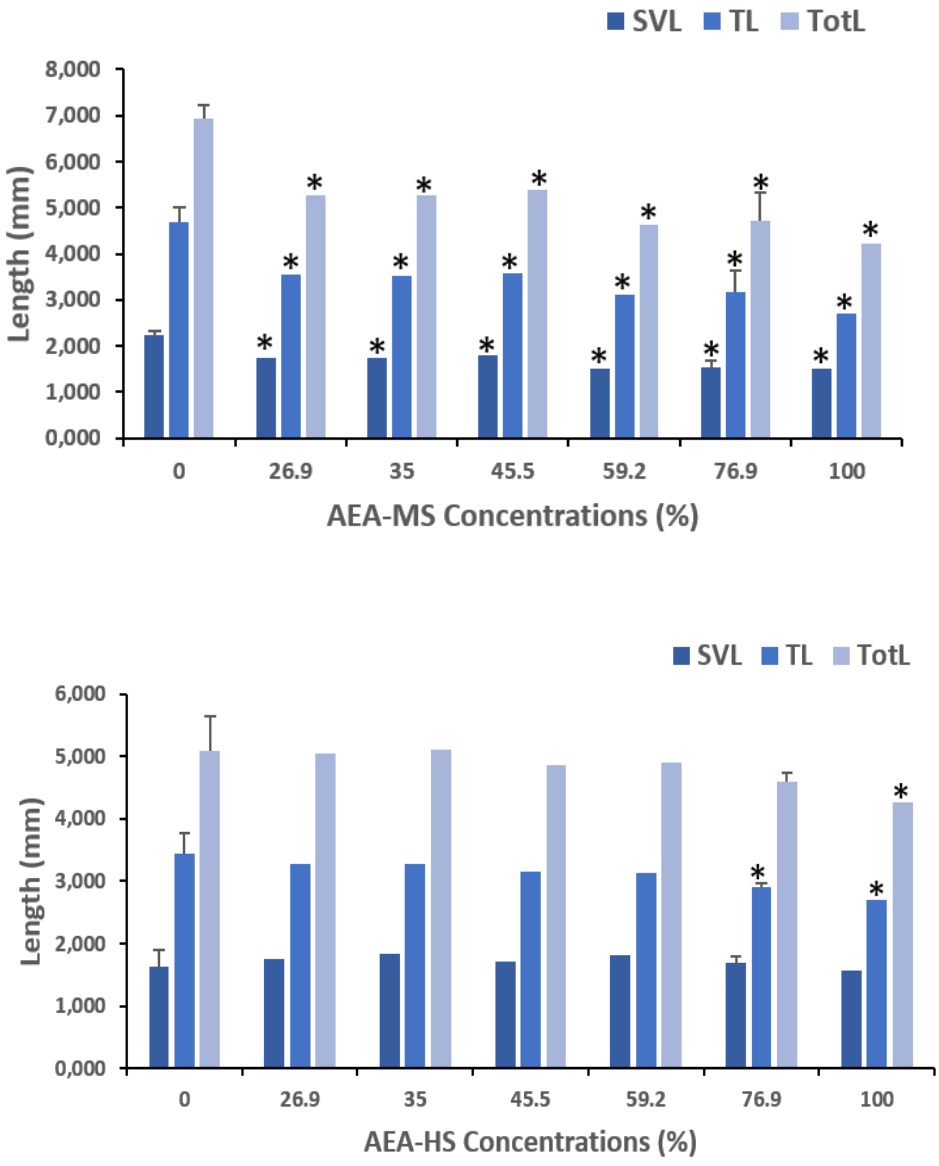

Figure 10: *Average length of snout-to-vent (SVL), tail (TL) and total (TotL) of tadpoles of* Xenopus laevis*, after being exposed, for 14 days, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (AEA-MS; up) or high (AEA-HS; down) severity. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.05).*

Regarding the body weight in tadpoles of *X. laevis*, a significant reduction was observed to all AEA-MS concentrations, while in AEA-HS the decrease in body weight was only observed for the high concentrations (100 %) (F= 14.15; F= 5.404; p< 0.05; Figure 11).

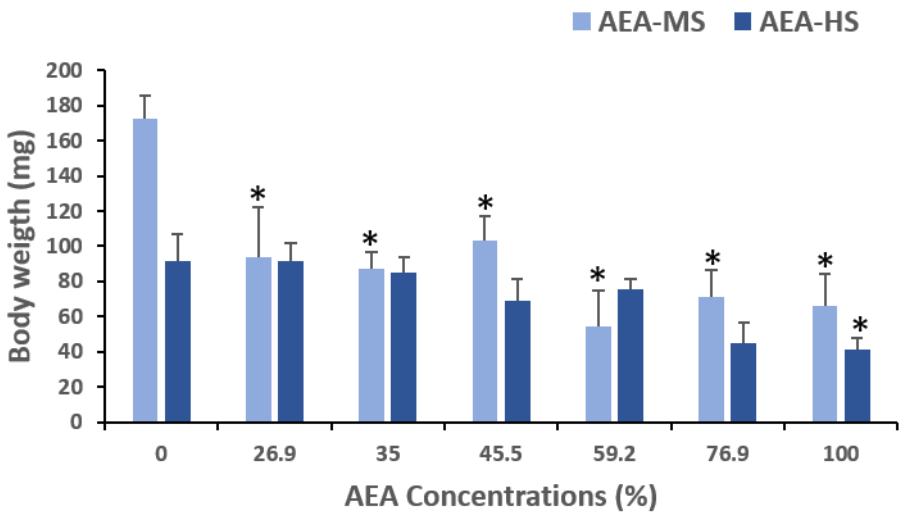

Figure 11: *Average body weight (mg) of tadpoles of* Xenopus laevis*, after being exposed, for 14 days, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (MS) or high (HS) severity. Error bars represent standard deviation. * indicates significant differences relatively to the control (p< 0.05).*

2.3.3.1 Biochemical biomarkers

The exposure of *Xenopus laevis* tadpoles demonstrated statistically significant results, with very high values, for 59.2, 76.9 and 100 % concentrations for AEA-MS Figure 12a. For CAT activity (Figure 12b), no statistically significant effect was observed for tadpoles exposed to AEA-MS and AEA-HS samples. For total glutathione activity (Figure 12c), only the 26.9 % concentration for AEA-MS revealed significant effects on tadpoles exposure, unlike for GST (Figure 12d), significant inhibitory effects were observed for 59.2 % for AEA-MS, and 26.9, 76.9 and 100 % for AEA-HS. Concerning the sub-individual endpoints, the exposure of *X. laevis* tadpoles to 100 % AEA-MS caused a significant induction in the AChE activity for tadpoles (F= 2.323; p= 0.0443), compared to the control treatment (Figure 12e). A significant induction response in ETS activity was observed at 45.5 % ash concentration for AEA-HS (F= 3.943, p= 0.0056; Figure 12f). For lipid activity,

induction of tadpoles exposure to AEA-HS was observed, with significant differences verified in the 100 % concentration; whereas for tadpoles exposed to AEA-MS the concentrations 35, 59.2 and 76.9 % concentrations reveals significant effects. AEA-MS exposure produced an inhibition in carbohydrates activity (Figure 12h). High concentrations of AEA-HS also caused a significant reduction in the total protein on tadpoles ($F= 4.614$; $p= 0.0022$; Figure 12i).

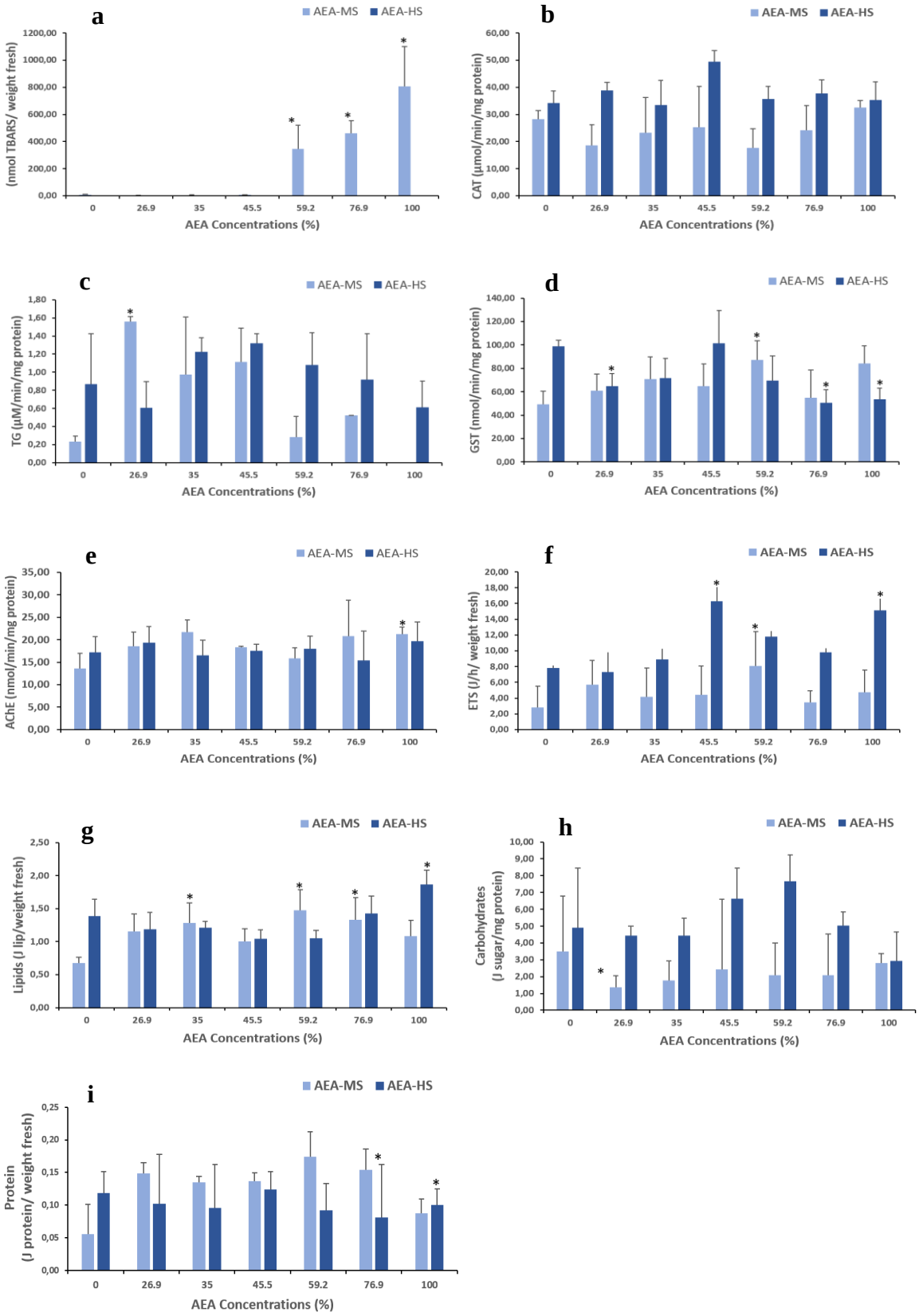

Figure 12: *Sub-individual effects in the tadpoles of* Xenopus laevis*, after being exposed, for 14 days, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (MS) or high (HS) severity.* ***a)*** *LPO= lipid peroxidation;* ***b)*** *CAT= catalase;* ***c)*** *TG= total glutathione;* ***d)*** *GST= glutathione S-transferase;* ***e)*** *AChE= acetylcholinesterase;* ***f)*** *ETS= electron transport system;* ***g)*** *Lipids;* ***h)*** *Carbohydrates;* ***i)*** *Protein activity. All values are presented as means ± SD. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.05).*

2.4 Discussion

After a wildfire, ashes containing compounds like metals, PAHS and nutrients are released and transported into aquatic systems. This study revealed the presence of several metals associated to MS and HS ashes and their corresponding extracts (AEA), which are considered priority substances by the USEPA (e.g. As, Zn, Ni, Cu, Cd, Pb and Cr) and by priority substances by the EC (e.g. Cd, Cr, Ni, Pb). Although the metals found in higher concentrations were the same in ash and AEA, it is notorious that their concentration in AEA were much lower than those found in the ashes, which could be due to their low solubility at the high pH values registered in the aqueous solutions (9.5-10.2) (Namieśnik & Rabajczyk, 2010). When comparing both ashes, high concentration of metals were found in AEA-MS, with exception for V and Pb. Silva *et al.,* (2015) studied the chemical composition of AEA, and when ashes and AEA matrices are compared, it 'clearly that the recovery/remobilization of chemical elements from ashes into AEA fluctuates within the different compounds, which it is due to its chemical specification, mineralogy of the ash and the pH of the solution (Silva *et al.,* 2015). The combustion of biomass, like other forms of incomplete combustion, produces substantial amounts of CH_4, volatile and semi-volatile organics and a variety of species containing nitrogen (N), including nitrogen oxides, as well as NH_3. Through forest fires, the release of NO_2 is triggered by the efficient oxidative combustion of nitrogen (N) contained in the biomass. Based on our results, in relation to nutrients, NO_2 was the element that most differed between the two AEA, with higher concentrations in AEA-MS, indicating a greater incomplete combustion of vegetation. Regarding the $CaCO_3$, higher values were found in the AEA-HS. According to Goforth *et al.,* (2005) and Ulery *et al.,* (1993), the presence of this chemical element in ashes is responsible for their brightness that increases with fire severity and was the major component of white ash. Ulery *et al.,* (1993) observed that light-coloured ashes collected after a high severity wildfire presented values of $CaCO_3$ ranging between 34 to 95 %, while the dark coloured ashes resulted from low to medium severity showed values of 12 % of $CaCO_3$.

The pH and conductivity levels were measured at the beginning and at the end of the embryonic teratogenicity tests and every 48 h, before and after changing the test solution, in the tadpoles toxicity assays, until the end of the tests (Annex I). Our results

revealed that there is a decrease in pH levels, in the 96 h exposure period, comparing the beginning and the end of the test. In tadpoles tests there was also a decrease in the pH values of the medium, when changing the solution medium (after 48 hours of exposure). As for conductivity values, they increased in both types of ash after tests for embryos and tadpoles. There is a strong relation with previous studies that recorded increases in pH following fire with the exposure temperature and severity (Certini, 2005; Neary *et al.,* 1999; Úbeda *et al.,* 2009; Úbeda *et al.,* 2010; Raison *et al.,* 1985; Raison & McGarity, 1980). Wildfires can cause increases in pH both by the combustion some carboxylic (acid) groups in the soil organic matter as well as through the release of base cations from the ash (Certini, 2005).

This study investigated the influence of fire severity on the ecotoxicity of the wildfires ashes to early life stages of *Xenopus laevis*. Therefore, embryos and tadpoles were exposed to aqueous extracts of ashes from two different severities: moderate (MS) and high (HS). Among the several studied parameters, mortality and tail length showed to be the most impaired, with statistical differences for both type of ashes. Exposure to AEA-MS and AEA-HS resulted in an increase in the mortality, with LC_{20} (95 % Cl) for embryos of 83.1 % (59.9-282.4) and 92.4 % (66.3-374.7), respectively. In fact, there are no published works reporting high mortality in embryos, which may indicate that embryos are not highly vulnerable to fire (Russell *et al.,* 1999). The similar mortality rate found in both ashes, suggests that the gelatinous layer that surrounds and protects the embryo, prevents the degradation of proteins essential to the development of the embryo (Przeslawski *et al.,* 2004) and thus constitutes an important barrier to the chemicals (Edginton *et al.,* 2007). However, comparing the embryonic development in both type of ashes, we verified that there is a greater variability between stages in *X. laevis* organisms in high concentrations (NF 44, 45 and 46) for the AEA-HS, whereas in the AEA-MS we found that most organisms are in NF 46 stage of development. Hence, our results showed that the high severity ashes affect the embryonic development of amphibians, delaying the stage of development. Moreover, other biometric parameters, particularly the tail length was also affected when the organism were exposed to both extracts. Due to a greater predominance of lower

stages of development in 76.9 % and 100 % of AEA-HS, there was also a decrease in the average of snout-to-vent and total body length.

Concerning the biochemical biomarkers, the significantly high values of lipid peroxidation for both *Xenopus laevis* embryos, observed for AEA-MS results from errors associated with biochemical analyses. An inhibition of AChE was observed in *X. laevis* embryos exposed to high concentrations of AEA-MS. A wide range of metallic compounds such as Cd, Cu, Hg and Zn have the potential to inhibit the cholinesterase enzyme (ChE) activity, however the inhibition mechanism is not fully known (Elumalai *et al.,* 2007; Jebali *et al.,* 2006; Labrot *et al.,* 1996; Payne *et al.,* 1996; Stefano *et al.,* 2008). Jebali *et al.,* (2006), in *in vivo* studies observed an inhibitory effects of Cd on the brain AChE of *S. dumerilli*. Hence, the AChE inhibition reported in this study could be attributed to the presence of pernicious metals found in the extracts, particularly in the AEA-MS. In turn, in embryos exposed to AEA-HS (for 100 % concentration), CAT activity was clearly induced. According to Napierska *et al., (*2018) the exposure to Pb triggers the oxidative stress. Consequently, with the increase of oxidative stress, in the presence of reactive oxygen species (ROS) there is an activation of catalase activity, which is an ROS protective enzyme. Even at low levels, Pb was capable of producing significant toxicological effects that are common to a large number of aquatic organisms (Mager, 2011; Napierska *et al.,* 2018; Nunes *et al.,* 2014; Sepe *et al.,* 2003). Consumption of energy yielding substrates such as carbohydrates and lipids were not significantly altered in *X. laevis* embryos exposed to any type of ashes. This was probably due to the short duration of exposure (96 h). Nevertheless, for AEA-MS and AEA-HS a no linear response to ETS activity was observed for *X. laevis* embryos exposed to high concentrations (≥ 59.2 %). Maiti *et al.,* (2010) observed that the mitochondria electron transport chain activity of the brain of fish *Clarias batrachus* can be increase due to the inhibition of mitochondrial electron transport chain during the oxidative stress response triggered by the exposure to Pb. This inhibition of the electron transport system may influences the level of ATP production (in this case leading to reduction) in brain mitochondria. The $Na^{+}K^{+}$ ATPase protein can remain inactive if ATP production decreases due to insufficient activity in electron transport systems (Caito & Aschner, 2015). Maiti *et al.,* (2010) also reported that Pb^{2+} concentrations induce toxicity in the form of oxidative

stress through the inhibition of Na^+K^+ ATPase protein activity, which in turn leads to inhibition of the mitochondrial electron transport system. The results for total protein content is in line with what was previously mentioned about the increase in ROS production through the electron transport chain, since there is a significantly reduced in 100 % of AEA-MS.

In what concerns the effects of the ash extracts on *Xenopus laevis* tadpoles, our results indicates that tadpoles may be differentially impacted by moderate and high ash extracts. Thus, exposure of tadpoles to AEA-MS resulted in a high mortality compared to AEA-HS (LC_{20} -25.0 in AEA-MS and 67.3 in AEA-HS). Although the mortality rate was found lower in the AEA-HS, in relation to the malformations observed, there was a greater number of individuals with abnormalities, namely bent notochord malformations and abdominal edema when exposed to the highest concentration of AEA-HS. According to Driscoll & Roberts (1997) and Russell *et al.,* (1999), eggs and larval amphibians mortality of aquatic life stages are rarely observed and when it happens may be insignificant and could result from thermal stress or rapid changes in water chemistry (Spencer & Hauer, 1991). Differences in the development stage were also observed between the two types of ash extracts. At high concentrations of AEA-MS the development stage NF 49 and NF 50 were the predominants, while in the initial treatments it was the stage NF 51 and NF 52. For AEA-HS was verified that NF 50 stage development has been found the predominant in most ash concentrations and NF 51 development stage was also observed in the lowest concentrations. Most amphibian larvae species modify their activity, morphology, differentiation rate, growth and development rate in response to environmental stimuli and pressures (Van Buskirk, 2002; Hua *et al.,* 2015). In this way, amphibian larvae can grow normally for long periods of time without advancing in the development stage.

Regarding the biochemical effects of tadpoles, the significantly high values of lipid peroxidation for tadpoles, observed for AEA-MS cannot be taken into account, since they result from errors associated with chemical analyses. The biochemical markers analysed, such as TG, GST and carbohydrates, reported similar linear responses to tadpoles exposure, for AEA-MS. In the exposure to AEA-HS, the activity of TG and carbohydrates decreased, in

contrast to the lipid activity that increased. For *Xenopus laevis* tadpoles when exposed to both ash extracts, an increase in the AChE activity was observed for the highest concentration, more evident in the AEA-MS. Several studies have demonstrated the effects of other classes of environmental contaminants on AChE activity of several aquatic species (Diamantino *et al.,* 2003; Labrot *et al.,* 1996; Payne *et al.,* 1996). Van Meter *et al.,* (2018) reported that in green frogs (*Lithobates clamitans*) subject to exposure to mixed pesticide treatments, such as atrazine, 2,4-D and metolachlor, the increase in AChE activity, associated with the positive regulation of tyrosine, caused changes in neurotransmitter functions, which can result in neural over-stimulation. Concerning the CAT, no significant effects were observed for both type of ashes. According to previously mentioned studies (Mager, 2011; Napierska *et al.,* 2018; Nunes *et al.,* 2017; Sepe *et al.,* 2003), the non-increase of activity of the antioxidant enzyme suggests that despite the distinct toxic metals that were found in the ash extracts the detoxification oxidative stress mechanism were possibly not activated. In contrast, the ETS activity of *X. laevis* tadpoles were significantly altered by the exposure to both ash extracts, with an increase in their activity, which is in line with most studies already carried out (Napierska *et al.,* 2018). For total protein content, a significant inhibition was observed for organisms exposed to 76.9 and 100 % AEA-HS. Proteins play an important role in the life of all organisms. Thus, the decrease in protein levels observed in this study can be attributed to the immobilization of some toxic compounds, such as lead, by oxidative stress (Begum & Vijayaraghavan, 1996). The metallic element lead, present in aqueous extracts of high severity ash in higher concentration, is associated with increased environmental toxicity and can induce oxidative damage through direct effects on the cell membrane of organisms (Ercal *et al.,* 2001).

Chapter III

Toxicity of ashes driven by wildfires of different severities on tadpoles of the anuran *Pelophylax perezi*

Chapter III- Toxicity of ashes driven by wildfires of different severities on tadpoles of the anuran *Pelophylax perezi*

3.1 Introduction

Wildfires occur in nature (e.g. burn of forests, grasslands, prairie, bush areas) as a consequence of natural phenomena (e.g. lightning, volcanic activity) or anthropogenic activities (e.g. burn farming, fireworks, intentional act of humans) (Pausas, 2004; Pausas *et al.,* 2008; WHO, 2020). Within the context of climate changes, the associated increase in temperatures, extremely dry conditions, and wind strength, are predicted to potentiate the frequency and severity of wildfires in some regions of the globe (Jones *et al.,* 2020; Sun *et al.,* 2019). Namely, the Mediterranean basin has been considered a hotspot for wildfires related with climate changes (Pausas 2004, 2008; Pausas & Fernández-Muñoz, 2011). As an example, Turco *et al.,* (2018) projected an increase of 40 % to 100% of the burned area in the Mediterranean Europe region, across scenarios of 1.5 to 3 °C increases in temperature. Adding to the well-known negative impacts that these wildfires may cause in terrestrial ecosystems (e.g. loss of soil structure and of net gross primary producers), severe impacts are also expected to occur in freshwater ecosystems located adjacent or downstream the burned areas (Bodí *et al.,* 2014; Santín *et al.,* 2015; Shakesby & Doerr, 2006; Sun *et al.,* 2019). In the course of a wildfire, large quantities of particles are released into the atmosphere and formed ashes accumulate on the soil. These ashes are made up of organic (e.g. polycyclic aromatic hydrocarbons) and/or inorganic compounds (e.g. metals), which after events of post-fire rain, through processes such as the surface runoff of ash, may be mobilized and released into the aquatic systems, compromising the ecological quality of these ecosystems (change in chemical composition; pH; dissolved oxygen conductivity; increase in turbidity, temperature, organic matter; high nutrient loads, for example phosphorus, nitrogen; and suspended sediments, among others) (Campos *et al.,* 2012; Leite, 2011; Nogueira, 2013; Nunes *et al.,* 2017; Pradhan *et al.,* 2020; Ré *et al.,* 2020b). The type and quantity of chemicals being released into the water is dependent on several factors, namely on the severity of the wildfire. Pereira *et al.,* (2012) reported that ashes originated from low to medium-severity wildfires exhibited greater

concentrations of total carbon and nitrogen, and water-extractable Ca, Mg and K; while increased pH values, $CaCO_3$ levels and water extractable total sulphur were observed for ashes released from high-intensity wildfires. These physical and chemical changes that are induced in water bodies, may cause severe ecotoxicological effects in the aquatic biota. Actually, an increasing number of works have been published reporting both lethal and sublethal effects caused by wildfire ashes in species belonging to different taxonomic and functional groups, including producers, primary consumers, secondary producers (Buddle *et al.,* 2006; Cooper *et al.,* 2015; Malison & Baxter, 2010; Persoone & Guillett, 1990). Although, all this toxicity data that has been generated, knowledge gaps still exist regarding the adverse effects that ashes release from wildfires may pose to amphibians. This is a group of vertebrates that may be especially vulnerable to this type of contamination, as: (i) most species reproduce in the aquatic environment, (ii) the breeding season of many species overlap with the wildfire season, (iii) early life stages are totally aquatic, (iii) tadpoles feed on organic matter at the surface of the sediment (where ashes are expected to deposit); (iv) their skin is highly permeable, and (v) by exhibiting a bi-phasic life-stage, including aquatic early-life stages and terrestrial adult stages, makes them prone to exposure to this type of contamination both in aquatic and terrestrial compartments (Katzenberger *et al.,* 2012; Maravalhas *et al.,* 2018; Pilliod *et al.* 2003). Very few works have evaluated the toxicity of wildfire ashes on aquatic life stages of amphibians. Lyon *et al.,* (1978), Driscoll & Roberts, (1997) in Pilliod *et al.,* (2003) reported that mortality, as a direct effect of wildfire, is rarely recorded on aquatic life stages of amphibians, because they are more protected from direct contact with wildfire. Pilliod *et al.,* (2003) and Spencer & Hauer, (1991) reported, as an example of indirect effect of wildfires, that mortality in adults and tadpoles tailed of the amphibian species of *Ascaphus montanus* could result from thermal stress or rapid changes in water, since smoke diffusion increases ammonium toxicity.

Accordingly, this study aimed at assessing the influence of wildfire severity on the ecotoxicity of aqueous extracts of the released ashes on tadpoles of the anuran species *Pelophylax perezi*. This species was chosen as a model species because it is very common in the Southern part of the Mediterranean region (as mentioned above, considered a hotspot of wildfires), its geographic distribution occurs from Portugal to southern France

(García-Muñoz *et al.,* 2010; Pausas & Fernández-Muñoz, 2011). Furthermore, it has a conservation status of least concern and is capable of colonizing a wide diversity of habitats, enabling to easily obtain large number of organisms in the field to perform laboratorial experiments.

3.2 Material and Methods

3.2.1 Collection of ashes and preparation of aqueous extracts of ash (AEA)

Ashes were collected in March 2019, immediately after a wildfire that consumed an area of 320.39 ha, from two hillslopes with different severities: moderate (MS; 40°78'37.48"N 8°45'39.35"W) and high (HS; 40°78'37.48"N 8°45'39.35"W). The two hillslopes, corresponding to LMS and HS, were located in Nespereira de Cima (Oliveira de Azeméis, Aveiro) in north-central Portugal (Figure 1; Chapter II), and were mainly covered by stands of Maritime pines (*Pinus pinaster*). Taking into account the spatial heterogeneity of the ashes, on each hillslope, five equidistant points were sampled along a transect established from top to bottom of entire length of the hillslope. At each of the five sampling points, a grid with 50 x 60 cm was used to sample the ashes. In each grid, the entire ash layer was collected with a brush and spoon, to avoid mixing with the soil, and then the ash samples were sieved separately in a 2 mm mesh and transported to the laboratory in plastic bags, under dark conditions. In the laboratory, the sieved ash samples from MS and HS wildfires were air dried and then mixed in a container for the production of a single composite. Subsequently, the samples stored at -20 °C in dark plastic bags (to reduce microbial activity) until the preparation of the aqueous extracts (Campos *et al.,* 2016; Silva *et al.,* 2015).

Aqueous ash extracts (AEA) from MS and HS were prepared for further chemical analysis and ecotoxicity assays. For the stock solution of each ash (100 % of AEA), 10 g of ash were weight and added to 1 L of FETAX medium (Dawson & Bantle, 1987). The following additional six ash concentrations were prepared by diluting the stock solution of AEA with FETAX medium: 26.9 %, 35 %, 45.5 %, 59.2 %, 76.9 %.

The procedure of physicochemical analysis of AEA samples were described in section 2.2.2 (please see chapter II).

3.2.2 Test Organisms

The amphibian species *Pelophylax perezi* (López-Seoane, 1885) was selected as the model species of amphibians to run this study. Egg masses of *P. perezi* less than 36-h old, were collected in a freshwater pond located near the city of Aveiro (40°36´16´´N, 8°41´48´´W; Quinta da Boavista, Aveiro, Portugal). After arrival to the laboratory, the eggs were transferred to aquaria containing FETAX medium, and were maintained with constant aeration, at a constant temperature (23± 2 °C) and photoperiod (14:10 h light: dark), until hatching and reaching Gosner developmental stage G 25 (Gosner, 1960). At this stage, tadpoles were used to perform the toxicity assays.

3.2.3 Tadpoles toxicity assays

Tadpoles of *P. perezi*, at developmental stage G 25, were exposed to a control (consisting of Fetax medium; Dawson & Bantle (1987)) and to several concentrations of AEA obtained from the ashes of wildfires with different severities (26.9 %, 35 %, 45.5 %, 59.2%, 76.9 % and 100 %), by following the guideline of ASTM (2002) with some modifications, namely the duration of the assay was extended for 14 days. Exposure occurred by introducing four tadpoles in high-density 500-mL plastic vessels filled with 200 mL of the test solution and with constant aeration, to avoid hypoxia conditions during the assay. Five replicates were performed per treatment, which were placed, for 14 days, in a climatic chamber with controlled temperature (23 ± 1 °C) and photoperiod (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™. At the start and end of the assay, the following parameters were measured: temperature (°C), pH, dissolved oxygen (mg/L) and conductivity (µS/cm), by using a WTW multiparameter equipment (Multi 3410 SET C). Regarding the monitored endpoints, mortality, malformations and hatching were checked

daily; dead organisms were removed from the test vessels to avoid the growth of microorganisms, which could impair the viability of the alive tadpoles. At the end of the assay, adding to the endpoints mentioned previously, developmental stage, body length (snout-to-vent, tail and total length) and body weight were assessed for the surviving organisms. Furthermore, biochemical responses were also evaluated. For this, after the identification of developmental stage and biometric measurements, surviving tadpoles were immediately deep-frozen in liquid nitrogen and stored at -80 °C, until further analysis. Given the diversity of chemicals that may be present in the aqueous extracts of the ashes, biochemical responses related with oxidative stress [lipid peroxidation (LPO), catalase (CAT), total glutathione (TG), glutathione S-transferase (GST)], neurotoxicity [acetylcholinesterase (AChE)] and energetic metabolism [electron transport system (ETS), total lipids, total carbohydrate and total protein] were evaluated.

3.2.4 Biochemical marker analyses

To proceed with the biochemical determinations, tadpoles were thawed on ice and afterwards homogenized with a sonic homogenizer (Y stral homogenizer) (for 10 seconds) in 1700 µL of ultra pure water. From each replicate, aliquots of 300 µL were taken for lipids, sugars and protein contents and electron transport system (ETS) activity analysis. An aliquot of 200 µL was used for determination of LPO, whereas the remaining homogenate tissue from each sample (~ 500 µL) was diluted with 500 µL of 0.2 M K- phosphate buffer (pH 7.4), centrifuged at 10,000 g for 20 minutes at 4 °C to isolate the Post-Mitochondrial Supernatant (PMS). Those PMS samples fraction was then divided into 5 microtubes and kept in -80 °C until further analyses of biomarkers. All the reactions were carried out in 96-wells microplates at 25 °C and determined in a microplate reader spectrophotometer (Multiskan Spectrum, Thermo Fisher Scientific, Waltham, USA) by following the methodologies briefly described below.

The procedure of determination of LPO, CAT activity, TG, GST, AChE activity, ETS, lipids, carbohydrates and total protein were described in section 2.2.5 (please see chapter II).

3.2.5 Data analyses

The concentration of AEA inducing 20 % of mortality in the tadpoles of *P. perezi* was computed through the Probit regression, using the Priprobit software (Lewis & Finney, 1972). To determine significant differences between the responses measured in tadpoles exposed to the AEA with those exposed to the control, one-way analysis of variance, followed by the multicomparison Dunnett's post-hoc test were performed in the Grahpad Prism 8 software. Following these tests, the lowest observed effect concentrations (LOEC) and non-observed effect concentration (NOEC) were determined. The normal distribution of data was checked through the Shapiro-Wilk's test and the homoscedasticity of variances through the Bartlett's test. The results obtained from the tadpoles toxicity assays did not allow the determination of an EC_{50}, this only the 20 % lethal concentration was calculated. A significance level (α) of 0.05 was used in all statistical tests.

3.3 Results

3.3.1 Physical and chemical characterization of AEA

The results obtained regarding the concentrations of arsenic, cadmium, cobalt, chromium, copper, manganese, nickel, lead, vanadium and zinc were quantified in ash and aqueous extracts of ash and are presented in section 2.3.1 of chapter II (please see Table I).

3.3.2 Tadpoles toxicity assays

The average levels of pH, conductivity (µS/cm), dissolved oxygen (mg/L) and temperature (°C), for AEA-MS and AEA-HS ash concentrations (0 %, 26.9 %, 35 %, 45.5 %, 59.2 %, 76.9 % and 100 %) are reported in Table VII and Table VIII.

and Table VII for the beginning and end of tadpoles toxicity assays. A general increase at pH and conductivity values was recorded over the 14 days exposure period, with the measurement of values every 48 hours, when changing the test solution. The smaller value

was verified in control treatment for AEA-MS, as well as for AEA-HS. Dissolved oxygen levels did not differ much from the beginning to the end of assays for both types of ashes.

Table VI: *Physico-chemical parameters measured in the samples of AEA from the moderate severity (MS) wildfire, at beginning (0 h) and after changing medium solution (48 h) of tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	566	543	8.8	8.8	19
26.9 %	9.2	8.1	625	776	8.7	8.7	19
35 %	9.3	8.2	638	784	8.7	8.7	19
45.5 %	9.4	8.2	654	820	8.8	8.7	19
59.2 %	9.5	8.2	681	857	8.8	8.6	19
76.9 %	9.7	8.3	704	899	8.8	8.7	19
100 %	9.7	8.3	751	982	8.7	8.6	19

Table VII: *Physico-chemical parameters measured in the samples of AEA from the high severity (HS) wildfire, at beginning (0 h) and after changing medium solution (48 h) of tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.6	598	619	8.9	8.9	18
26.9 %	9.6	8.3	701	811	8.8	8.7	18
35 %	9.8	8.6	736	884	8.8	8.8	18
45.5 %	9.9	8.7	778	909	8.8	8.8	18
59.2 %	10.0	8.7	818	974	8.8	8.7	18
76.9 %	10.1	8.8	896	998	8.9	8.7	18
100 %	10.2	8.9	989	1097	8.9	8.5	18

The survival of *P. perezi* tadpoles was significantly affected by the exposure to 100 % of the AEA-MS and AEA-HS, a 30 % mortality was registered for both types of ashes (F= 6.167; p< 0.05; F= 5.222; p< 0.05, respectively, Figure 13). Regarding the incidence of malformations, only one surviving individual exposed to 100 % of AEA-MS and AEA-HS exhibited tail curvature (Figure 13).

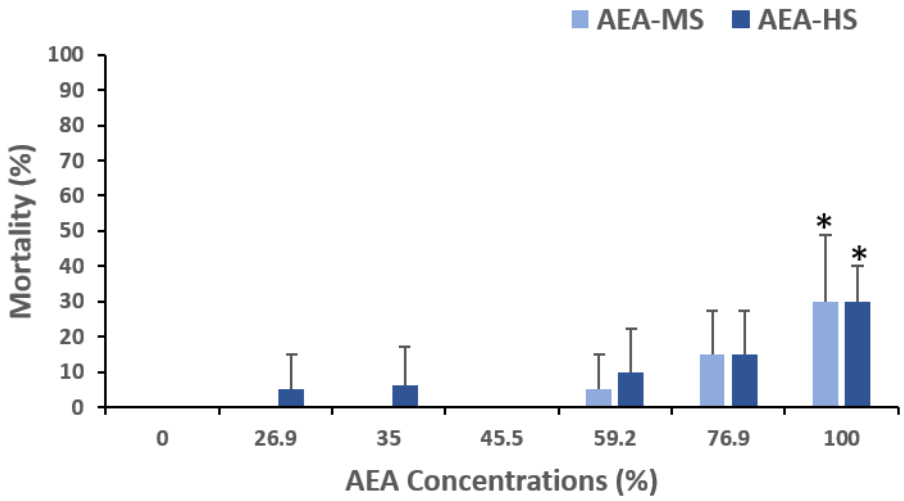

Figure 13: *Average mortality of* Pelophylax perezi, *after being exposed, for 14 days, to several concentrations of aqueous extracts (AEA) of ashes obtained from wildfires of moderate (MS) or high (HS) severity. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.05).*

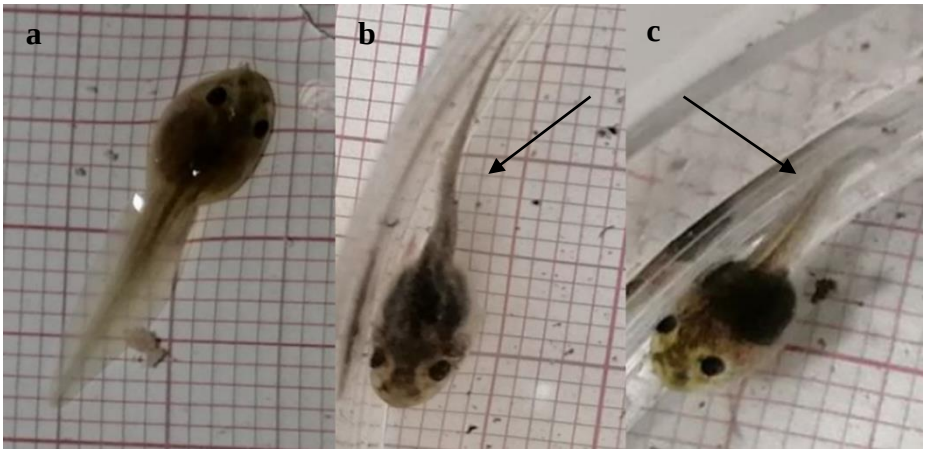

Figure 14: *Tadpoles of* Pelophylax perezi *after being exposure, for 14 days, to several concentrations of aqueous extracts (AEA) of ashes obtained from wildfires of moderate (MS) or high (HS) severity.* ***a)*** *control tadpole without malformations;* ***b)*** *tadpole exposed to 100 % AEA-MS concentration, showing a bent notochord;* ***c)*** *tadpole exposed to 100 % AEA-HS concentration, showing a bent tail.*

Exposure to the AEA also influenced the developmental stage of the tadpoles (Figure 15). For organisms exposed to AEA-MS, most were at developmental stage G 28 (> 65 %), except for the group exposed to 100% MS that were mainly at developmental stage G 27 (70 %). On average, tadpoles exposed to 100 % AEA-MS exhibited a significant delay in development stage comparatively to the control group (which were mainly at G 28-55 % and G 29-20 %) ($Q = 3.098$; $p < 0.05$). Concerning tadpoles exposed to AEA-HS, a significant delay in developmental stage was registered for organisms exposed to 59.2, 76.9 and 100% ($Q \leq 4.214$, $p < 0.05$). Most of the organisms exposed to 26.9 and 35% were at G 28 (≥ 85 %), while those exposed to 45.5, 59.2, 76.9 and 100% were mainly at G 27 (> 55 %).

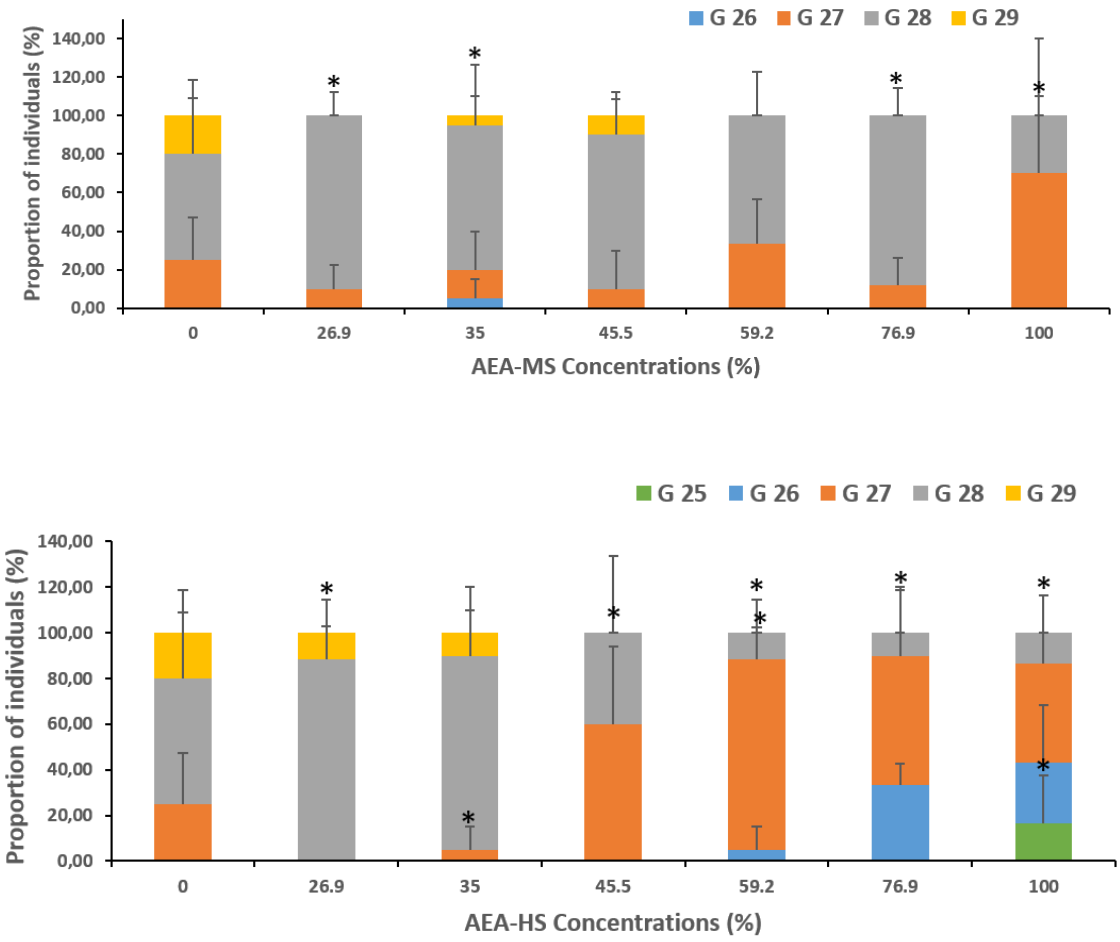

Figure 15: *Average proportion (%) of tadpoles of* Pelophylax perezi *at Gosner developmental stages G 25, G 26, G 27, G 28 and G 29, after being exposed, for 14 days, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (AEA-MS; up) or high (AEA- HS; down) severity. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.05).*

The tail length of tadpoles exposed to 59.2, 76.9 and 100 % of AEA-MS was significantly decreased, while a significant reduction on the body and total length was observed at concentrations ≥ 59.2 % ($Q \leq 4.382$; $p < 0.05$; Figure 16). The AEA-HS only caused a significant reduction on tail, body and total length at concentrations ≥ 59.2 % ($q' \leq 5.174$; $p \leq 0.011$; Figure 16).

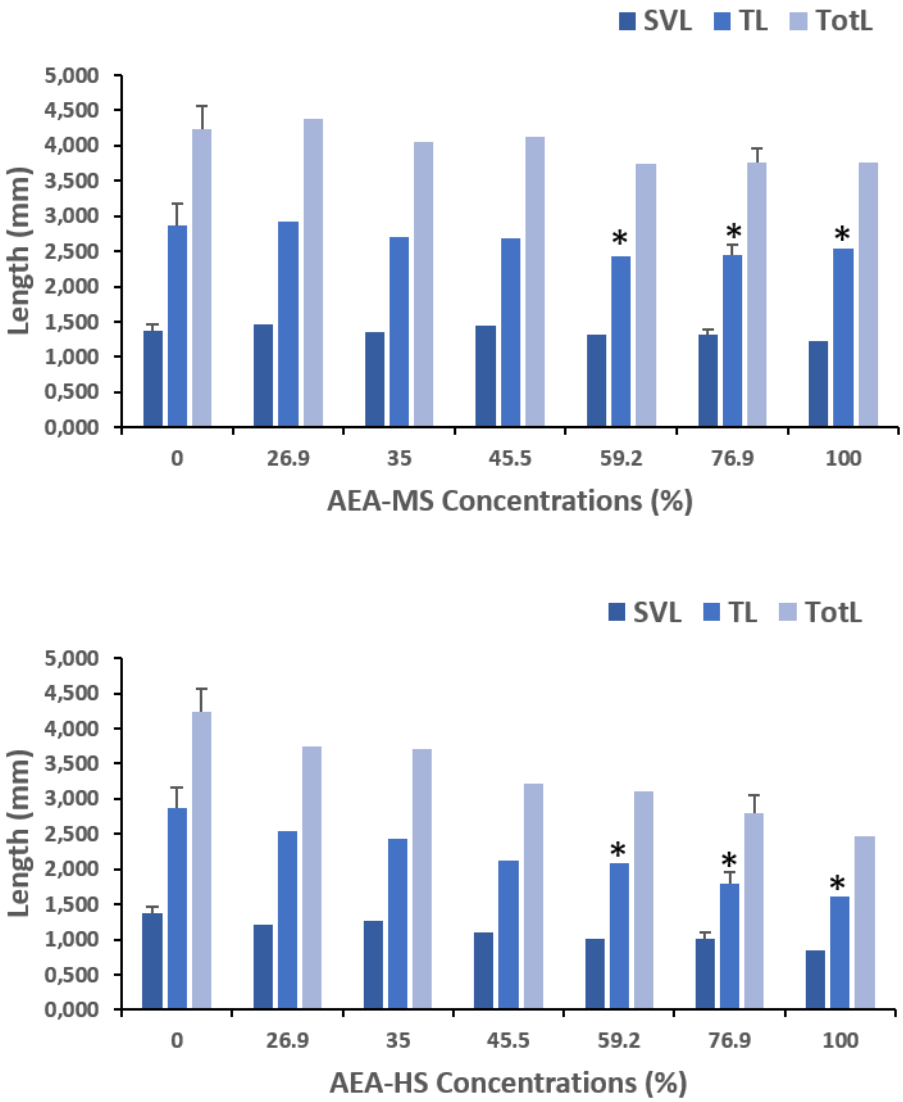

Figure 16: *Average length of snout-to-vent (SVL), tail (TL) and total (TotL) of tadpoles of* Pelophylax perezi, *after being exposed, for 14 days, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (AEA-MS; up) or high (AEA-HS; down) severity. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.05).*

The body weight of tadpoles was significantly increased (in 30 %) after being exposure to 26.9 % of AEA-MS, and a significantly reduction after exposure to concentrations ≥ 59.2 % of AEA-HS (in more than 27 %) (q' ≤ 7.213; p ≤ 0.004; Figure 17).

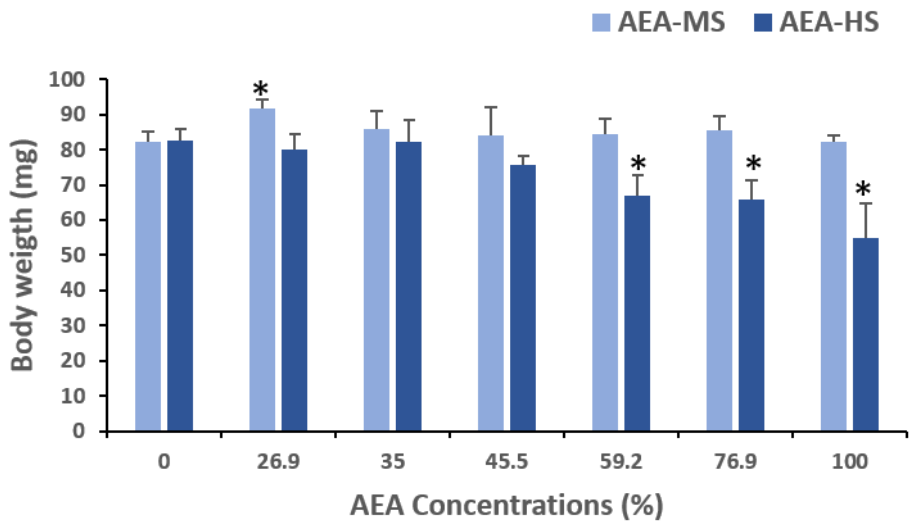

Figure 17: *Average body weight (mg) of tadpoles of* Pelophylax perezi, *after being exposed, for 14 days, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (MS) or high (HS) severity. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.05).*

3.3.2.1 Biochemical marker analyses

For lipid peroxidation, the *Pelophylax perezi tadpoles* only revealed significant effects on AEA-HS, for a concentration of 26.9 % (Figure 18a). Significant alterations were observed for CAT activity (Figure 18b) at 26.9 % AEA-MS concentration (F= 3.093; p= 0.0188) and at 26.9 %, 35 %, 45.5 % and 59.2 % AEA-HS concentrations (F= 5.532; p= 0.0007) with an induction in activity. Regarding the activity of total glutathione (Figure 18c), no significant effects were observed, despite its slight inhibition for AEA-MS. GST activity was also increased on tadpole exposed to AEA-MS, while in AEA-HS there was a non-linear response pattern with a tendency for decreasing GST activity after 76.9 % ash dilution (Figure 18d). The AChE activity of *Pelophylax perezi* exposed to AEA-MS and AEA-HS was inhibited, being registered a significant difference in 100 % ash eluate for both type of ashes (F= 4.850; p= 0.0004; F= 3.379; p= 0.0129; respectively, Figure 18e), when compared to the control treatment. A linear response was obtained for ETS activity levels (Figure 18f), lipids

content (Figure 18g) and carbohydrates (Figure 18h) for both types of ashes. Ash concentrations at concentration of 100 % to AEA-MS ($F= 5.401$: $p= 0.0008$), and 76.9 and 100 % to AEA-HS ($F= 7.478$; $p< 0.0001$) significantly decreased the protein content (Figure 18i).

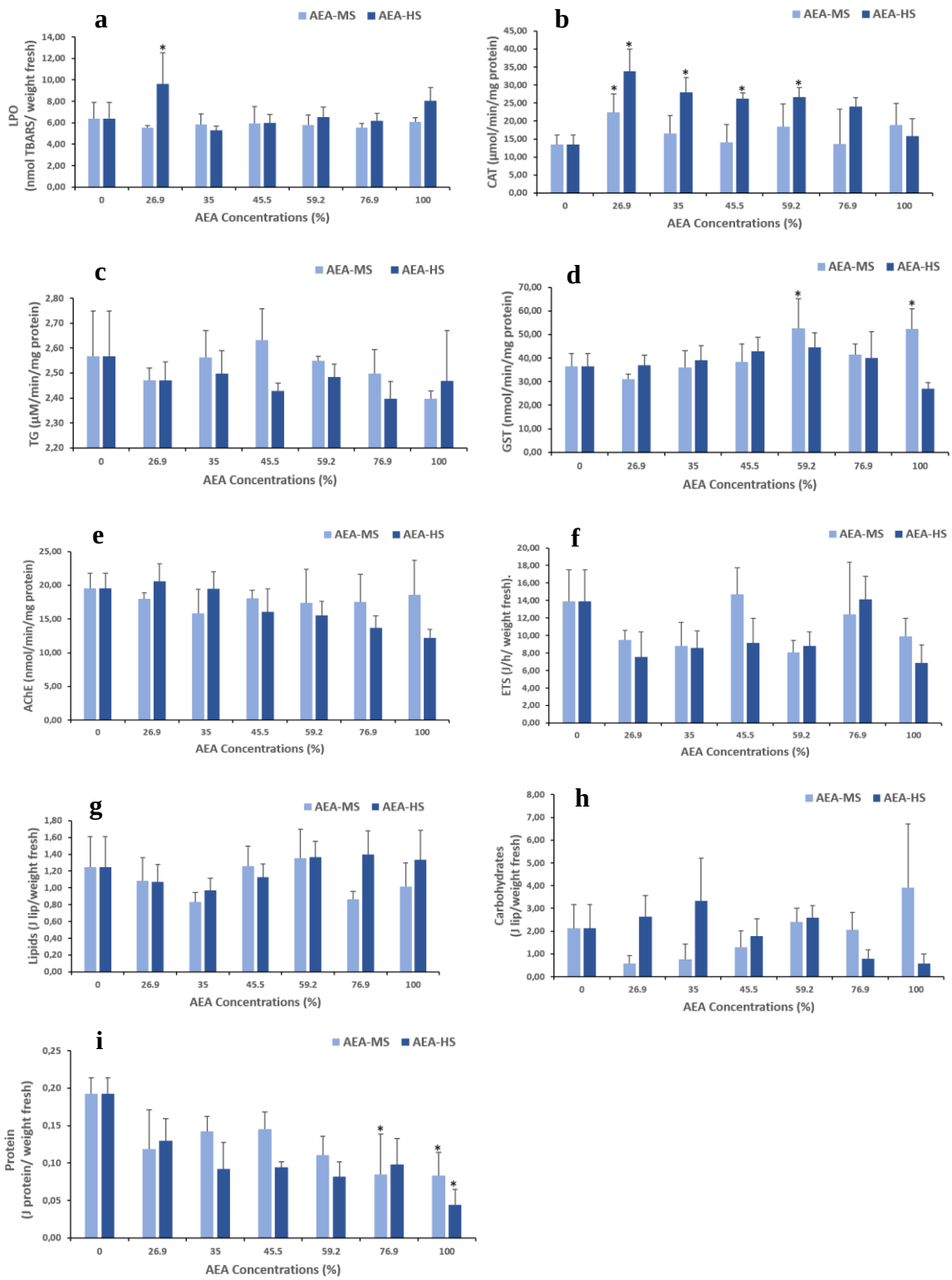

Figure 18: *Sub-individual effects in the tadpoles of* Pelophylax perezi, *after being exposed, for 14 days, to several concentrations of aqueous extracts of ashes (AEA) obtained from wildfires of moderate (MS) or high (HS) severity.* ***a)*** *LPO= lipid peroxidation;* ***b)*** *CAT= catalase;* ***c)*** *TG= total glutathione;* ***d)*** *GST= glutathione S-transferase;* ***e)*** *AChE= acetylcholinesterase;* ***f)*** *ETS= electron transport system;* ***g)*** *Lipids;* ***h)*** *Carbohydrates;* ***i)*** *Protein activity. All values are presented as means ± SD. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.05).*

3.4 Discussion

Wildfires can be considered as one of the biggest sources of ash contamination in aquatic systems. These ashes are constituted by many metals that may be released into the superficial water, as they enter freshwater ecosystems, changing its physicochemical parameters (Campos *et al.,* 2012, 2016; Silva *et al.,* 2015). The chemical analysis performed to AEA of MS and HS in chapter II clearly demonstrate the release of several elements into the test media (artificial surrogate of superficial freshwater), corroborating that ashes are a source of chemical substances to superficial waters.

The physico-chemical parameters measured in the MS and HS AEA of this work, revealed high pH values (within the alkaline range: 9.7 to 10.2). Several authors have studied the influence of ashes on water pH, but there is still some inconsistency between the studies. While Hall & Lombardozzi (2008) reported that the pH of running waters was not significantly affected by fire ashes; Bodí *et al.,* (2014) and Neary *et al.,* (2005) noticed an increment of pH following overland ash flow immediately after a wildfire. Furthermore, for ashes originated from wildfires of conifer forests, Goforth *et al.,* (2005) reported values of pH of 12 and 13, respectively, which is in line with the results obtained in the present work (Annex II). It was also observed that the pH of AEA-HS was higher than that of AEA-MS. These results match the knowledge available in the literature. Pereira *et al.,* (2012) compared chemical composition of ashes originated from wildfires of different severities and reported that pH values were higher in ashes originated from high severity wildfires (approx. 8.4). Such increases in pH could be related with higher contents in calcium carbonate, as the concentration of this compound is also positively associated with the severity of wildfires (Pereira *et al.,* 2012). Concomitantly to the pH increase, it was also observed an increase in conductivity of AEA relatively to the test media, which could be due to the release of several inorganic elements into the FETAX medium (27 and 41 mgL^{-1}, respectively; please see results in Chapter II). As with the pH values, the conductivity of the two AEA also increased due to the solubilisation of major elements contained in ashes, as has been reported in other works (Campos *et al.,* 2012, 2016; Mansilha *et al.,* 2019; Silva *et al.,* 2015). The conductivity of MS was slightly lower than that of HS, though most of the analysed elements were present at higher concentrations in MS AEA (please see Chapter

II). Such higher conductivity of HS could be due to the presence of other elements that were not here analysed, as for example Mg^{+} and K^{+} that are elements commonly appearing at high levels in ashes (Liodakis *et al.,* 2009; Pereira *et al.,* 2012; Silva *et al.,* 2015).

The performed ecotoxicity assays revealed that the two types of ashes exerted both lethal and sublethal effects on tadpoles of *P. perezi*. The high pH values as well as the presence of several inorganic element were most probably responsible for the observed effects. Aquatic life stages of some amphibian species were reported to be sensitive to high pH. Wijethunga *et al.,* (2015) observed a significant decrease in survival and hatching rate of embryos of *Rhinella marina* exposed to pH equal or above 8.5. Adding to this, Fominykh (2008) observed that prolonged exposure (> 10 days) to pH values above 9.0 in larvae of *Rana temporaria* (close species to *P. perezi*), *Lissotriton vulgaris* and *Salamandrella keyserlingii* compromised their survival. As well, the adverse effect of inorganic elements on amphibians have been demonstrated in several works. Specifically, for P. perezi tadpoles, Santos *et al.,* (2013) computed an LC_{20}, 96 h of 0.5 (0.29–0.63) Cu mg L^{-1}, which is a value close to the copper levels quantified in AEA MS (0.294 mg L^{-1}; Chapter II) and HS (0.259 mg L^{-1}; Chapter II). In tadpoles of another species of the Genera Pelophylax (*P. nigormaculata*), Huang *et al.,* (2014) showed a significant effect of lead in the body length and time to reach metamorphosis at concentrations equal or above 40 µg/L (below the concentrations found in AEA of MS and HS). In the present work, delayed developmental stage and body lengths, of *P. perezi* tadpoles, were the most sensitive endpoints for both MS and HS. Though, it is recognised that the observed effects result from the exposure to a mixture of chemical substances, it is hypothesised that Pb may play an important role on these effects as it is known to retard growth in amphibians by interfering with thyroxin, and the synthesis of new tissues, among others (Carey & Bryant , 1995). The smaller body length and delayed development may seriously affect the fitness of *P. perezi* tadpoles. By reaching later the metamorphosis, they experience a higher cumulative risk of death as these vulnerable stages will be exposed for a longer period to the chemical contamination. Although some of the metals present in AEA are known to cause mutagenic effects (e.g. Pb, Cd), no malformations were registered in tadpoles, this could be due to the fact that

exposure occurred after the completion of embryonic development or to concentrations of metals that are below the levels capable of inducing such effects.

At the biochemical level, effects were observed in tadpoles exposed to AEA-HS, an increase in catalase was observed in all concentrations except the highest (100 %); in tadpoles exposed to AEA-MS, where an inhibition in TG activity was noticed; an induction in GST activity for AEA-MS, and an inhibition in protein activity for both types of ashes. This result is not surprising due to the presence of some metallic species such as As, Cd, Pb, Hg, Cr, Ni, Mn and Fe that are known to increase the production of reactive oxygen species (ROS) (Jadhav *et al.,* 2007). As a protective enzyme for the production of reactive species, catalase is induced to cope with the increased ROS production during oxidative stress.

To our knowledge this (jointly with chapter II) is the first study demonstrating the ecotoxicity of aqueous extracts of wildfire ashes to aquatic life stages of amphibians. Nevertheless, some works have been published on the effects of AEA to freshwater organisms, Silva *et al.,* (2015, 2016) reported EC_{20} (of an AEA obtained with a 1:4 (v/v) ratio) of 46 % for *L. minor* and > 100 % for *D. magna*. Though these AEA resulted from different wildfire ashes, comparing the results with the ones here obtained it is suggested that tadpoles are more sensitive to this type of contamination. But, further comparative studies should be performed in order to understand if invertebrates may be used as adequate surrogate for the risk assessment of wildfire ashes on aquatic life stages of amphibians.

Overall, the present work provides a better understanding on the adverse effects that AEA from MS and HS wildfires pose to larvae of *Pelophylax perezi*. These results gain further relevance as this species is autochthonous of a region that is considered a hotspot for wildfires.

Chapter IV

Discussion

and Final Conclusions

Chapter IV- Discussion and Final Conclusions

Wildfires are considered a major cause of disturbances in freshwater ecosystems due to the input of ashes by overland flow. Besides the increase in particles loading and therefore in turbidity, ashes contain several toxic compounds (e.g. metals and PAHs), which impair the biota of aquatic ecosystems (Campos *et al.,* 2012, 2016; Silva *et al.,* 2015, 2016). Fire severity influences the degree in which an ecosystem is affected, as it will involve different temperatures, ashes pH, loading of chemical substances entering the aquatic system (Certini, 2005; Keeley, 2009; Neary *et al.,* 1999; Rugenski & Minshall, 2014; Úbeda *et al.,* 2009; Verkaik *et al.,* 2015). Studies assessing the wildfire ashes pH, described inconsistent changes in pH as a result of wildfires: while Hall & Lombardozzi (2008) reported that pH of freshwater systems was not affected by runoff ashes, Neary *et al.,* (2005) reported an increased in pH levels following overland ash flow immediately after a wildfire. The increase in ashes concentration caused the pH to increase proportionally, for both MS and HS (Annex I and II). Even comparing the measurements after 48 h of exposure (which coincided with the change of medium) it was found that a decrease in pH values occurs. For *Pelophylax perezi* tadpoles exposure the difference at the beginning and after 48 h medium solution was high when compared to *X. laevis* tadpoles exposure for the same period exposure. Studies by Bodí *et al.,* (2014) and Santín *et al.,* (2015) found that water quality was affected by the entry of particles (from the vegetation and soil organic matter burned) contributing to an increase in TSS, with a consequent increase in turbidity from suspended solids. According to the results obtained in the present work, TSS levels were higher for AEA-HS when compared to AEA-MS. These results agree with literature evidences (Santín *et al.,* 2015), as in the wildfires with higher severity the fuel load (i.e. dead and live available biomass) affected is greater, which may lead to a positive relationship between the severity of the forest fire and the total ash load observed. Wood & Armitage (1997) and Yu *et al.,* (2019) documented that increases in TSS levels in aquatic freshwater systems, through surface runoff of compounds derived from the combustion of biomass from wildfires, limit light penetration, the photosynthesis rate and consequently poorer growth rates of macrophytes. As expected, electrical conductivity also showed

higher values at the highest concentration (100 %) compared to the control (FETAX medium solution), essentially due to the solubilisation of major chemical elements contained in ashes (Pereira *et al.,* 2012; Ulery *et al.,* 1993). Hall & Lombardozzi (2008) and Mansilha *et al.,* (2019) evaluated and compared conductivity values in burnt streams to control streams, even one year after the wildfire, and the pre- and post-fire conditions, respectively, and both, reported an increase in electrical conductivity. The ash produced by wildfires is a complex matrix, which when reaching the water bodies by surface runoff results in an increase in organic and inorganic compounds, such as PAHs, metals and nutrients, such as phosphorus, nitrogen and carbon, changing the water chemistry (Bixby *et al.,* 2015; Bodí *et al.,* 2014; Francos *et al.,* 2017; Nunes *et al.,* 2017; Smith *et al.,* 2011). Chemical elements such as Cu, Mn and Zn plays a very important biological function role in animals, contrary to nonessential metals, like Cd, Pb and Ni that even at low concentrations can be toxic to organisms (Ré *et al.,* 2020a, b). This study identified Cu, Mn, Ni, V and Zn as the principal elements in ashes from a pine stand burnt at moderate severity (MS); and Cu, Mn, Pb, V and Zn as the main elements in ashes from a pine stand burnt at high severity (HS). Regarding the aqueous extracts of ashes (AEAs) Cu, Mn, Ni, V and Zn were the chemical elements with the highest concentrations in the AEA-MS; and Cu, Mn, Pb and Zn in the AEA-HS. Some studies (Kristensen *et al.,* 2014; Odigie & Flegal, 2014) reported post-fire lead remobilization in ash during wildfires events, as like Mn that was reported as the element with highest remobilization in post-fire soil environment (Campos *et al.,* 2016; Costa *et al.,* 2014). According to Parra *et al.,* (1996), results for Mn concentration on soil after a wildfire in the Sierra de Gredos mountain in Spain, revealed a great increase of total Mn in post-fire soil samples when compared to the reference samples, essentially due to the accumulation in their leaves vegetation. Similarly, Costa *et al.,* (2014) after analysing the post-fire soil and ash samples collected 5 months after wildfire, verified an increase in Mn concentration in ash samples (compared to the underlying soil). Kabata-Pendias (2010) also found that the high concentration of Mn in ash was related with the high Mn concentration in the needles of the resinous species. In fact, our study burnt area was covered by resinous *P. pinaster* trees, and Mn was the chemical element present at highest concentration in both ash and AEA, for moderate and high severities. The Zn was the

second chemical element with the highest concentration for both AEA (MS and HS). Pereira & Úbeda (2010) reported in a study on a small burnt plot in Portugal, that ash contained high concentration of metals, such as Al, Mn, Fe and Zn; and that factors like plant species distribution and burning severity influences the metal presence. Odigie & Flegal (2014) and Plumlee *et al.,* (2007) observed that immediately after a wildfire Zn was the element with major concentration in ashes from 28 sites affected by wildfires in southern California during November 2007. In 1977, Auclair examined Cu, Zn and Mn concentration in wetland plant tissues and in the soils of Carex medow, in the Quebec Province of Canada and reported that these metals were significantly mobilized by burning (Auclair, 1977). Similarly, during the severe fire occurred in Southern California in 2003, the atmospheric deposition rates increased Zn concentrations by a factor of 6 when compared with the unburned areas, which could have impacted the soil and water resources through rain (Sabin *et al.,* 2005). According with our study, the high concentration of Zn is likely due to the easy absorption and accumulation of this metal by plants (Driscoll *et al.,* 2013; Obrist, 2007; Rutter *et al.,* 2011). Concerning Cu, as mentioned before is one of the most important metals intervening in biological functions. However, according to Abrantes *et al.,* (2017) and Plumlee *et al.,* (2007), the metallic element copper in certain concentrations becomes more toxic to biota, with a tendency for persistence and bioaccumulation in the food chain (Abrantes *et al.,* 2017; Campos *et al.,* 2012, 2016; Nunes *et al.,* 2017; Silva *et al.,* 2015). Campos *et al.,* (2016) reported Cu as one of the compounds at highest concentration in ash burnt soil post-fire, which is in agreement with the results obtained in the present work, as Cu was present in both type of ashes and AEA. The remaining elements (e.g. Ni, Pb and V) were observed at lower concentrations, however even at such concentrations they can be toxic to the biota (Abrantes *et al.,* 2017; Napierska *et al.,* 2018). As shown in Table VIII, some metals are considered priority pollutants according to the United States Environmental Protection Agency (US EPA) and the European Community (EC). Maximum concentrations of metals such as As, Cd, Cr, Cu, Pb, Ni and Zn are shown in Table VIII, due to the fact that they are one of the most worrying metals at an environmental level with high environmental toxicity.

Table VIII: *Ash components and their respective environmental quality standards (EQS) for aquatic life biota. These concentrations are according to (USEPA, 2017): National Recommended Water Quality Criteria - Aquatic Life Criteria. EQS expressed as Criterion maximum concentration (CMC).*

	As	**Cd**	**Cr**	**Cu**	**Pb**	**Ni**	**Zn**
EQS (μL^{-1})	340	1.8	570	-	65	70	120

Initial aquatic stages of amphibians are very sensitive to contamination (Kerby *et al.,* 2010; Todd *et al.,* 2011) driven by wildfires ashes and, for this reason, our work aimed to assess the influence of ash toxicity according to the severity of forest fires in the development of embryos and tadpoles of two species of anurans: *Xenopus laevis* and *Pelophylax perezi*.

Embryonic and larval amphibian survival, for both species, was affected by both types of ashes (AEA- MS and AEA-HS). Analysing and comparing the exposure of *X. laevis* embryos and tadpoles to the two AEA (MS and HS), we verified that there was a higher mortality in tadpoles of this species for both severities when compared to embryos. Differences in sensitivity of distinct life stages to pollutants have been reported often and is attributed to the jelly coat matrix that involves embryos and to the complete tissue and organ differentiation in larvae and tadpoles (Berrill *et al.,* 1998; Pauli *et al.,* 1999). However, despite the degree of protection afforded by the jelly coat, some portions of the chemical may become available and may influence growth development (Edginton *et al.,* 2007; Marques *et al.,* 2008) and in this way, the jelly coat can react with available chemical elements and become toxic (Marquis *et al.,* 2006; Räsänen *et al.,* 2003). In tadpoles, mouth is functional (promoting exposure through ingestion of contaminated food items), gills are developed and do not present a jelly coat, characteristics that favours an higher contact with water contaminants (Mitchell *et al.,* 2005). Thus, according to our results, for 96 h embryos exposure for AEA-MS and AEA-HS the stage of development observed at the end of the assay was similar for most individuals (NF 46); contrary to the exposed to tadpoles to AEA-MS and AEA-HS in which the developmental stages were not similar among individuals, with lower stage of development being verified in the AEA of high severity. *Pelophylax perezi* tadpoles exposure indicate that mortality rate was significantly affected by the 14 days of exposure to the two AEA severity wildfires. Several studies indicate that

tadpoles exposed to concentrations of metals, separately or together, affect their survival and growth. Lefcort *et al., (*1998) proposed two possible consequences of metals on tadpoles. First, metals such as zinc are toxic to anuran larvae and cause tadpoles dead. According with our chemical analyses, zinc was the third chemical element with a highest concentration, 1.88 mg L^{-1} and 1.32 mg L^{-1} for AEA-MS and AEA-HS, respectively. And for that reason, Zn may have contributed to the 30 % mean of mortality present in both type of ashes. Secondly, metals can temporarily inhibit larval development. Wei *et al.,* (2015) studied the toxicity of *R. zhenhaiensis* tadpoles to metallic elements Cu^{2+}, Pb^{2+} and Zn^{2+} in a chronic study over a period of 18 days, with exposure to 1/10 LC_{50} concentration of metal ions, and found that the metallic elements significantly affected the growth of tadpoles compared to the control, after period exposure. Growth retardation is a very common strategy used when organisms are exposed to contaminants, namely metals (Haywood *et al.,* 2004). Similar results were reported for body length, where it was found that for embryos exposed to AEA there was a slight decrease in this endpoint compared to the control, while for tadpoles exposed to AEA a significant decrease in body length was observed relatively to the control, for both type of ashes. Malformation were also observed (bent and notochord curvature, and edemas) and this sub-lethal effect may also contribute to the lower reaction of organisms to mechanical stimulations (Marques *et al.,* 2008). According to our results for stage of development, a delay in development was also observed for *P. perezi* tadpoles. This may have significant effects in the fitness of organisms, as they will take a longer time to reach metamorphosis and consequently reproduction.

Regarding biochemical effects on *Xenopus laevis* embryos and tadpoles exposed to AEA-MS similar responses for CAT and ETS activity were observed. The AChE activity showed contradictory responses: an inhibition of its activity in the embryos exposure, and an induction in the tadpoles exposure. Responses in the AChE activity can be explained by the differential exposure to chemical in the two life stages (Pradhan *et al.,* 2020). Concerning proteins, an inhibition was observed for embryos, whereas for tadpoles a linear response was verified. Biomarkers response for embryos and tadpoles exposed to AEA-HS revealed an AChE activity linear for both type of assays. CAT activity was induced in 96 h embryo exposure and for tadpoles a linear response was obtained. Paulino *et al.,* (2012)

proposed that the increased activity of other antioxidant enzymes, such as glutathione peroxidase (GPx) or other forms of antioxidant defence, could explain the linear response of CAT activity to AEA-HS. The CAT enzyme is a protective enzyme that is induced when an increase in the production of ROS occurs during oxidative stress (Napierska *et al.,* 2018; Nunes *et al.,* 2014). Despite not being analysed, the chemical elements Fe and Mn, among others, are active redox metals involved in the cellular oxidative state. The element Fe plays an important role in oxidative stress through hydroxyl radicals in the reactions of Fenton and Haber-Weiss; on the other hand, Mn plays its role in cellular adaptation to oxidative stress. Veronez *et al.,* (2016) demonstrated that chronic exposure to Fe and Mn concentrations, alone or in combination, causes an increase in oxidative stress markers in *L. catesbeianus* tadpoles, accompanied by an increase in ROS production. In fact, Mn was the element that obtained the highest concentration for both types of AEA.

On *P. perezi* tadpoles biochemical analyses, similar responses was obtained for the major biomarkers analysed, namely CAT, for both type of AEA samples. The response of AChE activity was the opposite for both types of severity: for MS there was an inhibition, whereas an induction was observed for HS. AChE activity was inhibited due the present of some metals such as As, Cd, Cr, Mn, Ni and Pb, that can increase the production of ROS (Jadhav *et al.,* 2007). This response of AChE activity of tadpoles exposed to aqueous extracts of ash from moderate severity coincides with the induction of CAT activity, which consequently is increased when production of ROS are induced with oxidative stress (Hansen *et al.,* 2007; Napierska *et al.,* 2018; Nunes *et al.,* 2014; Radwan *et al.,* 2010). Some studies (Kim & Kang, 2015) reported a significant reduction in AChE activity in fish, when exposed to various toxic substances, like metals. However, little is known about the action of some metals or their action effects when mixture on AChE in amphibians Cholinesterase activity as potential biomarkers: characterization in bullfrog tadpole's brian after exposure to metals (Pradhan *et al.,* 2020). ETS activity also presented a similar non-linear response for both type of ashes, that is, in lower treatments an induction in tadpoles activity was observed, with subsequent stabilization in intermediate treatments and an inhibition in final treatments, associated with increased ROS production. For proteins, in both ashes, an inhibition as a response was showed in the tadpoles exposure.

In conclusion, the obtained results showed that the entrance of wildfire ashes in freshwater ecosystems causes changes in their physical and chemical parameters (e.g., pH, conductivity, metal concentrations) and that these changes depend on the severity of the wildfires. Furthermore, it is concluded that the ashes and the chemicals that are released into the surface water are toxic to early life stages of amphibians. The level of the toxicity depends on the severity of the wildfire, on amphibian species and on developmental life stage. These results suggest that extrapolation of the effects of wildfire ashes among amphibian species and early life stages may be associated with a high uncertainty and, therefore, an ecotoxicity dataset regarding the effects of wildfire ashes to aquatic life stages of several amphibian's species is needed to promote an accurate risk assessment for this taxon. Furthermore, it is suggested that future studies should include ecotoxicity assessment under more realistic exposure scenarios. Namely, (i) by performing in situ exposures as they include natural variations in daily temperature and other physical, chemical and biological parameters, that under laboratorial experiments are set to optimal conditions; (ii) assessing the capacity of tadpoles to escape areas impacted with wildfire ashes. This is a relevant scenario to be explored since in lotic systems, tadpoles may move upstream the ashes affected area to avoid the contaminated environment; (iii) assess long term-effects. Since the results here obtained revealed that exposure to AEA delays development, it is of most relevance to understand how such delays may impair metamorphosis in the organisms.

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***Table IX**: Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (07/02/2020) and after changing medium solution (48 h) (09/02/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.6	557	608	8.8	8.8	20
26.9 %	9.2	7.8	638	768	8.8	8.7	20
35 %	9.3	7.8	642	785	8.8	8.7	20
45.5 %	9.4	7.9	654	781	8.7	8.7	20
59.2 %	9.5	7.9	680	831	8.7	8.6	20
76.9 %	9.5	8.0	703	845	8.7	8.7	20
100 %	9.5	8.0	755	956	8.7	8.6	20

***Table X:** Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (09/02/2020) and after changing medium solution (48 h) (11/02/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.7	560	592	8.7	8.8	20
26.9 %	9.3	7.8	620	778	8.7	8.7	20
35 %	9.3	7.9	641	770	8.7	8.7	20
45.5 %	9.4	7.9	668	815	8.7	8.7	20
59.2 %	9.5	8.1	700	865	8.7	8.7	20
76.9 %	9.6	8.1	735	906	8.8	8.7	20
100 %	9.7	8.2	794	1031	8.7	8.6	20

Table XI: *Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (11/02/2020) and after changing medium solution (48 h) (13/02/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	565	641	8.7	8.5	19
26.9 %	9.3	7.8	647	812	8.7	8.5	19
35 %	9.3	8.2	649	781	8.7	8.6	19
45.5 %	9.5	8.2	666	803	8.7	8.4	19
59.2 %	9.6	8.3	667	853	8.8	8.5	19
76.9 %	9.6	8.4	717	887	8.7	8.5	19
100 %	9.7	8.4	758	976	8.7	8.6	19

Table XII*: Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (13/02/2020) and after changing medium solution (48 h) (16/02/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.7	570	680	8.7	8.7	20
26.9 %	9.3	8.1	639	783	8.7	8.7	20
35 %	9.3	8.1	648	803	8.7	8.7	20
45.5 %	9.4	8.2	665	862	8.7	8.6	20
59.2 %	9.5	8.3	671	885	8.6	8.6	20
76.9 %	9.6	8.4	714	925	8.6	8.6	20
100 %	9.6	8.4	762	977	8.6	8.6	20

***Table XIII**: Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (16/02/2020) and after changing medium solution (48 h) (18/02/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	572	623	8.8	8.8	19
26.9 %	9.2	8.0	641	768	8.8	8.7	19
35 %	9.3	8.0	667	821	8.8	8.7	19
45.5 %	9.4	8.1	675	834	8.8	8.7	19
59.2 %	9.5	8.2	700	879	8.8	8.6	19
76.9 %	9.6	8.3	724	906	8.8	8.7	19
100 %	9.6	8.3	759	967	8.7	8.6	19

***Table XIV:** Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (18/02/2020) and after changing medium solution (48 h) (20/02/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.8	7.8	564	608	8.8	8.6	20
26.9 %	9.2	8.1	596	741	8.8	8.7	20
35 %	9.3	8.1	611	788	8.8	8.7	20
45.5 %	9.4	8.1	629	808	8.8	8.6	20
59.2 %	9.5	8.2	648	816	8.8	8.6	20
76.9 %	9.6	8.3	783	874	8.8	8.7	20
100 %	9.6	8.4	733	966	8.8	8.6	20

Table XV: *Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (20/02/2020) and after changing medium solution (48 h) (21/02/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.8	7.8	570	623	8.8	8.7	20
26.9 %	9.2	8.0	614	737	8.7	8.6	20
35 %	9.3	8.0	619	735	8.7	8.7	20
45.5 %	9.4	8.0	636	789	8.7	8.7	20
59.2 %	9.4	8.1	662	832	8.7	8.7	20
76.9 %	9.6	8.1	692	895	8.7	8.6	20
100 %	9.7	8.2	721	945	8.7	8.6	20

Table XVI: *Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (14/01/2020) and after changing medium solution (48 h) (16/01/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.7	584	622	8.8	8.8	19
26.9 %	9.8	8.8	710	869	8.8	8.7	19
35 %	9.9	8.8	734	933	8.8	8.7	19
45.5 %	10.0	8.9	778	997	8.8	8.7	19
59.2 %	10.1	9.0	863	1010	8.8	8.7	19
76.9 %	10.2	9.1	942	1078	8.8	8.7	19
100 %	10.3	9.1	986	1104	8.7	8.6	19

Table XVII*: Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (16/01/2020) and after changing medium solution (48 h) (18/01/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.7	579	625	8.8	8.7	20
26.9 %	9.6	8.8	709	880	8.8	8.7	20
35 %	9.8	8.8	739	929	8.8	8.7	20
45.5 %	9.9	8.9	780	978	8.8	8.7	20
59.2 %	10.0	8.8	833	1001	8.8	8.7	20
76.9 %	10.1	9.0	927	1061	8.8	8.7	20
100 %	10.2	9.1	991	1144	8.8	8.7	20

Table XVIII: *Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (18/01/2020) and after changing medium solution (48 h) (20/01/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.7	574	604	8.7	8.7	20
26.9 %	9.7	8.5	720	866	8.6	8.7	20
35 %	9.7	8.4	729	885	8.6	8.6	20
45.5 %	9.9	8.7	755	923	8.7	8.6	20
59.2 %	10.1	8.8	884	986	8.7	8.6	20
76.9 %	10.1	8.9	898	1007	8.7	8.6	20
100 %	10.2	8.9	997	1039	8.7	8.6	20

Table XIX: *Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (20/01/2020) and after changing medium solution (48 h) (22/01/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.8	7.7	559	615	8.8	8.8	19
26.9 %	9.7	8.2	710	869	8.7	8.7	19
35 %	9.8	8.2	732	905	8.7	8.7	19
45.5 %	9.8	8.3	769	935	8.8	8.7	19
59.2 %	10.0	8.5	833	1016	8.8	8.6	19
76.9 %	10.1	8.5	909	1040	8.8	8.7	19
100 %	10.2	8.7	1005	1216	8.7	8.6	19

***Table XX**: Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (22/01/2020) and after changing medium solution (48 h) (24/01/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.7	552	615	8.8	8.8	19
26.9 %	9.7	8.2	710	899	8.7	8.7	19
35 %	9.8	8.2	732	945	8.7	8.7	19
45.5 %	9.9	8.3	769	935	8.8	8.7	19
59.2 %	10.0	8.4	833	1066	8.8	8.6	19
76.9 %	10.1	8.4	909	1159	8.8	8.7	19
100 %	10.2	8.6	1005	1225	8.7	8.6	19

***Table XXI:** Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (24/01/2020) and after changing medium solution (48 h) (26/01/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.7	702	658	8.7	8.7	20
26.9 %	9.7	8.2	727	906	8.7	8.7	20
35 %	9.7	8.3	772	940	8.7	8.7	20
45.5 %	9.9	8.3	798	1011	8.6	8.7	20
59.2 %	10.1	8.4	824	1037	8.6	8.7	20
76.9 %	10.2	8.5	888	1098	8.6	8.6	20
100 %	10.2	8.7	990	1209	8.7	8.6	20

Table XXII: *Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (26/01/2020) and after changing medium solution (48 h) (28/01/2020) of* Xenopus laevis *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	589	598	8.7	8.7	20
26.9 %	9.7	8.2	689	858	8.7	8.7	20
35 %	9.8	8.2	737	932	8.7	8.7	20
45.5 %	9.8	8.3	758	947	8.7	8.7	20
59.2 %	10.1	8.3	813	1024	8.7	8.7	20
76.9 %	10.1	8.4	872	1088	8.7	8.7	20
100 %	10.2	8.7	957	1163	8.7	8.6	20

Table XXIII: *Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (05/06/2020) and after changing medium solution (48 h) (07/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	564	543	8.8	8.8	20
26.9 %	9.3	8.2	620	770	8.8	8.7	20
35 %	9.3	8.2	641	781	8.8	8.7	20
45.5 %	9.4	8.2	655	820	8.8	8.7	20
59.2 %	9.5	8.3	689	858	8.8	8.7	20
76.9 %	9.7	8.3	707	894	8.8	8.7	20
100 %	9.8	8.3	760	975	8.8	8.6	20

Table XXIV: *Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (07/06/2020) and after changing medium solution (48 h) (09/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	558	539	8.8	8.8	20
26.9 %	9.2	8.1	624	775	8.7	8.7	20
35 %	9.3	8.2	638	780	8.7	8.7	20
45.5 %	9.4	8.2	660	818	8.8	8.7	20
59.2 %	9.5	8.2	684	855	8.8	8.6	20
76.9 %	9.7	8.3	710	890	8.8	8.7	20
100 %	9.7	8.3	755	982	8.7	8.6	20

Table XXV: *Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (09/06/2020) and after changing medium solution (48 h) (11/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.9	560	545	8.8	8.8	19
26.9 %	9.3	8.2	625	776	8.7	8.7	19
35 %	9.3	8.2	637	783	8.7	8.7	19
45.5 %	9.4	8.2	651	824	8.8	8.7	19
59.2 %	9.5	8.3	679	851	8.8	8.6	19
76.9 %	9.6	8.3	700	892	8.8	8.7	19
100 %	9.7	8.4	749	978	8.7	8.6	19

Table XXVI*: Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (11/06/2020) and after changing medium solution (48 h) (13/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	568	545	8.8	8.8	20
26.9 %	9.2	8.2	629	779	8.7	8.7	20
35 %	9.2	8.2	633	785	8.7	8.7	20
45.5 %	9.4	8.3	650	824	8.8	8.7	20
59.2 %	9.5	8.3	679	860	8.8	8.6	20
76.9 %	9.7	8.3	702	894	8.8	8.7	20
100 %	9.7	8.3	752	981	8.7	8.6	20

Table XXVII: *Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (13/06/2020) and after changing medium solution (48 h) (15/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	560	546	8.8	8.8	20
26.9 %	9.2	8.0	621	780	8.7	8.7	20
35 %	9.3	8.1	637	783	8.7	8.7	20
45.5 %	9.5	8.2	655	822	8.8	8.7	20
59.2 %	9.5	8.2	680	856	8.8	8.6	20
76.9 %	9.6	8.3	701	896	8.8	8.7	20
100 %	9.8	8.3	751	983	8.7	8.6	20

Table XXVIII: *Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (15/06/2020) and after changing medium solution (48 h) (17/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	568	540	8.8	8.8	19
26.9 %	9.2	8.1	621	777	8.7	8.7	19
35 %	9.3	8.1	632	787	8.7	8.7	19
45.5 %	9.4	8.2	656	823	8.8	8.7	19
59.2 %	9.5	8.3	689	851	8.8	8.6	19
76.9 %	9.7	8.3	714	895	8.8	8.7	19
100 %	9.8	8.4	762	980	8.7	8.6	19

Table XXIX: *Physico-chemical parameters measured in the samples of AEA from the moderate severity wildfire, at beginning (0 h) (17/06/2020) and after changing medium solution (48 h) (19/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (MS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.9	566	543	8.8	8.8	20
26.9 %	9.2	8.1	629	772	8.7	8.7	20
35 %	9.2	8.1	631	780	8.7	8.7	20
45.5 %	9.4	8.2	656	824	8.8	8.7	20
59.2 %	9.6	8.2	681	861	8.8	8.6	20
76.9 %	9.6	8.3	700	903	8.8	8.7	20
100 %	9.7	8.3	758	989	8.7	8.6	20

Table XXX: *Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (05/06/2020) and after changing medium solution (48 h) (07/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.7	598	619	8.7	8.96	20
26.9 %	9.6	8.3	701	810	8.7	8.7	20
35 %	9.8	8.7	735	880	8.7	8.7	20
45.5 %	9.9	8.7	778	907	8.7	8.7	20
59.2 %	10.0	8.8	815	974	8.7	8.6	20
76.9 %	10.1	8.9	896	998	8.7	8.6	20
100 %	10.2	8.9	988	1088	8.7	8.6	20

Table XXXI: *Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (07/06/2020) and after changing medium solution (48 h) (09/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	599	620	8.8	8.8	20
26.9 %	9.7	8.3	697	808	8.7	8.7	20
35 %	9.8	8.6	730	881	8.7	8.7	20
45.5 %	9.9	8.7	777	905	8.8	8.7	20
59.2 %	10.0	8.8	818	976	8.8	8.7	20
76.9 %	10.1	8.9	890	999	8.7	8.7	20
100 %	10.2	8.9	990	1095	8.7	8.7	20

Table XXXII: *Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (09/06/2020) and after changing medium solution (48 h) (11/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.7	596	618	8.7	8.7	19
26.9 %	9.6	8.3	702	811	8.7	8.7	19
35 %	9.7	8.7	738	885	8.7	8.7	19
45.5 %	9.9	8.7	780	908	8.7	8.7	19
59.2 %	10.0	8.8	819	974	8.7	8.6	19
76.9 %	10.1	8.9	895	1000	8.7	8.7	19
100 %	10.2	8.9	991	1095	8.6	8.6	19

Table XXXIII: *Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (11/06/2020) and after changing medium solution (48 h) (13/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	600	621	8.8	8.8	20
26.9 %	9.7	8.3	699	815	8.7	8.7	20
35 %	9.7	8.6	736	884	8.7	8.7	20
45.5 %	9.8	8.7	775	909	8.8	8.8	20
59.2 %	10.1	8.8	820	977	8.8	8.6	20
76.9 %	10.1	8.8	900	998	8.8	8.7	20
100 %	10.2	89	989	1078	8.7	8.6	20

Table XXXIV: *Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (13/06/2020) and after changing medium solution (48 h) (15/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	599	622	8.8	8.7	19
26.9 %	9.7	8.3	703	809	8.7	8.7	19
35 %	9.9	8.7	733	886	8.7	8.7	19
45.5 %	9.9	8.7	789	913	8.8	8.7	19
59.2 %	10.0	8.7	815	975	8.8	8.7	19
76.9 %	10.1	8.8	886	990	8.7	8.7	19
100 %	10.2	8.9	991	1095	8.7	8.7	19

Table XXXV: *Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (15/06/2020) and after changing medium solution (48 h) (17/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.7	598	617	8.8	8.7	19
26.9 %	9.7	8.3	701	811	8.7	8.7	19
35 %	9.8	8.7	734	881	8.7	8.7	19
45.5 %	9.9	8.7	787	910	8.7	8.7	19
59.2 %	9.9	8.8	811	974	8.7	8.6	19
76.9 %	10.0	8.8	898	1001	8.7	8.6	19
100 %	10.1	8.9	990	1091	8.7	8.6	19

Table XXXVI: *Physico-chemical parameters measured in the samples of AEA from the high severity wildfire, at beginning (0 h) (17/06/2020) and after changing medium solution (48 h) (19/06/2020) of* Pelophylax perezi *tadpoles toxicity assay.*

Physico-chemical parameters (HS)							
Ash Concentrations (%)	**pH (0h)**	**pH (48h)**	**Conductivity (0h) (µS cm^{-1})**	**Conductivity (48h) (µS cm^{-1})**	**Dissolved oxygen (0h) (mg L^{-1})**	**Dissolved oxygen (48h) (mg L^{-1})**	**T (°C)**
0 %	7.9	7.8	599	619	8.8	8.8	20
26.9 %	9.7	8.3	698	813	8.8	8.7	20
35 %	9.9	8.7	731	888	8.8	8.7	20
45.5 %	9.9	8.8	783	912	8.8	8.7	20
59.2 %	10.1	8.8	819	984	8.7	8.6	20
76.9 %	10.1	8.8	894	990	8.7	8.7	20
100 %	10.2	8.9	989	1088	8.7	8.6	20