UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

The impact of wildfires on subterranean aquatic organisms

Maria Isabel Abreu Dias

Mestrado Biologia Humana e Ambiente

Dissertação orientada por:
Professora Doutora Ana Sofia Reboleira
Doutor Nelson Abrantes

2024

Acknowledgments

First, a special thank you to my advisor, Ana Sofia Reboleira, for her teachings and all the enthusiasm she gave me along the way, contributing to all the learning I will take with me for life. It was incredible to be part of this adventure and discover this invisible world, the subterranean world. I would also like to thank my supervisor, Nelson Abrantes, for his constant help and support in writing this dissertation and the University of Aveiro for this opportunity. Thank you for this great opportunity to contribute to the growth of Science!

I could not fail to thank Professor Carlos Gravato, who accompanied me tirelessly during most of this journey. I am entirely grateful for his immense support!
To my family, who was always ready to listen to all the adventurous episodes of trips to the caves at dinner time and for all the accompaniment.

To many friends! To Guadalupe, who always accompanied me and encouraged me always to give my best during this work. To Leonor, the faithful companion of (some) moments of frustration in the writing of the thesis, but many more of joy and laughter. Thank you for your support and for exchanging many messages: “Please, be proud of me: I wrote a paragraph today!”. To Carlota, who was an essential company in this work, even when we spent the weekend at college. To Tiago, who contributed with his support and reading of the 1500 versions of the thesis, and to all my other friends, I thank you immensely!

Finally, I could not fail to thank the professors who marked this journey from the first day I entered college and who contributed to my growth. To Professor Carlos da Câmara, thank you for the countless conversations about Science in the college corridors and all the teachings that helped shape this path. To Teacher Teresa Rebelo, who encouraged me from the first day of the second cycle and from whom I learned a lot! Thank you for your patience and perseverance in adapting me to this different chapter of my path and for the enthusiasm for Biology that you taught me.

I learned that Science requires a dose of curiosity and how beautiful it is to do it!

Resumo

É escasso o conhecimento que se tem sobre os ecossistemas subterrâneos, tal como sobre os organismos que neles habitam. Estes ecossistemas possuem condições distintas dos da superfície e providenciam diversos serviços ao ser humano e a toda a biodiversidade.

As grutas são estruturas geológicas compostas por cavidades naturais podendo ser formadas pela reação química entre calcário e água, ou de atividade vulcânica pela consolidação de material vulcânico. Albergam aquíferos e são influenciadas pela geologia do terreno, condições climatéricas, propriedades químicas da água e pelas interações biológicas que nelas existem. São caracterizadas por serem um habitat afótico, com valores de temperatura anuais quase constantes e próximos dos da temperatura média anual à superfície, de humidade relativa elevada e de reduzida disponibilidade de alimento. Como consequência da escuridão total, os ciclos circadianos são inexistentes, assim como a fotossíntese, verificando-se uma grande dependência da infiltração de carbono oriundo da superfície.

Os ecossistemas subterrâneos albergam grupos faunísticos muito especializados resultantes das pressões seletivas impostas por este ambiente que moldaram os organismos habitantes ao longo do tempo. Existem várias classificações ecológicas para categorizar a fauna subterrânea tendo como base as adaptações morfo-fisiológicas e na dependência aos ecossistemas subterrâneos. Designam-se estigóbios os organismos que completam o seu ciclo de vida em águas subterrâneas e que apresentam troglomorfismos, características como a despigmentação, redução das estruturas oculares, alongamento corporal, e desenvolvimento de apêndices. Para além da adaptação morfológica, a escassez de carbono e nutrientes limita a densidade populacional, obrigando a que as espécies se adaptem a um ambiente pouco energético, observando-se reduzidas taxas metabólicas e de reprodução. Como representam um sistema semi-fechado, é verificada uma tendência para a aglomeração de espécies cavernícolas únicas levando a uma taxa de endemismo elevada e a uma maior vulnerabilidade.

Estes habitats subterrâneos fornecem serviços de ecossistemas essenciais para a biodiversidade e para o ser humano, como serviços de regulação (qualidade da água), de provisão (consumo de água) ou culturais (turismo, educação e espeleologia recreativa), e suporte a ecossistemas dependentes deste. Aproximadamente 95% da água consumível mundialmente provém de aquíferos subterrâneos contando com microorganismos e invertebrados subterrâneos para a sua manutenção ecológica. Embora passe despercebido trata-se de um ecossistema crucial para a biodiversidade, contudo, é muito afetado por fontes de contaminação de origem antropogénica e natural. A poluição subterrânea está associada à infiltração oriunda das descargas industriais e urbanas, dos compostos químicos intensamente utilizados na agricultura, entre outros, podendo ser rápida e difícil de controlar. No contexto das alterações climáticas, a temperatura é um fator ambiental nocivo a considerar, uma vez que investigações preveem impactos significativos nas populações subterrâneas comprometendo a sustentabilidade ecológica destes ecossistemas.

Os fogos florestais são acontecimentos naturais e, por um lado, benéficos para o ecossistema florestal, pois mantêm e moldam a dinâmica do mesmo promovendo a biodiversidade. Contudo, são considerados o maior problema ambiental afetando com regularidade grandes áreas do Mediterrâneo e de forma cada vez mais intensa como consequência das mudanças climáticas. Tomam uma forma não controlada ardendo em vegetação selvagem, frequentemente em zonas rurais, impactando os ecossistemas através da produção, mobilização e dispersão de uma mistura de compostos orgânicos e inorgânicos que é resultado destes fenómenos. São substâncias de grande preocupação devido à elevada persistência, toxicidade, propensão para a bioacumulação e por exibirem propriedades mutagénicas e cancerígenas

em organismos de espécies aquáticas. Os Hidrocarbonetos Policíclicos Aromáticos (HPAs) são compostos orgânicos formados pela combustão incompleta de combustíveis fósseis como o carvão ou o petróleo, assim como outras substâncias. Na constituição das cinzas também se incluem metais, compostos inorgânicos, que se apresentam em maior quantidade e que contribuem para a elevada toxicidade e propensão para a bioacumulação na cadeia trófica. Foi demonstrado que a precipitação pós-incêndio e consequente escoamento superficial têm um impacto considerável na qualidade da água constituindo um problema para o ser humano, uma vez que é dependente desta diretamente para consumo. Para além disso, produz um espetro vasto de efeitos nas propriedades dos solos, da vegetação e na atmosfera, levando a consequências para o ambiente consoante a severidade de cada ocorrência. Coloca-se a hipótese de que, para além de serem considerados um exemplo de fonte de poluição para os ecossistemas aquáticos, também o sejam para os subterrâneos.

Os organismos subterrâneos possuem funcionalidades que contribuem para a manutenção da boa qualidade da água subterrânea. Como tal, é necessário potenciar a investigação de modo a promover a conservação de todo o ecossistema, minimizar os impactos nos ecossistemas adjacentes e cobrir a lacuna de conhecimento que existe sobre a vida subterrânea. Pouco se sabe sobre os efeitos ecotoxicológicos nestes organismos, no entanto, existem análises biomoleculares em organismos aquáticos de superfície que demonstraram modificações pró-oxidativas evidenciando uma resposta defensiva aos contaminantes a que foram submetidos. Considera-se que estes elementos associados ao escoamento pós-incêndio contribuem para a ocorrência de efeitos subletais nocivos e comprometem o bom desempenho biológico destes organismos.

A presença destes compostos em águas subterrâneas tem vindo a ser detetada na proximidade de incêndios florestais. Contudo, até ao presente continua a ser indispensável analisar os efeitos letais e sub-letais em espécies com maior ou menor adaptação aos sistemas aquáticos subterrâneos para se poder estimar a sensibilidade das diferentes espécies e as suas implicações ecológicas. Outro objetivo desta investigação passa por avaliar, pela primeira vez, uma bateria de biomarcadores de dano e de defesa determinando os principais processos fisiológicos causados pelas cinzas de incêndios florestais em organismos adaptados à vida em águas subterrâneas. Duas espécies aquáticas subterrâneas de crustáceos, *Proasellus lusitanicus* e *P. assaforensis* (Isopoda: Asellidae), endémicas do Maciço Calcário Estremenho e dos calcários da zona de Sintra-Cascais, respetivamente, foram expostas a seis concentrações diferentes da mesma amostra de cinzas. A bateria de biomarcadores inclui a análise de funções fisiológicas associadas à capacidade antioxidante não-enzimática (níveis de glutationa total; TG) e enzimática (atividade de catalase; CAT), fase II de conjugação e destoxificação (atividade de glutationa S-transferase; GST), stress oxidativo (níveis de peroxidação lipídica; LPO), e metabolismo aeróbico (atividade da cadeia transportadora de eletrões; ETS) e anaeróbico (atividade de lactato desidrogenase; LDH). Esta bateria foi complementada com as análises da atividade de acetilcolinesterase (biomarcador de danos neurofisiológicos e um proxy da atividade dos organismos), níveis de proteína e peso dos organismos ambos como biomarcadores de stress ao nível do indivíduo nos processos de biossíntese e de manutenção da massa corporal durante o período experimental.

Os resultados deste estudo revelam que as cinzas dos incêndios florestais afetam negativamente espécies de crustáceos de águas subterrâneas, demonstrando sensibilidades distintas nas suas respostas em função das alterações fisiológicas por adaptação ao meio. No caso dos organismos de *P. lusitanicus* expostos a todas as concentrações de cinzas verificou-se um aumento dos níveis de LPO associado à inibição significativa da atividade de CAT e a uma diminuição da proteína sendo os valores de NOEC (No Observed Effect Concentration) estimados inferiores a 1.25 g/L. Nesta concentração de cinzas também se observou em *P. lusitanicus* um aumento significativo da atividade de ETS. No entanto, a taxa de ventilação diminuiu significativamente apenas para concentrações de cinzas iguais ou superiores a 5 g/L. Nos organismos de *P. assaforensis* verificou-se um aumento significativo dos níveis de LPO quando expostos a concentrações de cinzas iguais ou superiores a 2.5 g/L. Contudo, este biomarcador de dano oxidativo em *P. assaforensis* está associado a um aumento significativo da atividade de LDH observado nos organismos expostos a concentrações iguais ou superiores a 1.25 g/L. A comparação dos danos oxidativos nas duas espécies mostra, de forma inequívoca, que *P. lusitanicus* é mais sensível do que *P. assaforensis.*

A espécie *P. assaforensis*, morfológica e fisiologicamente menos adaptada às condições do ecossistema subterrâneo, demonstrou capacidade para ativação do metabolismo anaeróbico produzindo energia suficiente para impedir o dano oxidativo quando exposta a uma concentração de cinzas de 1.25 g/L, mas não para concentrações superiores. Contrariamente, os indivíduos da espécie *P. lusitanicus* revelaram elevados níveis de stress oxidativo e incapacidade para ativação do metabolismo anaeróbico para todas as concentrações testadas. Também se verifica a ativação da produção aeróbica de energia induzida pelas cinzas apenas para uma exposição de 1.25 g/L o que, concomitantemente com a inibição da capacidade antioxidante enzimática, não impede dano oxidativo dos organismos expostos. Além do mais, verifica-se uma diminuição na ventilação como estratégia de poupança de energia, consequente da toxicidade elevada induzida pelas cinzas, mas também a uma diminuição da biossíntese proteica que estará associada a uma tendência para a perda de peso dos organismos expostos a concentrações de 5, 7.5 e 10 g/L. Os resultados obtidos evidenciam que os fogos florestais, embora ocorram à superfície, geram condições de stress em comunidades de águas subterrâneas e produzem impactos nestes mesmos ecossistemas, causando disfunção do seu normal funcionamento ecológico e dos serviços que prestam à humanidade. Os ecossistemas de águas subterrâneas constituem um dos recursos naturais mais desprotegidos e desconhecidos do nosso planeta inteiro e por isso mais estudos são precisos para que se possa entender a sensibilidade destes organismos à pressão de qualquer fonte de contaminação, natural e antropogénica e de que forma irão responder aos seus efeitos.

Palavras-chave: grutas, cinzas de incêndios florestais, biodiversidade subterrânea, ecotoxicidade, conservação.

Abstract

Subterranean ecosystems are distinct due to their unique characteristics and specialized organisms. Caves, known for their ecological stability, are increasingly vulnerable to anthropogenic and natural contamination sources, including wildfires. Wildfires produce and disperse harmful organic and inorganic compounds, such as polycyclic aromatic hydrocarbons and metals, which pose significant environmental risks due to their toxicity and bioaccumulation ability in the food chain. This dissertation focuses, for the first time, on the impact of wildfire-related compounds on two subterranean aquatic crustaceans, *Proasellus lusitanicus* and *Proasellus assaforensis* (Isopoda: Asellidae), which play a critical role in maintaining groundwater quality. The organisms were exposed to varying concentrations of ash dilutions, and biomolecular analysis was conducted using total glutathione, glutathione S-transferase, catalase, lipid peroxidation, electron transport system, lactate dehydrogenase, acetylcholinesterase, and total protein. The results indicate an increase in lipid peroxidation levels in both species, reflecting oxidative stress. A rise in lactate dehydrogenase was observed only in *P. assaforensis,* which produced enough energy to prevent oxidative damage when exposed to a concentration of 1.25 g/L. Still, insufficient for higher concentrations, no significant ventilation differences were recorded. *P. lusitanicus* showed significant inhibition in catalase levels to all the tested concentrations and a decrease in ventilation at the highest concentrations, as a consequence of the high toxicity induced, given their inability to produce energy aerobically or anaerobically. This limitation, tied to a lack of anaerobic metabolism, suggests better adaptation to the underground environment, increasing the vulnerability of *P. lusitanicus* to stress, unlike *P. assaforensis*, which can adapt by producing anaerobic energy. These findings suggest that wildfire-related compounds may create stressful conditions in groundwater communities, potentially impacting these ecosystems and disrupting their ecological functioning. The potential loss of these species may threaten the ecosystem's ability to maintain water quality, leading to broader ecological imbalances.

Keywords: caves, wildfire ashes subterranean biodiversity, ecotoxicity, conservation

Table of contents

List of Figures

List of Tables

Acronyms and Symbols

AEA – Aqueous Extract of Ashes

ATP - Adenosine triphosphate

AChE – Acetylcholinesterase

BuChE - Butyrylcholinesterase

CAT – Catalase

ChE – Cholinesterase

EC – European Commission

ETS – Electron Transport System

GDEs – Groundwater Dependent Ecosystems

GST – Glutathione S-Transferase

LDH – Lactate dehydrogenase

LOQ – Limit of Quantification

LPO – Lipid Peroxidation

PAHs – Polycyclic Aromatic Hydrocarbons

PCBs – Polychlorinated Biphenyls

PROT – Protein

ROS – Reactive Oxygen Species

USEPA – United States Environmental Protection Agency

1 Introduction

1.1 Caves and subterranean fauna

The subterranean ecosystem is an invisible world to those who live on the surface. However, it is well known that this is a vast, unique, and susceptible ecosystem characterized by excessive humidity, an almost constant average annual temperature, lack of permanent light, and oligotrophic conditions (Gibert & Deharveng, 2002). These habitats, with essential abiotic factors for biocoenoses, serve as unique evolutionary laboratories for the study of adaptation and natural selection (Reboleira et al., 2011). The peculiar environmental conditions enable the organisms that inhabit it to develop distinctive morphological characteristics compared to surface species (Hose et al., 2022).

Caves are among the less well-known ecosystems because of the difficulty in studying them due to their limited accessibility (Ficetola et al., 2019), which poses challenges to their conservation (Castaño-Sánchez et al., 2020a). There is still much knowledge to be acquired, and consequently, specialized subterranean species continue to be among the least documented fauna on the planet (Nanni et al., 2023). Understanding the distribution, life history, phylogenetic relationships, interactions within different subterranean communities, and sensitivity to environmental disturbances represent essential steps toward consolidating scientific knowledge to support conservation planning (Mammola et al., 2019a).

Subterranean habitats vary widely in size from small holes to large caverns and in depth from a few centimeters to several meters below the surface (Moldovan et al., 2018). Although they can be found in a wide variety of rocks and substrates, the most well-known examples are solution caves in limestone and volcanic caves in basaltic lava (Lauritzen, 2018; Pipan & Culver, 2013).The majority and most well-known caves are karst caves due to their high accessibility and stability (Lauritzen, 2018), which are produced by the solvent action of water from the heavy rain events on limestone formations (Moldovan et al., 2018). Limestone rocks are rich in calcium carbonate ($CaCO_3$) and slightly acidified rainwater, when it encounters a fractured limestone massif, will slowly dissolve the rock, producing cracks and channels through which water flows (Gillieson et al., 2022 ; Sousa & Soares, 2015) The calcium carbonate, when in contact with the water containing CO_2, forms carbonic acid H_2CO_3. The acid water penetrates the cracks in the limestone and collides with the rock, producing bicarbonate $Ca(HCO_3)_2$which is soluble and easily transported by water. Caves gradually form as the fractures gradually deepen and dissolve the calcium bicarbonate (Culver & Pipan, 2009). This type of rock is made up of sedimentary elements of high solubility and is very porous, which can generate large cavities due to calcium carbonate dissolution reactions caused by the infiltration and percolation of acidic water (Sousa & Soares, 2015). This porosity generated by chemical corrosion considerably weakens the massif, making it less resistant and more permeable (Sousa & Soares, 2015).

Fauna in subterranean ecosystems is composed of a vast diversity of microbial and metazoan species, many of which are endemic and support essential functions and processes (Saccò et al., 2024). They present a suite of morpho-physiological and behavioral adaptations to accompany the peculiar environmental conditions (Howarth & Moldovan, 2018a; Mammola et al., 2019b). Loss of vision, lack of pigment, frail appearance, development of appendages and sensory organs, reduction of wings among insects and weakening of the cuticle in arthropods, reduced metabolism, and a long life span with a low reproduction rate, are some traits of troglomorphisms resulting from the selective pressure of the underground environment (Pipan & Culver, 2013). In caves, food supplies are typically dispersed and difficult to find, making it hard for animals to be selective and adapt to various resources (Kováč, 2018). However, with the elongated appendages, multiplied sensory areas, and chemoreception, cave animals can improve their food-finding ability by detecting food faster and at a more considerable distance from their bodies and spending less energy seeking food (Kováč, 2018). This is important for the survival of organisms, as it helps them locate food through mechanical reception, avoid danger, and reproduce (Howarth & Moldovan, 2018b). The absence of sunlight means the absence of daily dark-light cycles and implies the loss of circadian rhythm (Culver & Pipan, 2009; Culver & Pipan, 2016; Howarth & Moldovan, 2018a). It also contributes to the lack of photosynthetic producers, and consequently, carbon and oxygen rates are lower than at the surface, causing a high dependence on carbon (Culver & Pipan, 2009). Consequently, cave-dwelling species are forced to adapt to low available energy levels and reduce metabolic rates (Hervant & Renault, 2002; Howarth & Moldovan, 2018a).

Several ecological classifications categorize subsurface fauna based on their morpho-physiological adaptations and dependence on subterranean ecosystems. Terrestrial organisms that complete their life cycle underground and exhibit troglomorphisms are called troglobionts. Troglophiles, on the other hand, inhabit subterranean ecosystems but complete their life cycle on the surface, and trogloxenes are species that appear sporadically in caves without forming permanent connections to this environment (Deharveng & Bedos, 2018; Howarth & Moldovan, 2018a). Similarly, stygobionts are obligate groundwater organisms, as they live exclusively in groundwater, whereas stygophiles inhabit both groundwater and surface environments, and stygoxenes appear in groundwater only occasionally (Galassi, 2001; Howarth & Moldovan, 2018a).

In total, there are more than 7.000 species of animals living in groundwater, most of them are representatives of old lineages currently extinct at the surface (Stein et al., 2012). There are various species crucial to preserving its quality (Mammola et al., 2019a; Nanni et al., 2023) as crustaceans of the family Asellidae (Isopoda) are widely distributed in groundwater; among them, the genus Proasellus is the richest in Europe, with several surface and subterranean species in Portugal (Saclier et al., 2024). *Proasellus lusitanicus* Frade, 1938 is a stygobiont crustacean endemic to the Estremenho Karst Massif, which has been used as a model in physiology and ecotoxicology studies (Di Lorenzo and Reboleira, 2022).

There are more than 14 known karst units in Portugal, the most significant of which are Jurassic limestones and dolomites found in Estremenho (Serras de Aire e Candeeiros), Algarve, Sicó-Condeixa-Alvaiázere, Arrábida, and Montejunto. Additionally, some Cambrian limestone and marble caves can be found in Estremoz and Adiça in the Alentejo, as well as in Vimioso in the northeast region (Reboleira et al., 2013b). The richest massif in Portugal is Algarve, followed by Sicó and Estremenho. However, they all harbor the most biogeographical relicts, with troglobionts representing 81 % and stygobionts 19 % of the cave-dwelling organisms from Portugal´s karst regions (Reboleira et al., 2013b).

1.2 Ecosystem services provided by subterranean ecosystems

Groundwater is the largest reserve of freshwater in the world (Foster & Chilton, 2003; Shiklomanov, 1998). It interacts with the five global surface aquatic biomes (coastal water, oceans, estuaries, rivers, and lakes) and, together with the atmosphere and oceans, is responsible for the function of the global water cycle (Saccò et al., 2024). Underground habitats provide essential ecosystem services, including biogeochemical processes, such as the cycling of organic carbon and nutrients, the biodegradation of contaminants, and the eradication of viruses and pathogenic microorganisms (Griebler et al., 2014), regulating (water quality), provisioning (water consuming), supporting (soil formation for agricultural production), and cultural (tourism, education, recreational caving) (Nanni et al., 2023). Moreover, 97% of all the unfrozen water is stored underground and constitutes the largest source for human consumption, agricultural activities, and industrial purposes, while surface lakes and rivers represent only 2% (Gibert & Deharveng, 2002). Being intimately interconnected with other ecological systems (including the surface), underground services can be beyond the ones mentioned. Caves, specifically karst ones, serve as organic carbon dioxide sinks, helping reduce climate change's effects (Gillieson et al., 2022). Beyond that, bats form large congregations of mammals roosting in caves, playing critical functions such as pollinators, seed dispersers, and insect pest controllers (Kunz et al., 2011; Nanni et al., 2023). All these illustrate the importance of the subterranean ecosystem to all groundwater-dependent ecosystems (GDEs) and how humankind's survival is likely to depend more on maintaining healthy subterranean environments than is generally recognized (Mammola et al., 2019a).

1.3 Contamination sources

Subterranean ecosystems are susceptible to surface contamination, which can disrupt their delicate balance in several ways. These impacts affect habitat stability, hydrology, biological communities, and structural integrity by infiltration from the surface (Castaño-Sánchez et al., 2020a). Sources of disruption include deforestation, industrial and urban discharges, tourism, which induces changes in water chemistry, composition, and temperature of air masses, the introduction of exotic species, and agriculture (Böhlke, 2002; Castaño-Sánchez et al., 2020a; Moldovan, 2013; Nanni et al., 2023; Nicolosi et al., 2023).

Emerging contaminants, including pharmaceuticals (Duarte et al., 2023), veterinary products, fertilizers, food additives, and engineering nano-materials (Lapworth et al., 2012), as well as metals and polycyclic aromatic hydrocarbons (PAHs) (Mansilha et al., 2014), volatile organic compounds (Castaño-Sánchez et al., 2020a), microplastics are the most commonly detected contaminants in subterranean ecosystems coming from the most diverse sources of pollution mentioned above (Reboleira et al., 2013a).

Agriculture, through the use of fertilizers and pesticides, has a significant impact on subterranean ecosystems (Nanni et al., 2023). The nutrients found in the greatest concentration are nitrogen and phosphorus, which form part of the fertilizer mix and are essential for plant growth (Castaño-Sánchez et al., 2020a). However, excessive use contributes to altering the microbial community and the biochemical cycle of nitrogen in aquifers (Marschner, 2003).

Caves are very balanced environments with minimal contact with the outside world, and this balance can be corrupted through tourism (Cigna, 2016, Moldovan, 2003). Visitors can alter the cave temperature (air and water) (Šebela & Turk, 2014), increase the CO_2 air concentration, resulting in a rise of corrosion processes rather than the deposition of new formations (Cigna, 2016; Sánchez-Moral et al., 1999), carry dust pollutants, and propagules of microorganisms (Saiz-Jimenez et al., 2012). Beyond that, visitors' noise and artificial lighting can negatively affect the bats' reproducing or overwintering (Mann et al., 2002; Piano et al., 2022). Green plants (usually algae, ferns, and mosses) can grow favorably on walls with intense artificial light (Cigna, 2016). These plants produce a greenish layer on surfaces, which can become covered by the calcite deposit and become irremovable (Cigna, 2016).

Deforestation is another source of ambiental impact that contributes to the loss of surface vegetation, which can lead to more surface erosion and more exposure of bedrock on karstic zones, habitat alterations, changes in the water balance, and the subterranean atmosphere temperature (Nanni et al., 2023; Van Beynen & Townsend, 2005). Wildfires also contribute to these effects, as does contamination through the metals and PAHs that constitute the ash (Mansilha et al., 2014; Nanni et al., 2023).
Metal contamination in subterranean environments includes natural and anthropogenic sources, such as mining activities, where pollution can persist after those activities have ended (Duruibe & Egwurugwu, 2007), the application of sludge and biosolids to land, urban and agricultural runoff, wind-borne soil

particles, volcanic activity, and ashes from wildfires (Castaño-Sánchez et al., 2020a; Mansilha et al., 2014; Nriagu, 1989).

Subterranean ecosystems are affected by anthropogenic factors and environmental factors, such as temperature (Castaño-Sánchez et al., 2020a; Medina et al., 2023). It is estimated that the increase in temperature could cause damage to the cave community since it is ectothermic and it is used to little or no thermal variations in the environment (Castaño-Sánchez et al., 2020b). Compared to surface aquatic species, subterranean organisms tend to maximize their physiological performance in a low-temperature range. They suffer a deficit in the performance of physiological processes, which they try to counteract through response mechanisms. These defense mechanisms are effective at low temperatures, but at higher than optimal values, the survival of some underground species is already compromised (Di Lorenzo & Reboleira, 2022)

1.4 Conservation status of groundwater ecosystems

Subterranean habitats are essential for supporting rare types of life and should be prioritized in conservation programs despite being neglected when considering global conservation challenges (Mammola et al., 2019a). Directive 2006/118/EC (*Directive 2006/118/EC of the European Parliament and of the Council of 12 December 2006 on the Protection of Groundwater against Pollution and Deterioration - EU Monitor*, n.d.) of the European Parliament included the goal of achieving water quality levels that do not give rise to significant harmful effects on human health and the environment, so it is necessary to avoid, prevent, and reduce concentrations of pollutants in the underground environment, as well as including measures to prevent and control groundwater pollution. The assessment of water quality presupposes using reliable methods for monitoring water, establishing limit values for all polluting substances and indicators that characterize groundwater as at risk of not presenting a good chemical level of groundwater. According to Directive 2000/06/EC (Smith, 2015), these thresholds will be established taking into account the extent of interactions between groundwater and associated aquatic and terrestrial ecosystems, the pollutants that pose a risk to groundwater bodies, their origin, their toxicology and dispersion tendency, their persistence and bioaccumulation trend, as well as hydrogeological characteristics that include information on the concentrations of substances and the water balance. To ensure sustainable, fair, and balanced water use, this framework helps ensure an adequate supply of high-quality surface water and groundwater (Smith, 2015). Just as the protection and sustainable management of terrestrial and aquatic surface ecosystems is essential, there is no argument for excluding underground ecosystems and their biodiversity (Boulton, 2005; Mammola, et al., 2019a). Groundwater and GDEs must be conserved and managed jointly under a “one water” framework (Saccò et al., 2024). Current efforts to protect surface ecosystems are based on the awareness that human beings depend on the services provided by communities, which is also true for underground ecosystems due to the numerous services they provide (Griebler et al., 2023).

Naturally, subterranean ecosystems will react to human-induced changes that negatively impact the surface environment (Mammola, et al., 2019a). Subterranean biodiversity is at risk of extinction due to its geographic restriction and high rate of endemism, low densities of individuals, and low reproductive output, as well as the lower possibility of recovery from adverse environments (Castaño-Sánchez et al., 2020a; Mammola et al., 2019a). For that, there is an urgent need to deepen our knowledge of the communities and ecosystem functions, as well as the susceptibility to anthropogenic sources of this type of habitat, to be able to define mitigation measures leading to its conservation and to avoid the risk of extinction (Mammola et al., 2019a). In addition, there is a need to develop risk assessment systems and monitoring schemes worldwide and raise awareness of these ecosystems' great importance (Boulton, 2005; Castaño-Sánchez et al., 2020a).

1.5 Wildfires

Wildfires are natural phenomena crucial in maintaining ecosystem dynamics and promoting biodiversity (Abrantes et al., 2017). However, despite their ecological benefits, they are increasingly viewed as a significant threat, particularly in the context of climate change. It is projected that the frequency of wildfires will rise due to increasing temperatures and more extended periods of drought (Carvalho et al., 2010; Nakicenovic et al., 2000). Often uncontrolled in rural areas, these fires can reach devastating scales, causing substantial environmental and socioeconomic impacts (Rodrigo-Comino & Salvati, 2024). Wildfires are the primary cause of hydrological and geomorphological change in fire-prone landscapes, leading to intense consequences for the environment depending on their severity (Campos et al., 2012). The ecological consequences are profound, affecting soil stability, structure, and properties, with an increase of erosion, ash deposition, and water supply reservoirs by contamination (Bladon et al., 2014; Mansilha et al., 2014; Smith et al., 2011), and biodiversity.

In addition to their effects on terrestrial environments, wildfires contribute to water quality degradation in aquatic systems. During rain events, ash and topsoil can be readily eroded and transported into water bodies, negatively impacting water quality (Costa et al., 2014; Mansilha et al., 2014; Smith et al., 2011). Also, inputs of ash and sediment can lead to increased turbidity, organic matter, alkalinity, pH, conductivity, temperature, and dissolved oxygen depletion in water (Costa et al., 2014; Rannalli, 2004; Smith et al., 2011). Wildfire ash is a significant source of contaminants, particularly PAHs and metals (Manoli & Samara, 1999). Both PAHs and metals are persistent, toxic, bioaccumulative, and classified as priority pollutants by the United States Environmental Protection Agency (USEPA) and the European Commission (EC) due to their potential health risks, which can cause significant damage to the biodiversity (Abrantes et al., 2017).

Although it is widely recognized that wildfires can impact water quality, their potential toxic effects on aquatic systems have only recently been explored. Several studies provided evidence that compounds released or mobilized by wildfires, such as PAHs and metals, are toxic to aquatic organisms, including periphytic, phytoplanktonic and zooplanktonic species, aquatic plants, aquatic macroinvertebrates, amphibians and fishes (Campos et al., 2012; Carvalho et al., 2019; Nunes et al., 2017; Pilliod et al., 2003; Pradhan et al., 2020; Ré et al., 2020; Silva et al., 2015; Vidal et al., 2021). All these studies have focused on surface water systems, such as streams, where the presence of ash is certain. However, ash has also been detected in groundwater (**Figure 1**), with studies confirming PAH contamination in aquifers within burned areas (Mansilha et al., 2014, 2020). Nonetheless, the impact of wildfire ash compounds on subterranean aquatic species remains largely unknown, and their ecological effects are still uncertain. Addressing this knowledge gap through further research is therefore essential.

Figure 1: Wildfire ashes found in the bottom of an intermittent subterranean river in a cave of the Estremenho karst massif, Portugal.

Mainland Portugal is divided into two regions of approximately the same size (Pereira et al., 2024). While the northern half is characterized by an irregular topography, a denser river network, and the predominance of forest and semi-natural areas, the southern half of the country is dominated by agricultural areas with mixed forests (Tonini et al., 2017). With a Mediterranean climate, Portugal is characterized by warm and dry summers as well as cool and wet winters, which promotes the growth of vegetation in winter and spring and the occurrence of fires during the summer due to water stress (Parente et al., 2016). Portugal is one of the most fire-prone countries in Europe due to a variety of variables, including the country's topography, vegetation, and favorable climate (Pereira et al., 2024). Regarding the vegetation, croplands and shrublands generally cover the south of the country, while the north and center are covered by eucalyptus and pines, which are very flammable forest types (Ermitão et al., 2023; Mateus & Fernandes, 2014).

The total area burned annually varies significantly from year to year and is strongly correlated with the severity of the weather and favorable conditions for occurrences, with maximum values being observed in 2003, 2005, and 2017, when Portugal faced extensive fire damage (**Figure 2**). According to climate change models, the severity and intensity of drought events is expected to rise in the Mediterranean regions (Bowman et al., 2017). Due to a reduced wet season, Portugal's yearly precipitation will drop by half, and temperatures will generally climb, especially inland (Miranda et al., 2002). Climate change is expected to produce changes in the distribution patterns of forest ecosystems, as well as an increase in the danger of soil degradation, fire, and desertification. Longer fire seasons are correlated with hot, dry summers in the Mediterranean region and, as a result, there will be more frequent and intense large-scale fires (Mateus & Fernandes, 2014).

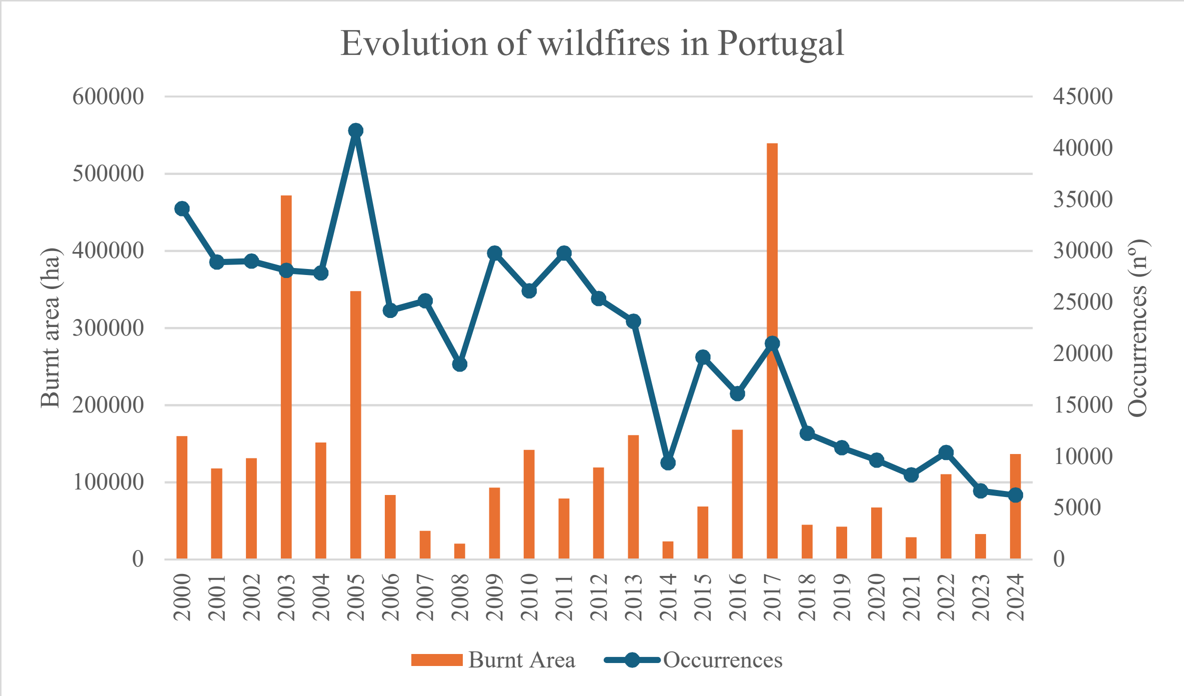

Figure 2: Evolution of wildfires in mainland Portugal by area burned and the number of occurrences between 2000 and 2024. (Source: ICNF, 2023,2024).

Karst regions and the nation's areas that have experienced fire from 2000 until 2023 are depicted in Figure 3. Karst systems are located in areas characterized by Mediterranean vegetation with the predominance of eucalyptus, pine, and croplands, which, together with the Mediterranean climate, makes them vulnerable to wildfire occurrences (Ermitão et al., 2023; Mateus & Fernandes, 2014). For that, the rise in the susceptibility of soils to erosion, coupled with the occurrence of hydrophobic soils, which reduces the infiltration rates and increases the overland runoff, contributes to the contamination of these ecosystems (Bladon et al., 2014). Due to their geological structure, karstic caves are vulnerable to all kinds of contaminants, particularly ashes, which easily penetrate the subsurface through open fractures that provide a direct exchange of surface water with groundwater, spreading rapidly in the aquifer network (Chen et al., 2017). One of the significant impacts in these regions is the alteration of hydrological systems with the loss of vegetation that accelerates soil erosion, resulting in more significant sedimentation and consequent contamination of water sources (Bian et al., 2019). These impacts are concerning, as karts reservoirs are essential for providing fresh water to the population (Goldscheider et al., 2020; Hartmann et al., 2014).

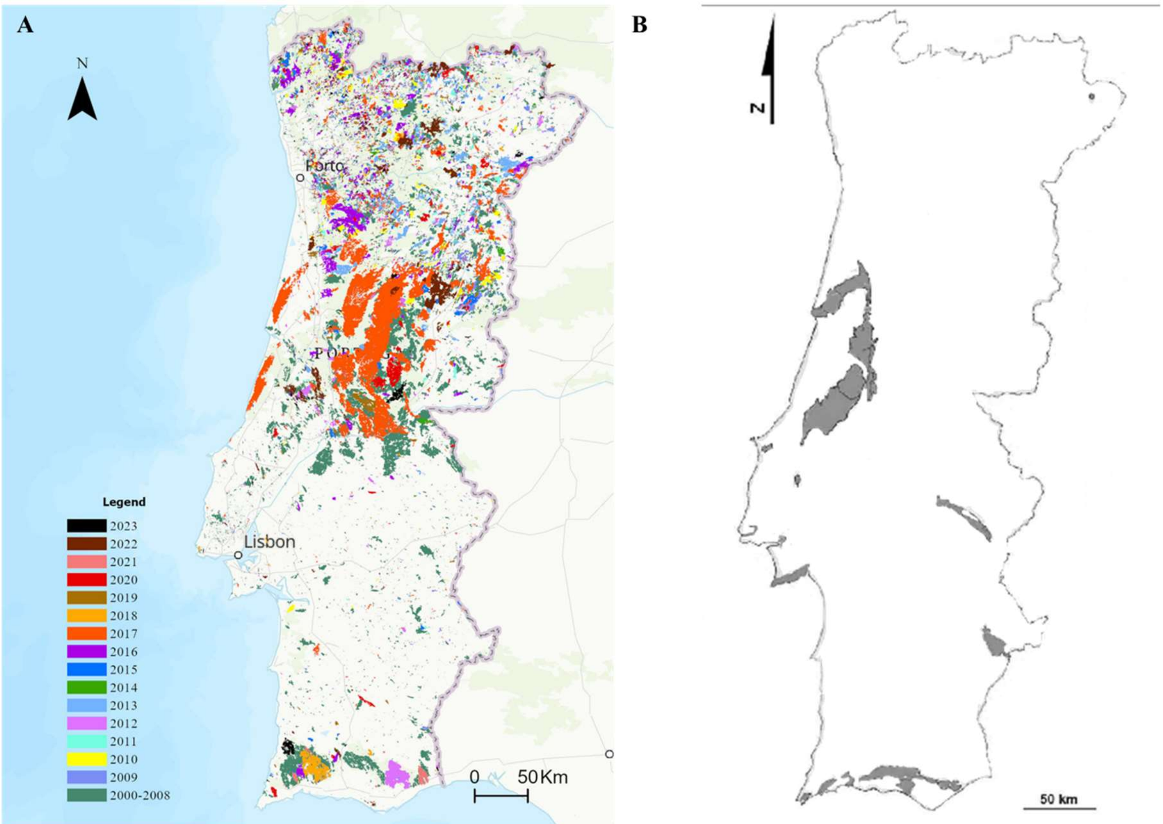

Figure 3: Information of the occurrence of wildfires in mainland Portugal from 2000 to 2023 (A) (ICNF, 2023). Map was generated using ArcGis Pro (v3.1.0, 2023); Geographical location of the main karst areas in mainland Portugal (B) (Reboleira et al., 2013b).

1.6 Ecological biomarkers

Anthropogenic activities are the main source of environmental contamination through industrial, domestic, and urban effluent sources (Amiard &Amiard-Triquet, 2013). The contamination process consists of an ecotoxicological triad: exposure, bioaccumulation in the organism, and the corresponding toxic effect (Amiard & Amiard-Triquet, 2013; Damiens & Minier, 2011). Once in the environment, chemical compounds, depending on their hydrophilic and lipophilic nature, can actively or passively cross any organism's biological membranes, respectively, reaching the cytoplasm of cells (Newman, 2003). Lipophilic compounds, such as polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs), pass through a biotransformation process with three main phases (Amiard & Amiard-Triquet, 2013; Damiens & Minier, 2011):

- Phase I: Oxidation, reduction and hydrolysis reactions;
- Phase II: The primary metabolites formed are generally electrophilic in phase I and are subsequently conjugated with endogenous functional groups, such as reduced glutathione, due to the action of conjugases, such as glutathione S-transferase, increasing solubility in water, which facilitates excretion and elimination;
- Phase III: The secondary metabolites resulting from phase II conjugation have endogenous functional groups that are recognized by the membrane proteins of the multi-xenobiotic transport system, which, at the expense of ATP, excrete the conjugated metabolites out of the cells.

In contrast, hydrophilic compounds do not pass through phase I biotransformation processes. Still, it might be excreted out of cells either indirectly conjugated with reduced glutathione and/or directly with this non-enzymatic antioxidant. Nevertheless, during phase III, those conjugated hydrophilic compounds might be recognized and excreted out of the cells at the expense of ATP (Newman, 2003).
The exhaustion of the previous processes might lead to the permanence of undesirable compounds inside cells, which provoke directly diverse harmful effects in macromolecules or indirectly through redox reactions that increase the levels of reactive oxygen species to abnormal levels, leading to oxidative damage. Such effects are detectable using the so-called biomarkers that show alterations in the expected levels of each organism when exposed to a stressful condition. These changes can be observed at various levels of biological organization, such as the molecular, cellular, individual, and population levels Amiard &Amiard-Triquet, 2013).

According to a new recent approach, a new trend classifies biomarkers only into two distinct groups, i.e., biomarkers of damage representing a deleterious change in the cell biology of exposed organisms and biomarkers of defense or tolerance showing reversible changes in the cell biology of an exposed organism and the adaptation processes to stress at the expense of energy (Amiard &Amiard-Triquet, 2013).

Glutathione S-Transferase (GST) is an enzyme that participates in the detoxification process of xenobiotic compounds to facilitate excretion from organisms (Regoli et al., 2011). After oxidation, it catalyzes the conjugation of the xenobiotics with glutathione to assist in this process (Newman, 2003; Regoli et al., 2011). GST activity is induced to neutralize the pollutants' toxicity, such as PAHs, oils, and pesticides (Newman, 2003). Glutathione is present in all organisms' cells, serving several functions, including amino acid transport and as a defense against oxidizing molecules and xenobiotic compounds (Newman, 2003). GSH (reduced glutathione) performs as the active form of glutathione that acts as an antioxidant and detoxifying agent, and GSSG (oxidized glutathione) is the form of glutathione that indicates oxidative stress levels (Damiens & Minier, 2011).

$$\text{Total Glutathione (TG)} = 2\text{GSH} + \text{GSSG} \quad (1)$$

GSH is a crucial component of TG, as it plays a role in neutralizing oxidative stress and maintaining cellular homeostasis and represents an essential element of the intracellular defense against ROS (Damiens & Minier, 2011). Its depletion and other oxidative reactions can lead to irreversible damage. The measurement of these two forms provides information about cellular defense and response to contamination (Baker et al., 1990).

Metabolic processes produce reactive oxygen species (ROS), including reactive molecules and free radicals from molecular oxygen (Amiard-Triquet, 2011). ROS are products of mitochondrial electron transport during aerobic respiration and play functions in intra and intercellular signaling (Amiard-Triquet, 2011). The structure of oxygen´s electron facilitates radical formation, and its sequential reduction leads to the creation of different types of ROS, like hydrogen peroxide (Regoli et al., 2011).

Catalase (CAT) is a biomarker enzyme that can be used to measure oxidative stress participating in the breakdown of H_2O_2 molecules into water and oxygen and is measured by the ability to decompose hydrogen peroxide (Gagné et al., 2018; Regoli et al., 2011; van der Oost et al., 2003).

Lipid Peroxidation (LPO) biomarker is closely associated with oxidative stress, resulting from an increase in ROS production due to insufficient levels of antioxidant, detoxification, and repair capacities (Halliwell & Aruoma, 1991; Regoli & Winston, 1999). Cells experience harmful oxidative reactions when there is an excess of reactive oxygen radicals that antioxidants and/or catalase activity cannot compensate for, for example (Gagné & André, 2014; Regoli & Winston, 1999). Reactive molecules, such as singlet oxygen, hydroxyl radicals, and other electrophilic species, initiate lipid peroxidation in polyunsaturated fatty acids, resulting in the removal of hydroxyl radical from it, causing membrane destruction (McGill & Jaeschke, 2013). Also, other macromolecules, such as proteins and DNA, can be damaged by ROS (Halliwell & Aruoma, 1991; Roméo & Giambérini, 2013; Regoli & Winston, 1999).

When an organism is subjected to chemical stress, more energy might be required for detoxification (Mouneyrac et al., 2011). Energy consumption is estimated by measuring the electron transport activity (ETS) at the mitochondrial level (Giusto et al., 2014). The ETS is a multi-enzyme complex found in the membrane of mitochondria, and its activity indicates the quantity of oxygen that would be consumed if every enzyme worked at maximum capacity (Giusto et al., 2014; Smolders et al., 2004). ETS plays an important role in cellular respiration, which is the process by which cells generate energy through adenosine triphosphate (ATP) (Cammen et al., 1990). It consists of a complex chain of protein complexes that transfer electrons through redox reactions (Minutoli & Guglielmo, 2009).

In contrast, LDH is involved in energy production through anaerobic metabolism and provides useful information concerning energy demand. It catalyzes the reversible reaction (Kantor et al., 2001):

$$\text{lactate} + NAD^+ \leftrightarrow \text{pyruvate} + NADH^+ + H^+ \quad (2)$$

Under normal conditions of oxygenation and lactate, absorption is high, the equation proceeds toward pyruvate (Kantor et al., 2001). When there is an oxygen debt, NAD and H+ accumulate and the pyruvate can't be metabolized further, the reaction proceeds toward lactate (González & Quiñones, 2000). Since aerobic metabolism can also be altered, the citric acid cycle enzymes, or Kreb's cycle, can provide information once it helps to convert back to NAD+, the NADH+ and H+ accumulated in earlier steps (Kantor et al., 2001).

The cholinesterase (ChE) enzyme is one of the most utilized biomarkers. The cholinesterase group (ChE) is related to neural and muscular functions (Howcroft et al., 2011). It plays an essential role in the neurotransmission process since it promotes the cleavage of acetylcholine, a neurotransmitter released at cholinergic synapses. This prevents overstimulation of the post-synaptic membrane and avoids continual nerve fringing, which is essential for adequately operating the sensory and neuromuscular systems. Two types of cholinesterases have been identified in vertebrates: Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) (Howcroft et al., 2011). BuChE is constituted in various organs such as the nervous central systems, kidneys, liver, muscles, heart, and lungs, but its role is not well understood (Pohanka, 2013). When AChE is inhibited, huge quantities of acetylcholine accumulate in the synaptic cleft, which results in the overstimulation of cholinergic receptors, causing acute consequences like death (Howcroft et al., 2011).

Each biomarker plays a role in organisms, so integrating different classes of biomarkers in the identification is more effective because, all together, it offers a complete analysis with an expected increase of relevance (Gagné & André, 2014). Moreover, the selected battery of biomarkers has been successfully used not only to determine the effects of PAHs (Almeida et al., 2012; Gravato et al., 2014; Oliveira et al., 2012; Silva et al., 2013) , and metals (Gravato et al., 2006; Oliva et al., 2012; Quina et al., 2023; Vieira et al., 2009), but also as
-warning tools associated with effects at higher levels of biological organization, such as growth, behavior, reproductive performance, and development (Amiard & Amiard-Triquet, 2013). In the particular case of the species *P. lusitanicus,* a battery of biomarkers was used to determine the sub-lethal effects of acetaminophen (Duarte et al., 2023).

1.7 Objectives of the dissertation and thesis structure

The principal objective

The primary purpose of this dissertation is to evaluate the sub-lethal effects of ashes generated by wildfires on two species of groundwater ecosystems, *Proasellus assaforensis* and *Proasellus lusitanicus*, endemic from Portugal of asellids, which exhibit different degrees of adaptation towards life in groundwater. The working hypothesis is that the complete adaptation of *P. lusitanicus* to groundwater life might be more sensitive to ash contaminants in water when compared with *P. assaforensis* due to its reduced physiological capacity to cope with abiotic fluctuations. In contrast, *P. assaforensis*, which inhabits both surface and subterranean environments, is more resilient because of its greater adaptability to environmental variability and stressors.

The specific objectives

1. Assess the sublethal stress responses of two groundwater crustacean species, *Proasellus assaforensis,* and *Proasellus lusitanicus*, when exposed to wildfire ash.

2. Analyze key physiological biomarkers, including antioxidant capacity, detoxification processes, energy metabolism (aerobic and anaerobic), and indicators of oxidative stress and cell damage in both species following ash exposure.

3. Compare the biomarker responses between the two closely related stygobiont species, which exhibit differing degrees of adaptation to groundwater environments.

4. Identify and differentiate the physiological mechanisms employed by the two species to cope with wildfire ash exposure.

The dissertation is structured into several key sections: an introduction that provides an overview of the research context, objectives, and structure of the study; a materials and methods section detailing the field and laboratory procedures undertaken; a results section presenting the experimental findings; a discussion interpreting the results; and final considerations summarizing the essential findings and their implications.

This study analyzes the effects of wildfire ash on two subterranean aquatic species using a comprehensive battery of biomarkers. It aims to compare and elucidate the response mechanisms of each species to the observed sublethal effects, considering their varying degrees of adaptation to the subterranean environment.

2 Material and Methods

2.1 Study area and ash sample collection

Ashes were collected in the Fátima municipality (39° 37'54.023" N, 8° 38'11.303" W) in the Estremenho Karst Massif, central Portugal, following a 2019 high-severity wildfire. The fire affected an area predominantly covered by *Arbutus unedo* Linnaeus, 1753, commonly known as the strawberry tree, a species from the Ericaceae family. Ash samples, approximately 3 L in volume, were collected at six points spaced 5 meters apart along a 25-meter transect, oriented along the steepest slope. The samples were gathered using a small spoon and brush to avoid soil contamination and then sieved through a 2 mm mesh. The samples were transported to the laboratory in plastic bags under complete darkness. The ash samples were air-dried in the lab and combined into a single composite sample. This composite sample was stored at -20°C in a cold room in opaque plastic bags to minimize microbial activity until aqueous extracts were prepared as per the methodology of Silva et al. (2015).

2.2 Preparation of aqueous extracts of ashes

Aqueous extracts of ashes (AEA) were prepared at a concentration of 10 g/L, following the total solids recorded in a surface water stream located in a burnt catchment area (**Figure 4**) (Santos et al., 2023). This concentration was selected to reflect surface ash deposition, considering that the caves under study, formed from karst material, exhibit high porosity and permeability.

To prepare the AEA, 10 g of the composite ash sample was dissolved in a 1 L beaker using mineral water (commercial bottled water) and stirred with a glass rod. The mixture was then transferred to a 1 L flask shielded from sunlight with aluminum foil and placed on a stirring plate for 2 hours to ensure complete homogenization. Following this procedure, dilutions were prepared according to the required ash extract volumes and mineral water ratios (**Table 1**). Five different concentrations of AEA were defined (100%, 75%, 50%, 25%, and 12.5%), plus a control, as detailed in **Table 1**, with each concentration prepared in 10 replicates. The pH and dissolved oxygen levels were measured at the beginning and end of the test.

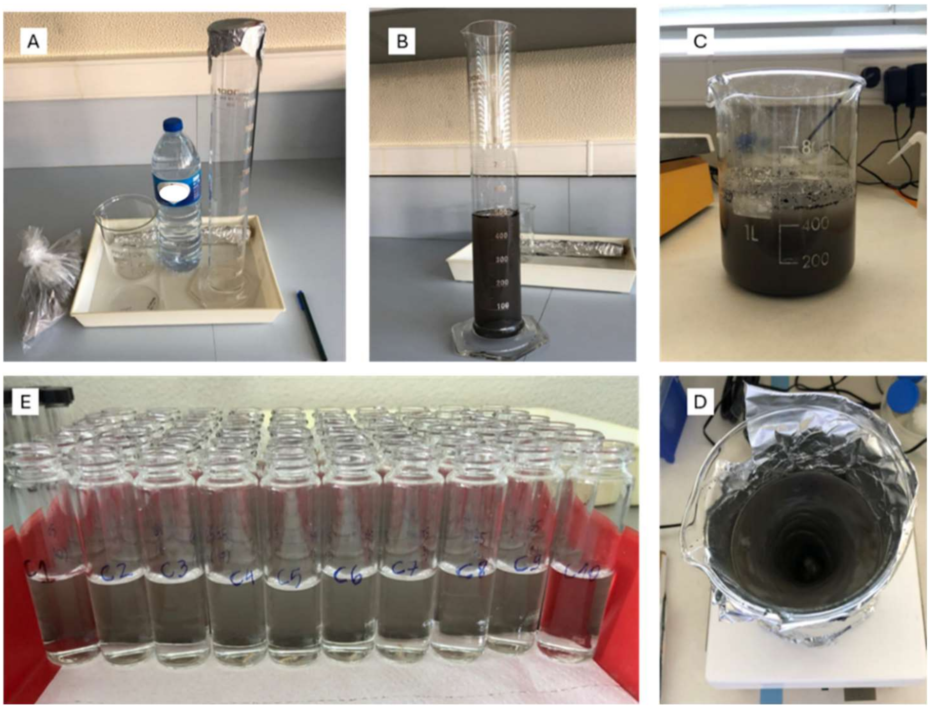

Figure 4: Preparation of aqueous ash extracts (AEA) and tested dilutions. A – Materials required for preparing the stock solution. B – Beaker with ash diluted in mineral bottled water. C and D – Homogenization of the stock solution. Preparation of aqueous ash extracts (AEA) and tested dilutions. E – Vials used for exposing organisms to the different dilutions and replicates.

Table 1: Concentrations to be tested and respective volumes of ash extract and mineral water.

	Control	12.5%	25%	50%	75%	100%
Ash extract (mL)	0	1.5	3	6	9	12
Mineral water (mL)	12	10.5	9	6	3	0

2.3 Chemical determinations

A subsample of the composite ash and a subsample of the aqueous extract of ashes (AEA), were analyzed for the quantification of polycyclic aromatic compounds (PAHs) and trace metals. Prior to chemical analysis, the ash subsample underwent microwave-assisted digestion (DIN 22022-1), while the AEA subsample was subjected to acid digestion using HNO_3/H_2O_2 (EN ISO 17294-2). A total of 3 L of ash extract was prepared from the composite sample, with 1 L allocated for metals analysis and 2 L for PAHs analysis. The solution for metals analysis was stored in a plastic bottle, while the PAH analysis samples were placed in two 1 L glass bottles, wrapped in aluminum foil to protect from sunlight, and sent to an external laboratory for testing.

The PAHs analysis focused on the 16 priority contaminants defined by the United States Environmental Protection Agency (Keith, 2014): naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, indeno(1,2,3-cd)pyrene, dibenz(a,h)anthracene, and benzo(g,h,i)perylene. PAH concentrations were determined by an external laboratory using gas chromatography coupled with mass spectrometry (GC-MS), following DIN 38407-F39 for the AEA subsample and DIN EN 15527 for the ash subsample. The limit of quantification (LOQ), representing the lowest concentration at which an analyte can be reliably detected, was 10 ng/L for the AEA subsample and 0.1 mg/kg for the ash subsample.

The trace metal analysis included the following elements: V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Cd, and Pb. For their quantification, inductively coupled plasma mass spectrometry (ICP-MS) was employed by an external laboratory, following the standards DIN EN ISO 17294-2 (E29) and DIN EN ISO 11885/DIN EN ISO 17294-2, for both the ash and aqueous ash extract (AEA) subsamples. Unlike the PAH analysis, the limit of quantification (LOQ) for each trace metal was specific to the analyzed element.

2.4 Test organisms and sampling

The genus *Proasellus* Dudich, 1925, part of the family Asellidae, comprises nine stygobiont species adapted to life in groundwater in Portugal, serving as excellent examples of European groundwater fauna. *Proasellus lusitanicus* is endemic to the Estremenho Karst Massif in central Portugal, an aquifer that was the primary water source for Lisbon during the 20th century (Frade, 1938, Magniez, 1967, Reboleira et al., 2013b). This species exhibits several key traits associated with subterranean life, and a metabolism adapted to higher temperatures (Di Lorenzo and Reboleira, 2022). Another species used as a model in this study is *Proasellus assaforensis*, a groundwater-adapted crustacean endemic to Assafora Cave, located in the karst area of Sintra-Cascais. Both caves are geologically characterized by their porosity and wide entrances (Reboleira et al., 2013a; Sousa and Soares, 2015).

According to Saclier et al., (2024), both species of *Proasellus* are phylogenetically close and derive from a common ancestor. Both species possess olfactory lamellae on their antennae for sensory navigation in the subterranean environment and robust mandibles for processing detritus and microorganisms in groundwater (Afonso, 1979; Frade, 1938). Males show pronounced sexual dimorphism, with stronger, spined pereopods compared to the females' slenderer ones, suggesting sexual selection influences (Afonso, 1979; Frade, 1938). Their pleopods support respiration and reproduction, vital adaptations for life in groundwater ecosystems (Afonso, 1979; Frade, 1938). Both species feed on organic materials and are predated by planarians leaches and *Pseudoniphargus* amphipods, which inhabit those groundwaters (Reboleira et al., 2013b) and play a crucial role in purifying aquifer water by feeding on biofilms or debris (Reboleira et al., 2013b). *P. lusitanicus* and *P. assaforensis* have shown resistance to certain pollutants such as acetaminophen, copper sulfate, and potassium dichromate while remaining sensitive to temperature increases (Duarte et al., 2023; Medina et al., 2023; Reboleira et al., 2013a). *P. assaforensis*, specifically, also demonstrated heightened sensitivity to NaCl, supporting its relevance as a model species in ecotoxicology (Castaño-Sánchez et al., 2021).

P. lusitanicus was collected from the Almonda Cave (39° 30' 16"N, 8° 36' 52"W) in the Estremadura Karst Massif, and *P. assaforensis* was collected in Assafora Cave (38°54'31.67"N 9°25'18.98"W) in the karst area of Sintra-Cascais (**Figure 5**). Specimens were collected following the recommendations by Di Lorenzo et al. (2019) and with the appropriate permits of the Instituto de Conservação da Natureza e das Florestas (ICNF). The individuals were collected using plastic pipettes and brushes and placed in plastic containers with groundwater and some sediment from the collection site (Duarte et al., 2023). All the containers were transported in a thermal box to the laboratory (Duarte et al., 2023). Adult specimens were then selected, excluding ovigerous females, and were kept in containers with commercial water in the laboratory for 24 hours in the total absence of light, at 17º C, a similar temperature to the collection site, in a climate chamber VWR INCU-Line 68R (Di Lorenzo et al., 2019; Duarte et al., 2023).

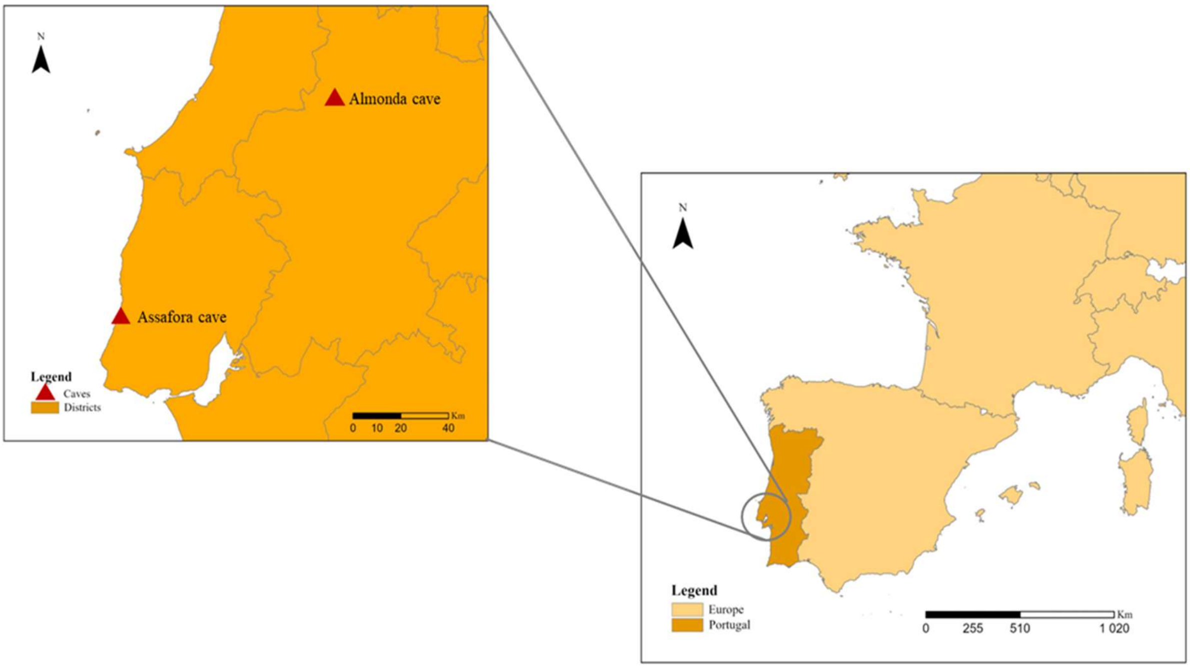

Figure 5: Geographical location of the sampled caves in Portugal. Map was generated using ArcGis Pro (v3.1.0, 2023).

2.5 Exposure conditions

The procedure followed the ecotoxicity testing guidelines for stygobitic crustaceans recommended by Di Lorenzo et al. (2019). Adult organisms were selected, excluding very small juveniles and ovigerous females, as ovigerous females can detoxify more effectively by accumulating toxins in their eggs (Di Lorenzo et al., 2019). Ten adult *Proasellus* spp. individuals were randomly exposed to each prepared ash solution and housed individually in glass vials with a test volume of 12 mL. Observations were made every 24 hours throughout the experimental period, and daily mortality was recorded.

Test validation followed the criteria outlined by Di Lorenzo et al. (2019): the experiment was considered valid if less than 20% of individuals in the control group died or became immobilized, and if the variation in dissolved oxygen concentration at the end of the test was within 20% of the initial value.

Exposure to various concentrations of the aqueous extract of ashes (AEA) lasted for 96 hours, following Di Lorenzo et al. (2019). After exposure, the organisms were carefully removed from each vial, dried carefully with absorbent paper, and individually placed in 2 mL microtubes. The specimens were frozen at -80ºC before the biomarker quantification, and weighed on an analytical balance, model VWRI611-226, LA 214i.

2.6 Ventilatory activity

After the essay, one-minute videos were taken of each individual registering the ventilatory activity (number of pleopod beats per minute), using iPhone 8 model MQ722LL/A. This movement is a possible proxy, so observation and counting assess the level of stress of the organisms and compare them with the results obtained in the biomarker analysis (Hervant et al., 1996).

2.7 Biomarkers

All the samples were homogenized in the 2 mL microtubes filled with 1200 µL of ultrapure water and 8 beads per microtube, using the Retsch MM400 bead mill for 2 min at 30 Hz. Each homogenized sample was divided into different microtubes to allow the following quantifications: Total Glutathione (TG), Glutathione S-transferase (GST), Lipid peroxidation (LPO), Electron Transport System (ETS), Total Protein (PROT), Lactate Dehydrogenase (LDH), Catalase (CAT), and Acetylcholinesterase (AChE). The samples were kept at -80ºC until the biochemical marker analysis was carried out (Duarte et al., 2023).

A series of biomarkers were analyzed after the essay to assess the sub-individual effects of the AEA on test species. Unlike in studies with aquatic animals, such as fish, where tissues from target organs such as the liver are used, the organisms were entirely used due to their small size (Rodrigues et al., 2023; Santos et al., 2021). All biomarkers were performed using the Rodrigues et al. (2022) protocol except for acetylcholinesterase.

Total glutathione (TG) was quantified by reading the reactions of reduced glutathione with DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) at an absorbance of 412 nm for 3 minutes every 30 seconds and standard curves (concentrations of 10000 µM, 100 µM, 10µM, 1µM and 0.1 µM) were made with reduced glutathione to determine the concentrations in the samples. 4 replicates were made for each pipetted sample.

The levels of glutathione S-transferase (GST) were determined by reading the reaction of reduced glutathione with CDNB (1-chloro-2,4-dinitrobenzene) at 340 nm for 5 minutes with a 30-second interval and expressed as mol/min/mg with a molar extinction coefficient of ε = 9.63×10^{3} M^{-1} cm^{-1}. Only 2 replicates were made for each pipetted sample.

Catalase (CAT) activity was measured by the hydrogen peroxide reaction at an absorbance of 240 nm for 3 minutes and 4 replicates of each sample were pipetted. The calculations were made using a molar extinction coefficient of ε = 40 M^{-1} cm^{-1} and the results were expressed as µmol/min/mg.

The reaction of thiobarbituric acid with the products resulting from lipid peroxidation was read at an absorbance of 535 nm, revealing the levels of lipid peroxidation (LPO). BHT (2,6-Di-tert-butyl-4-methylphenol 4% methanol) was previously added to the samples to prevent further lipid peroxidation. Each sample was pipetted four times. The calculations of concentrations were made with the molar extinction coefficient of ε = $1.56 \times 10^{5}\ M^{-1}cm^{-1}$ and the results were expressed as nmol/mg.

The activity of the electron transport system (ETS) was read for 6 min at an absorbance of 490 nm with 30-s intervals and shaking. A mixed solution of NADH-β-NADH and NADPH-β-NADPH with the Buffered substrate solution was made and pipetted to the microplate after the four samples replicates together with the Iodonitrotetrazolium chloride (INT) solution. The calculations were made with a molar extinction coefficient of ε = 1.59×10^{4} M^{-1} cm^{-1} and expressed as nmol/min/mg.

For the LDH activity, 2 replicates of each sample were pipetted, and the reading was made at an absorbance of 340 nm each 20 seconds for 5 minutes. Calculations were made with ε = 9.6×10^{3} M^{-1} cm^{-1} and expressed as nmol/min/mg.

Acetylcholinesterase (AChE) levels were obtained following the Silva et al. (2021) protocol by measuring the microplates, with 4 replicates, over a period of 3 minutes at an absorbance of 414 nm with

30-s intervals for shaking. The molar extinction coefficient of ε = 13.6×10^3 M^{-1} cm^{-1} as used for the concentration´s calculation and expressed as nmol/min/mg.

Protein levels were determined in sample homogenates adapted to microplates and using γ-globulin as a standard. The absorbance at 600 nm was measured at 25 ºC after 15 min incubation. The levels of proteins were expressed as mg/mL for calculation purposes and mg/g of organism's weight.

All absorbances were read at a temperature of 25 ºC using a Thermo Scientific Multiskan Sky microplate spectrophotometer.

2.8 Statistical analysis

All the data analysis was performed using the R studio program version 4.3.1 (Rstudio Team, 2023). The Shapiro-Wilk test was conducted to evaluate the normality and to test for homoscedasticity. As none of the data samples followed a Gaussian distribution, a Kruskal-Wallis test was used to compare the means of the different groups. If there were any significant differences, a posteriori Dunn's test assessed differences between groups. Significance was set at <0.05 for the p-value.

3 Results

3.1 Chemical characterization of the ash and ash extract (AEA)

The analyzed ash samples were characterized by their origin from a high-severity wildfire and their light coloration, which reflects their composition. Among the chemical elements present in the ash, the highest concentrations were observed for Fe, Mn, and Zn, ranging from 405 mg/kg (Zn) to 14000 mg/kg (Fe). In contrast, the Aqueous Extracts of Ashes (AEA) samples were dominated by Fe, followed by Mn and Zn, with concentrations of 86000 µg/L, 9300 µg/L, and 2757 µg/L, respectively.

Out of the 16 priority elements analyzed, all concentrations were below the Limit of Quantification (LOQ), except for naphthalene (NAP) and in the ash samples, which was detected at a concentration of 0.17 mg/kg. This suggests that the instruments could not detect concentrations of most elements below their respective threshold values while the sample was analyzed.

Table 2: Quantification of metals and PAHs in ash samples (mg/kg) and in the Aqueous Extracts of Ashes (AEA) (µg/l).

Elements	Ashes (mg/kg)	AEA (µg/l)
Metals		
V	49	301
Cr	26	165
Mn	1800	9300
Fe	14000	86000
Co	5.9	65
Ni	23	117
Cu	66	507
Zn	405	2757
As	7	46
Cd	2.3	11
Pb	90	540
PAHs		
Naphthalene (NAP)	0.17	<0.04
Acenaphthylene (ACY)	<0.04	<0.04
Acenaphthene (ACE)	<0.04	<0.1
Fluorene (FLU)	<0.04	<0.010
Phenanthrene (PHE)	0.06	<0.02
Anthracene (ANT)	<0.04	<0.01
Fluoranthene	<0.04	<0.02
Pyrene (PYR)	<0.04	<0.06
Benzo(a)anthracene (BaA)	<0.04	<0.04
Chrysene (CHR)	<0.04	<0.02
Benzo(b)fluoranthene (BbF)	<0.04	<0.05
Benzo(k)fluoranthene (BkF)	<0.04	<0.05

Benzo(a)pyrene (BaP)	<0.04	<0.03
Dibenzo(a,h)anthracene (DBA)	<0.04	<0.04
Benzo(g,h,i)perylene (BGP)	<0.04	<0.03
Indeno (1,2,3-Cd) pyrene (IND)	<0.04	<0.04
PAHs (Total)	<0.23	<0.9

3.2 Assays with *Proasellus assaforensis*

3.2.1 pH and dissolved oxygen

The dissolved oxygen and pH values were measured at the beginning and end of the experimental test in all the concentration groups, including the control (**Supplementary Table 2**). An average decline of 12% in dissolved oxygen was observed in the dilutions of the AEA.

3.2.2 Mortality

Figure 6 illustrates the daily mortality of *P. assaforensis* over the 96-hour test period. The recorded mortality of *P. assaforensis* exposed to ashes was consistently below 20% (equivalent to a maximum of 5 organisms). Notably, no mortality was observed in the low-concentration, high-concentration, or control groups throughout the experimental period.

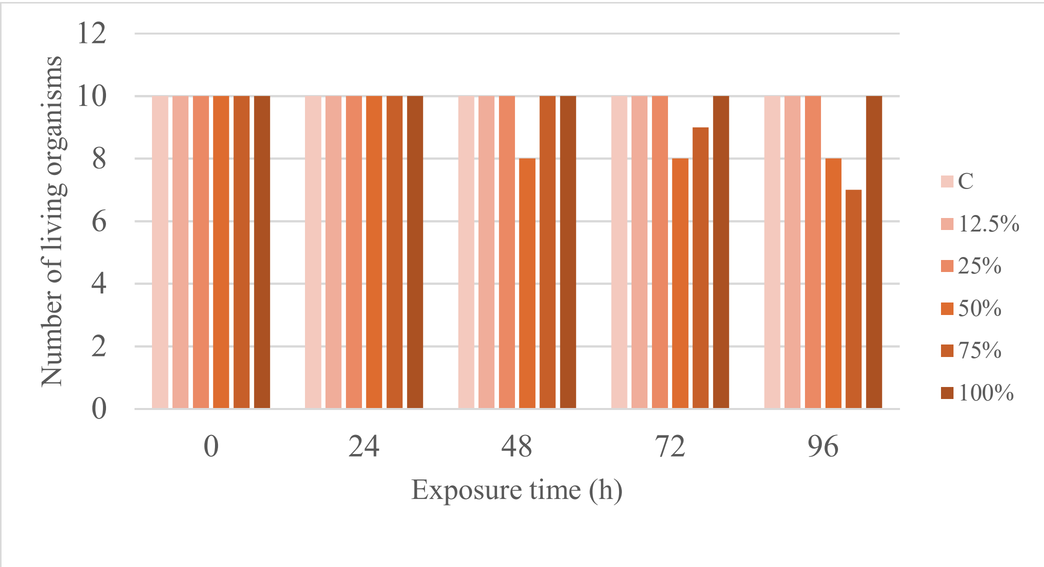

Figure 6: Daily mortality of *P. assaforensis* during the test period.

3.2.3 Biomarker analysis

Levels of lipid peroxidation (LPO) were significantly increased (χ^2= 14.7435, df =5, p = 0.0115) in organisms exposed to 2.5g/L (p = 0.0119), 5 g/L (p = 0.0058) and 10 g/L (p = 0.0008) compared to the control group (**Figure 7D**). In contrast, levels of total glutathione (TG) (χ^2= 3.5898, df = 5, p = 0.6098), glutathione S-transferase (GST) activity (χ^2 = 4.6928, df = 5, p = 0.4545) and catalase (CAT) activity (χ^2= 5.6298, df = 5, p = 0.3439) did not show significant differences across any of the tested AEA concentrations compared to the control group (**Figure 7A**, **Figure 7B**, **Figure 7C**, respectively).

The electron transport system (ETS) activity also showed no significant variation when compared to the control group (p = 0.1131) (**Figure 8A**). Lactate dehydrogenase (LDH) activity was significantly increased (χ^2 = 18.048, df = 5, p = 0.0028) with the rise in concentration and significant differences were detected between the control group and all the other test groups (1.25 g/L: p = 0.0114; 2.5 g/L: p = 0.0003; 5 g/L: p = 0.0012; 7.5 g/L: p = 0.0033; 10 g/L : p = 0.0001). Organisms exposed to 2.5 g/L and 10 g/L displayed higher levels of LDH than the other concentrations (**Figure 8B**).

Acetylcholinesterase (AChE) activity did not exhibit significant differences across all concentration groups compared to the control (p = 0.3184) (**Figure 9**). Similarly, protein levels (expressed as mg/g of weight) remained stable, with no significant variation observed during the test period (p = 0.3887) (**Figure 10A**).

3.2.4 Weight

Regarding organism weight, no significant changes were detected after exposure to AEA, as shown in **Figure 10B**. This consistency in weight validates the experimental test, as there were no adverse effects on the organisms' overall mass during the exposure period.

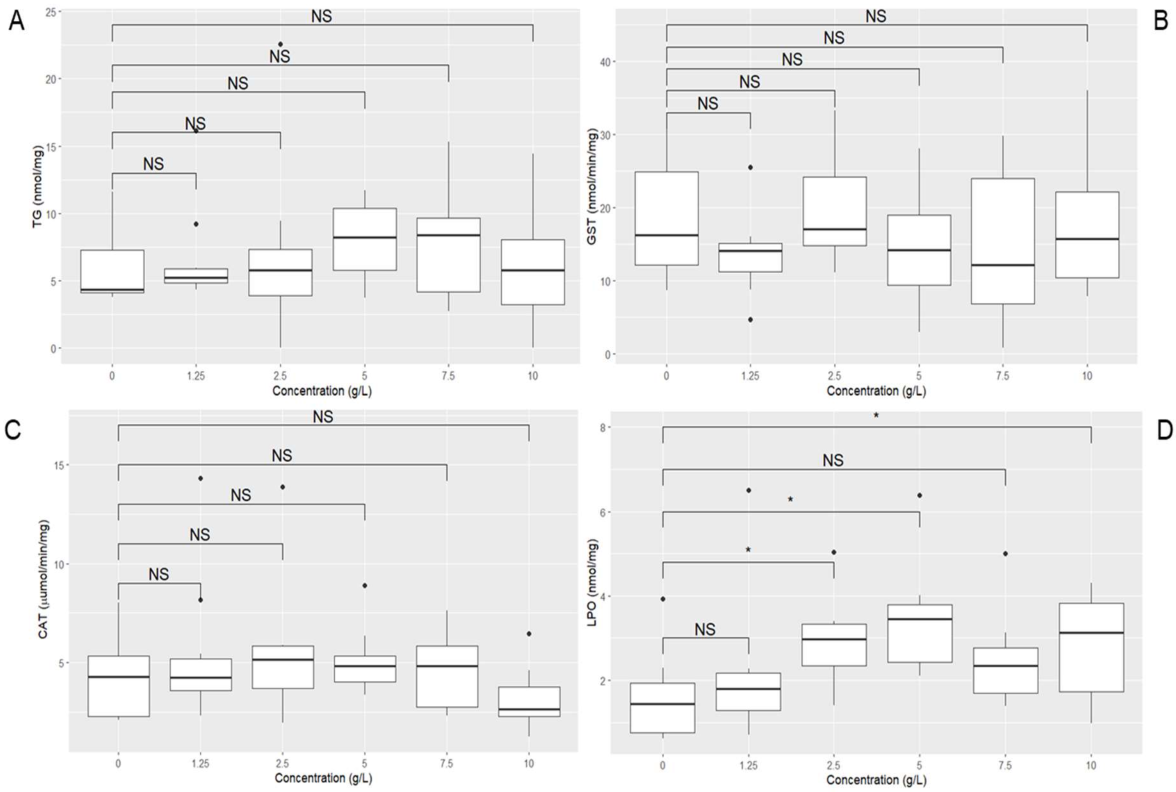

Figure 7: Total glutathione level (TG; A), glutathione S-transferase (GST; B) and catalase (CAT; C) activities, and lipid peroxidation (LPO; D) in *Proasellus assaforensis* after 96 h exposure to water containing 0 (Control), 1.25, 2.5, 5,7.5 and 10 g/L of wildfire ashes.

Values represent the average (± standard deviation) for 10 organisms per condition. *Asterisks* (*) indicate significant differences compared to the control group (p-value < 0.05), while "NS" denotes no significant differences. Black circles represent outliers.

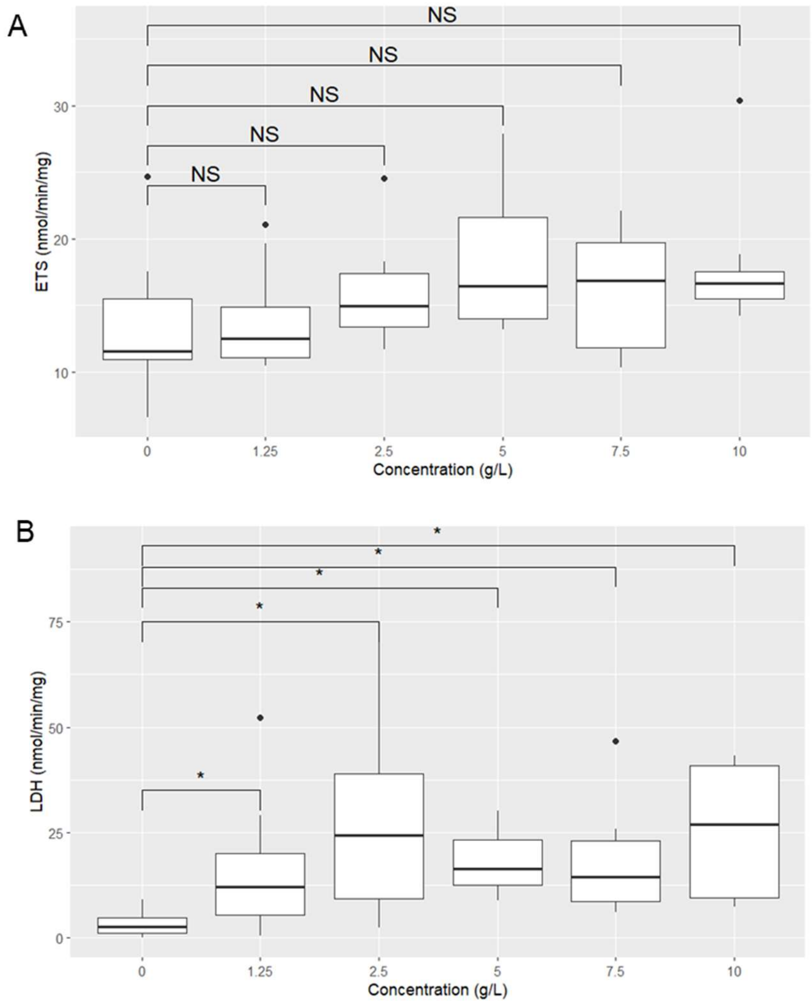

Figure 8: Electron Transport System activity (ETS; A), Lactate dehydrogenase (LDH; B) in *Proasellus assaforensis* after 96h exposure to water containing 0 (Control), 1.25, 2.5, 5,7.5 and 10 g/L of wildfire ashes.
Values represent the average (± standard deviation) for 10 organisms per condition. *Asterisks*(*) indicate significant differences compared to the control group (p-value < 0.05), while "NS" denotes no significant differences. Black circles represent outliers.

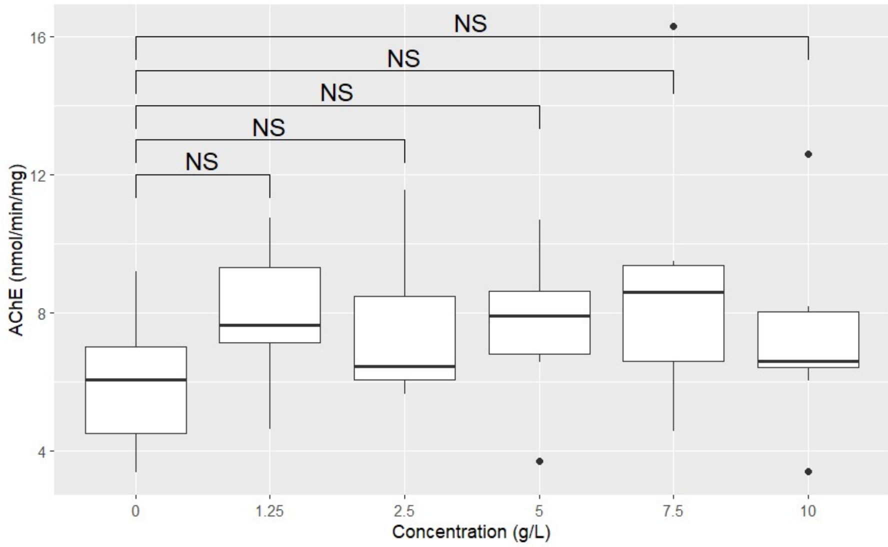

Figure 9: Acetylcholinesterase in *Proasellus assaforensis* after 96 h exposure to water containing 0 (Control), 1.25, 2.5, 5,7.5, and 10 g/L of wildfire ashes. The values represent the average (± standard deviation) for 10 organisms per condition. *Asterisks* (*) indicate significant differences compared to the control group (p-value < 0.05), while "NS" denotes no significant differences. Black circles represent outliers.

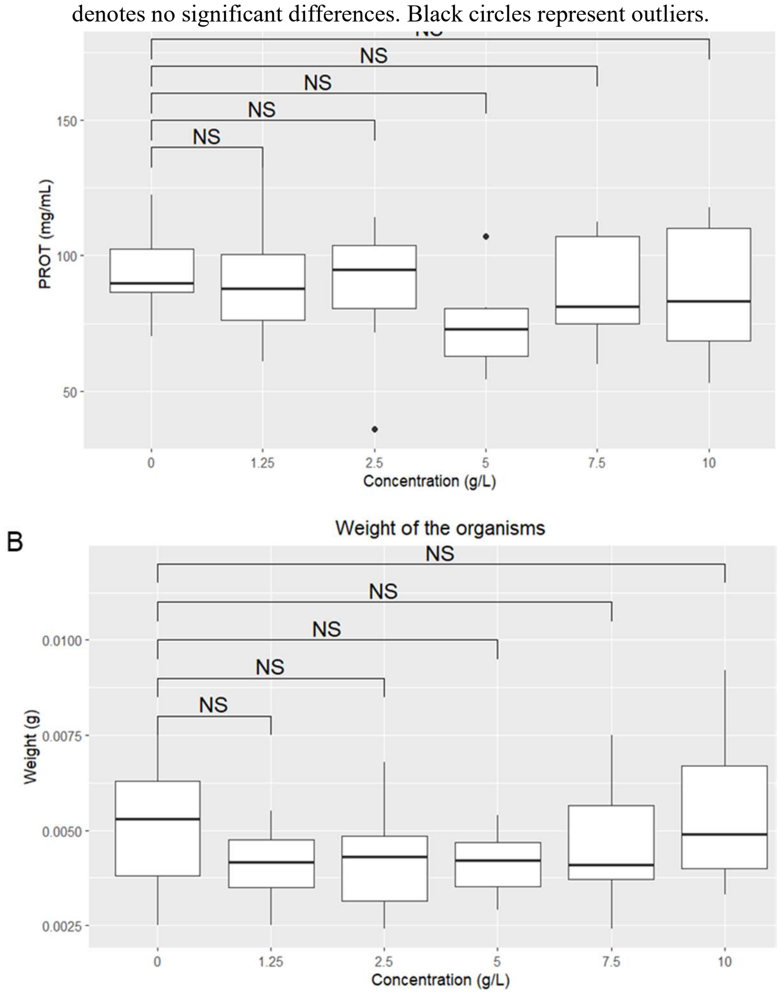

Figure 10: Protein levels (PROT; A), weight values (B) in *Proasellus assaforensis* after 96 h exposure to water containing 0 (Control), 1.25, 2.5, 5,7.5 and 10 g/L of wildfire ashes. Values represent the average (± standard deviation) for 10 organisms per condition. *Asterisks* (*) indicate significant differences compared to the control group (p-value < 0.05), while "NS" denotes no significant differences. Black circles represent outliers.

3.2.5 Ventilatory activity

This species had no significant differences in pleopod activity between the concentration groups and the control after exposure to ash, according to **Figure 11** .

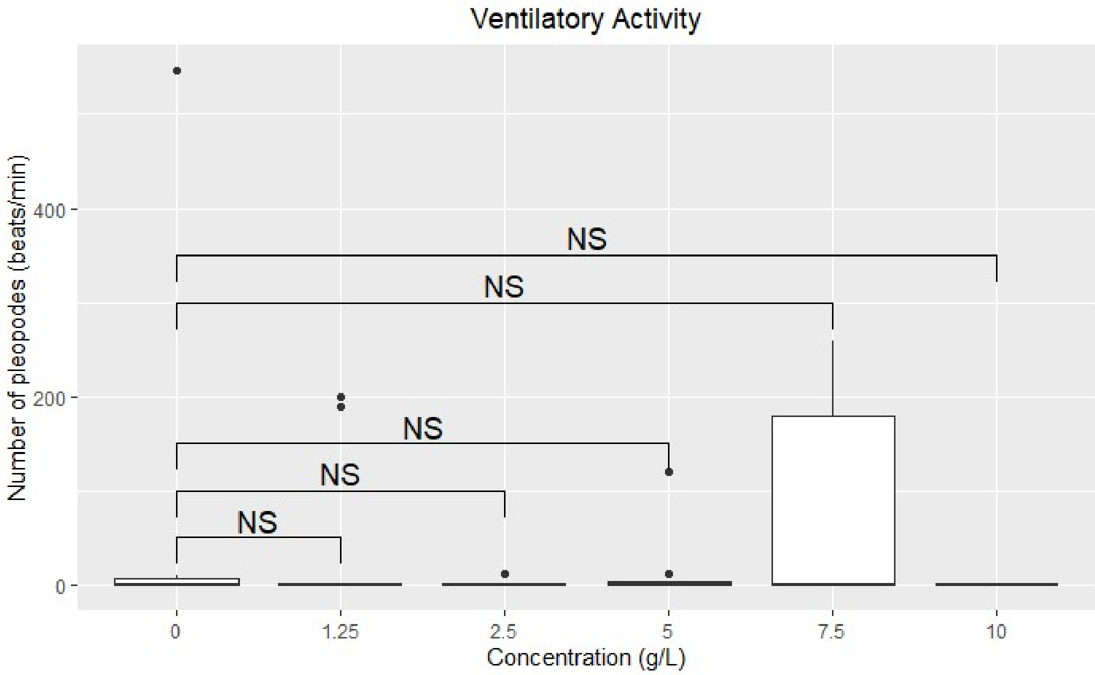

Figure 11: Ventilatory activity in *Proasellus assaforensis* after 96 h exposure to water containing 0 (Control), 1,25, 2,5, 5,7.5 and 10 g/L of wildfire ashes. Values represent the average (± standard deviation) for 10 organisms per condition. *Asterisks* (*) indicate significant differences compared to the control group (p-value < 0.05), while "NS" denotes no significant differences. Black circles represent outliers.

3.3 Assays with *Proasellus lusitanicus*

3.3.1 pH and dissolved oxygen

The dissolved oxygen and pH values were measured at the beginning and end of the experimental test in all the concentration groups, including the control (**Supplementary Table 3**). An average decline of 10% in dissolved oxygen was observed in the dilutions of the AEA.

3.3.2 Mortality

Figure 12 illustrates the daily mortality of *P. lusitanicus* over the 96-hour test period. The recorded mortality of *P. lusitanicus* exposed to ashes was consistently below 20% (equivalent to a maximum of 12 organisms). No mortality was observed in the control group throughout the entire experimental period. However, the 5g/L concentration group of ash extract had the highest levels of deaths, followed by the 7.5g/L and the 10g/L concentration groups during the entire period of 96 hours.

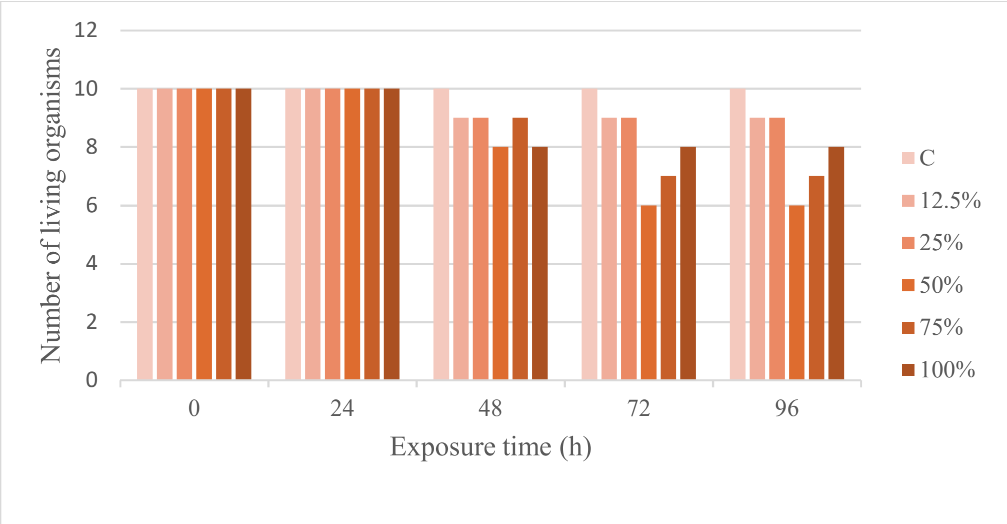

Figure 12: Daily mortality of *P. lusitanicus* during the test period.

3.3.3 Biomarker analysis

Catalase activity significantly decreased (χ^2= 22.4210, df = 5, p = 0.0004) in *P. lusitanicus* exposed to all ash concentrations compared to control (1.25 g/L: p = 0.0018; 2.5 g/L: p = 0; 5 g/L: p = 0.0103; 7.5 g/L: p = 0.0022; 10 g/L: p = 0.0018) (**Figure 13C**).

Levels of lipid peroxidation were significantly increased (χ^2= 22.0545, df = 5, p = 0) in organisms exposed to 1.25 (p = 0.0337), 5 (p = 0.0471), 7.5 (p = 0.0226) and 10 g/L (p = 0) when compared to control. No differences (p = 0.00051) were noted between the control and the group exposed to 2.5 g/L ashes (**Figure 13D**).

The levels of total glutathione and the glutathione S-transferase activity were not significantly altered (p = 0.1483 and p = 0.5949, respectively) in *P. lusitanicus* exposed to any of the concentrations of ash tested (**Figure 13A** and **Figure 13 B**).

The activity of the electron transport system showed a significant increase in organisms exposed to 1.25 g/L (χ^2=11.3377; df = 5; p = 0.0065) ashes, but not to higher concentrations when compared to the control group (**Figure 14A**). Also, the activity of lactate dehydrogenase was not significantly altered (χ^2 = 7.4109, df = 5, p = 0.1918) in any of the concentration groups tested (**Figure 14 B**).

The activity of acetylcholinesterase showed no significant changes across concentrations (p = 0.4094) (**Figure 15**).

Protein levels significantly decreased (χ^2=20.9235, df = 5, p = 0) in organisms exposed to 1.25 (p < .0001), 2.5 (p = 0.0083), 5 (p = 0.0028), and 7.5 g/L (p = 0.0175) compared to the control (**Figure 16A**).

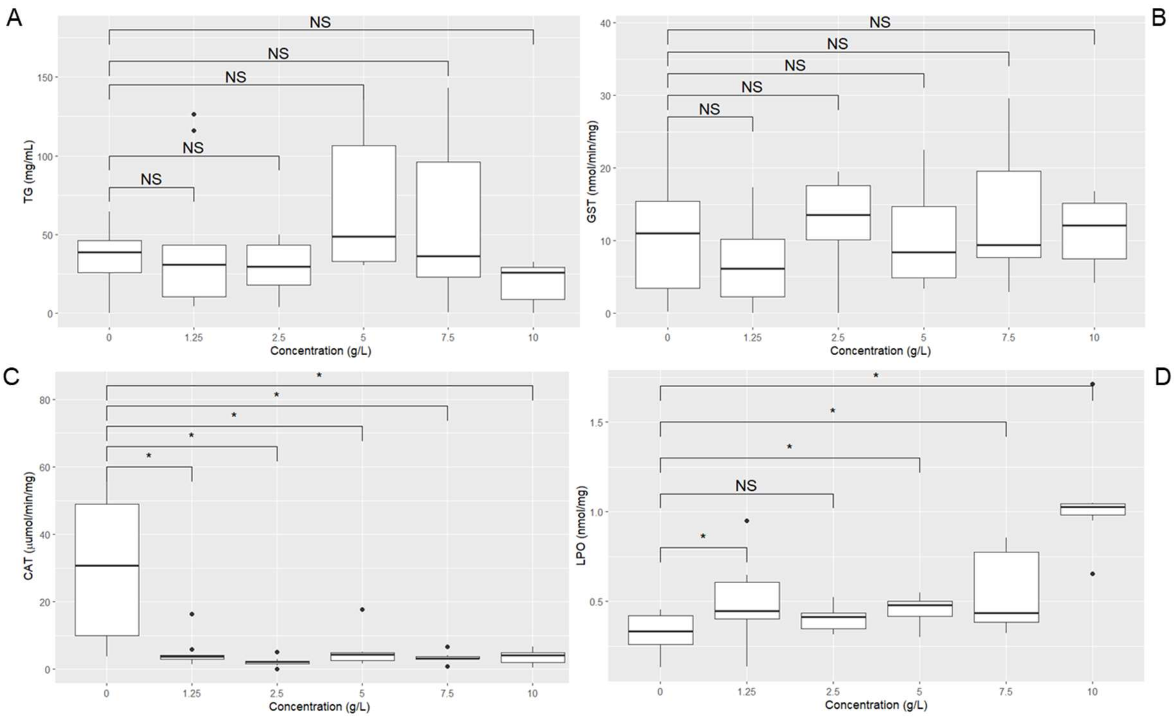

Figure 13: Total glutathione level (TG; A), glutathione S-transferase (GST; B) and catalase (CAT; C) activities, and lipid peroxidation (LPO; D) in *Proasellus lusitanicus* after 96 h exposure to water containing 0 (Control), 1.25, 2.5, 5,7.5 and 10 g/L of wildfire ashes.
Values represent the average (± standard deviation) for 10 organisms per condition. *Asterisks* (*) indicate significant differences compared to the control group (p-value < 0.05), while "NS" denotes no significant differences. Black circles represent outliers.

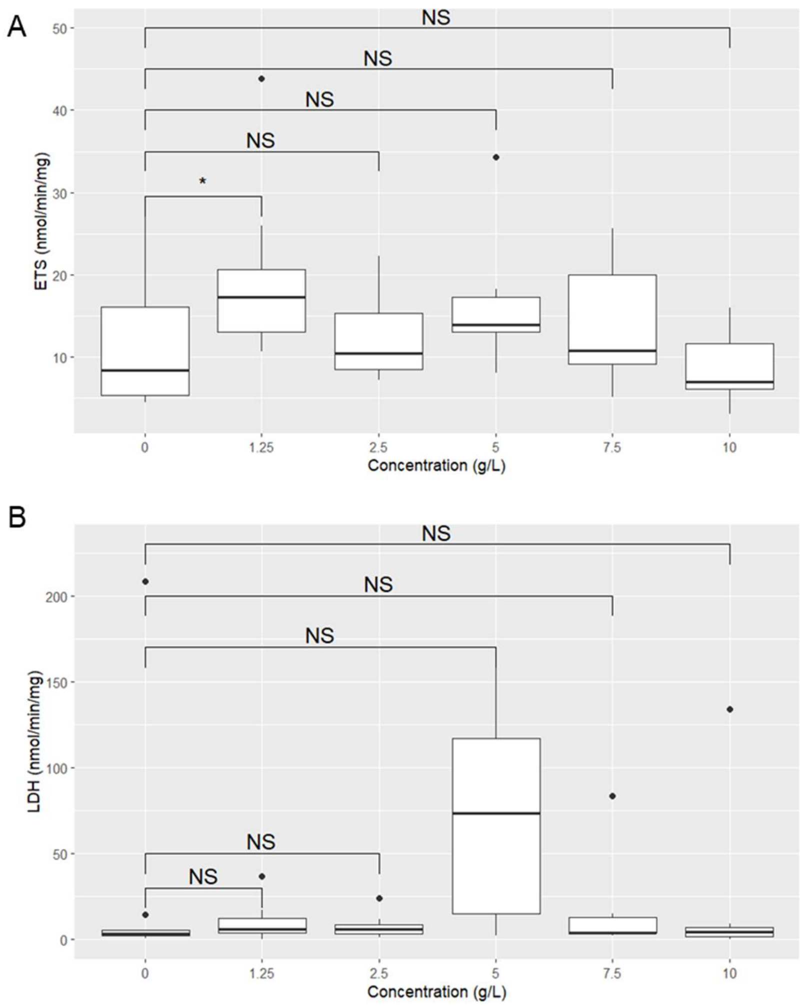

Figure 14: Electron Transport System activity (ETS; A), Lactate dehydrogenase (LDH; B) in *Proasellus lusitanicus* after 96 h exposure to water containing 0 (Control), 1.25, 2.5, 5,7.5 and 10 g/L of wildfire ashes.
Values represent the average (± standard deviation) for 10 organisms per condition. *Asterisks* (*) indicate significant differences compared to the control group (p-value < 0.05), while "NS" denotes no significant differences. Black circles represent outliers.

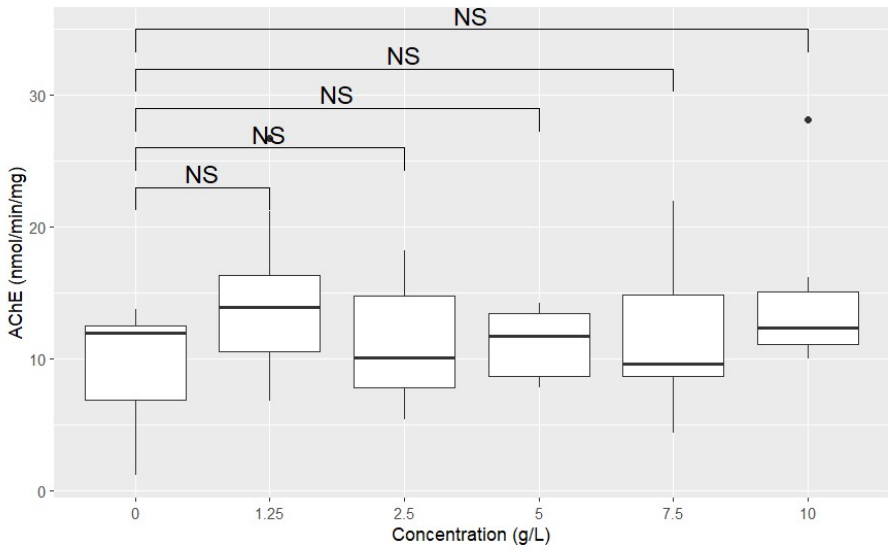

Figure 15: Acetylcholinesterase in *Proasellus lusitanicus* after 96 h exposure to water containing 0 (Control), 1.25, 2.5, 5,7.5 and 10 g/L of wildfire ashes. Values represent the average (± standard deviation) for 10 organisms per condition. *Asterisks* (*) indicate significant differences compared to the control group (p-value < 0.05), while "NS" denotes no significant differences. Black circles represent outliers.

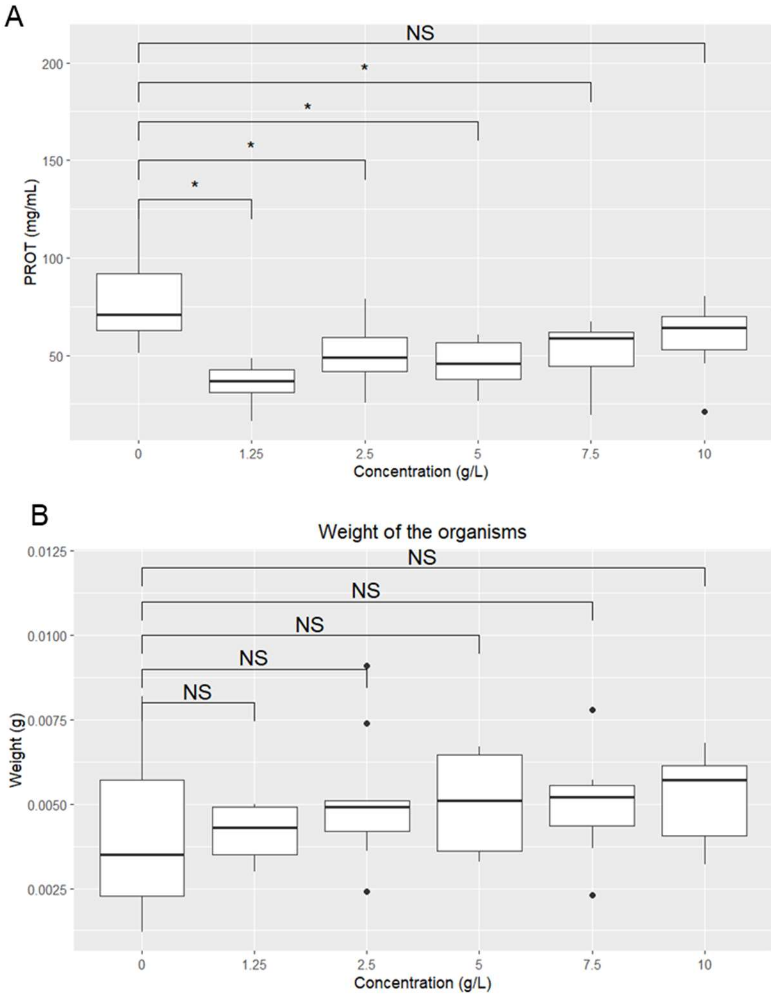

Figure 16: Protein levels (PROT; A), weight values (B) in *Proasellus lusitanicus* after 96 h exposure to water containing 0 (Control), 1.25, 2.5, 5,7.5 and 10 g/L of wildfire ashes. Values represent the average (± standard deviation) for 10 organisms per condition. *Asterisks* (*) indicate significant differences compared to the control group (p-value < 0.05), while "NS" denotes no significant differences. Black circles represent outliers.

3.3.4 Ventilatory activity

The pleopods' beating (ventilator activity) was significantly decreased in organisms exposed to 5, 7.5, and 10 g/L ($\chi^2 = 15.8931$, df = 5, p = 0.01) (**Figure 17**).

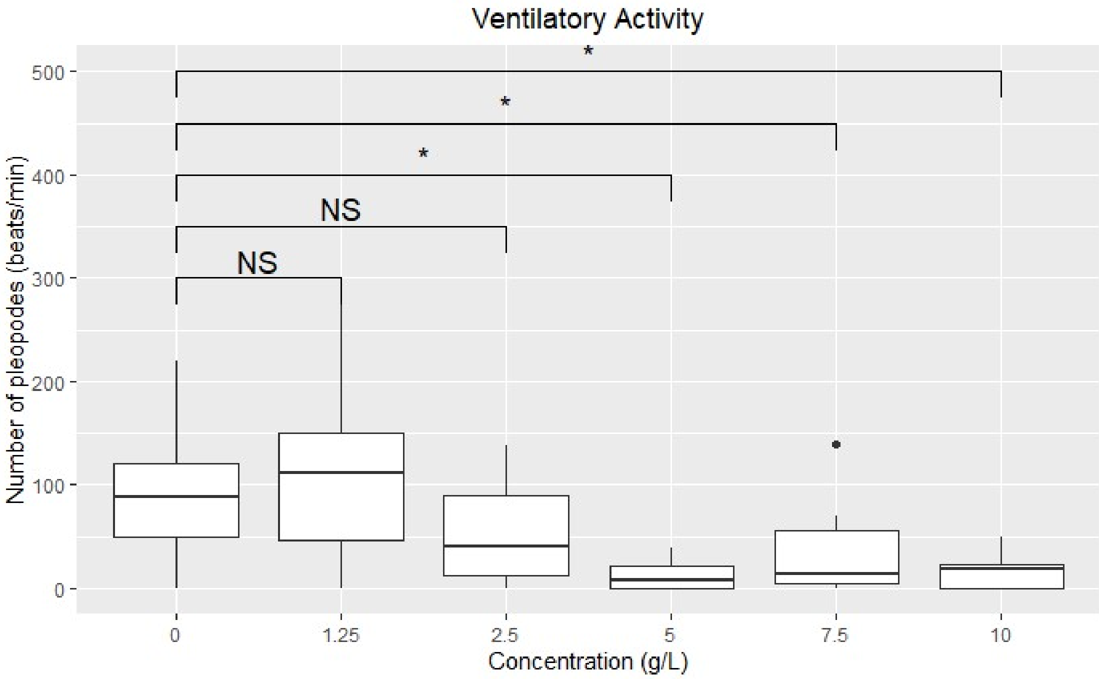

Figure 17: Ventilatory activity in *Proasellus lusitanicus* after 96 h exposure to water containing 0 (Control), 1.25, 2.5, 5,7.5 and 10 g/L of wildfire ashes. Values represent the average (± standard deviation) for 10 organisms per condition. *Asterisks* (*) indicate significant differences compared to the control group (p-value < 0.05), while "NS" denotes no significant differences. Black circles represent outliers.

4 Discussion

This study shows the pernicious effects of post-wildfire contamination in groundwater-adapted species. The biomarker analysis assessed on two sister species of stygobiotic crustaceans of the genus *Proasellus*, but with different degrees of adaptation to groundwater, revealed that both were facing an oxidative stress condition when exposed to ashes. The effect concentration of ashes inducing stress was lower in the most adapted species to groundwater, i.e., *P. lusitanicus*. Those differences in sensitivity presented by sister species after exposure to ashes contrast with a similar sensitivity pattern exhibited after lethal exposure to copper and potassium (Reboleira et al., 2013b). This study emphasizes the importance of accounting for the mixture of toxicity and ash particles' physical presence when comparing species sensitivities to wildfire ashes. Furthermore, the biomarker responses demonstrated distinct metabolic strategies of *P. lusitanicus* and *P. assaforensis* as a consequence of their degree of adaptation to the groundwater environment.

Ashes characterization

Wildfire ash can be defined as heterogeneous material that is influenced by multiple factors such as soil type, plant species, vegetation composition and burned part, fire temperature, and combustion completeness (Bodí et al., 2014; Demeyer et al., 2000.; Goforth et al., 2005). The post-wildfire runoff samples from *Arbutus unedo* showed higher concentrations of metals than PAHs in both particulate and dissolved phases, reflecting the predominance of metals typically observed in high-severity occurrences (Campos et al., 2012a; Etiégni & Campbell, 1991; Goforth et al., 2005). These facts are in line with the results obtained in this study. None of the 16 PAHs analyzed showed concentrations above the detection limit (DL) in the AEA samples, which showed that organic compounds were completely combusted due to the severity of the wildfire. In the analysis of high-severity occurrences of *Pinus* and *Eucalyptus,* Pradhan et al. (2020) observed a similar trend: only one compound was detected in the dissolved fraction samples, while the others remained below the DL.

Regarding the metal composition, Fe exhibited the highest concentration in the AEA samples, followed by Mn and Zn, as can be compared with the standard values in the **Supplementary Table 1**. These findings align with previous studies highlighting the prevalence of these metals in post-fire runoff (Mesquita et al., 2022; Muñiz González et al., 2023; Nunes et al., 2017; Pradhan et al., 2020; Santos et al., 2023). Mn, in particular, is commonly found in the form of oxides and derives from burnt vegetation (Parra et al., 1996). Its high concentration is often associated with the foliage of coniferous trees (Costa et al., 2014; Parra et al., 1996); however, this does not apply to the present study, as the ashes were collected from a shrubland area dominated by *A. unedo*.

It is important to note that while identifying individual toxic elements in ash samples is essential, the potential synergistic or antagonistic interactions between organic and inorganic compounds must also be considered (Crémazy et al., 2018). For that, the toxicity observed in this study cannot be attributed solely to the group of elements with the highest concentrations. Instead, it is crucial to recognize that a mixture of elements contributes to the overall harmful impact on organisms, even though little is known about the potential interactions and combined effects of these metals in aquatic animals (De Oliveira et al., 2018). Although very low concentrations of organic compounds were recorded in the present work, the interactions between all the elements and compounds in the mixture and their effects on the target organisms should not be overlooked. Moreover, other factors must also be considered, such as the presence of ashes as particles and/or the water-induced pH changes.

Effects of ashes on organisms

Studies have been carried out on various organisms, and all have concluded that the ashes from wildfires have a deleterious impact on the most diverse organisms from different ecosystems. Effects of post-fire runoffs on aquatic organisms were evaluated on microbial decomposer communities (Carvalho et al., 2019), aquatic primary producers (Campos et al., 2012b; Charette & Prepas, 2003; Cowell et al., 2006; Mesquita et al., 2022; Ré et al., 2020; Silva et al., 2015), amphibians (Pilliod et al., 2003; Santos et al., 2023) and fishes (Brito et al., 2017; Kwan et al., 2024; Nunes et al., 2017). Despite the objective of assessing the effects of ashes on two sister species with different degrees of adaptation to the groundwater ecosystem, this thesis aimed to determine why those species are susceptible to water contamination by ashes and whether the degree of adaptation influences their sensitivity. Therefore, the novelty of this research work bears on the physiological responses evaluated on those two stygobiotic species.

In the present study, the aqueous extract of ashes (AEA) significantly increased LPO in both species, showing evidence of oxidative stress and pessimal health due to increased reactive oxygen species (ROS) and limited antioxidant defenses. Metals such as Fe, Mn, Zn, Cu, Pb, V, Cr, Ni, Cd, and Co, as well as PAHs, are known for inducing oxidative stress in various organisms when the antioxidant capacity and/or repair mechanisms are not enough to cope with increased levels of ROS (Amiard-Triquet et al., 2012; Pinto et al., 2003). However, current results show that the physiological responses of both species linked to a disease state were very different.

P. lusitanicus, exposed to ashes, showed a decrease in CAT activity for all the tested concentrations, which might lead to oxidative damage due to an incapacity to cope with increased ROS. This agrees with previous reports (Pinto et al., 2003). Moreover, the inhibition of CAT activity and the increase in LPO levels may be attributed to the presence of metal elements in the ash samples, which contributed to the negative impacts on the functional responses of both species. Beyond that, this effect may also be attributed to the binding of metal ions to the enzyme's –SH groups, compromising its ability to decompose hydrogen peroxide, thus allowing ROS, including superoxide anions (O_2^-), to accumulate (Atli et al., 2006). A similar result was observed in a study with *Esomus danricus,* which was exposed to low concentrations of copper, showing alterations in CAT activity and elevated LPO levels (Vutukuru et al., 2006). The increased LPO activity at a later stage was attributed to the cumulative effects of oxidative stress induced by copper over the test duration (Vutukuru et al., 2006). The extended increase in LPO observed is inconsistent with the findings of this investigation since microcrustaceans, which are more susceptible than organisms such as *E. danricus,* were used. The same results were seen with the crustacean *Macrobrachium nipponense*, which registered an inhibition of CAT, with oxidative damage associated with the highest tested concentration of Cd (0.04 mg/l)(Wang et al., 2021). In addition, it has been reported that *Notopterus notopterus* exposed to metal-contaminated water (Fe, Cu, Ni, Cd, Pb, and Zn) exhibited elevated levels of LPO and protein carbonyls, which are indicative of lipid and protein oxidation, and heightened ROS levels in tissues, leading to oxidative stress (Mohanty & Samanta, 2016). Nevertheless, *P. assaforensis* exposed to the lowest concentration of AEA (1.25 g/L) could avoid oxidative damage to lipids by triggering the backup anaerobic energy production, as seen through an increased LDH activity. However, acquiring energy through anaerobic metabolism was insufficient to avoid oxidative stress when exposed to higher AEA concentrations. According to Sokolova et al. (2012), aerobic energy production serves as a backup mechanism that usually represents an extreme stress condition, which seems to be the case for *P. assaforensis* exposed to concentrations of ashes higher than 1.25 g/L. Thus, this species appears to employ a different strategy, which increases

LDH, indicating higher lactate levels in the internal fluids, but does not entirely prevent oxidative damage. Also, increased levels of LDH result from anaerobic energy production because of aerobic insufficient production to cope with the noxious effects of contaminants. In a study conducted with *Carassius auratus gibelio* exposed to various concentrations of Cu, a stimulation of LDH was observed, which may indicate a dependence on anaerobic metabolism (Teodorescu et al., 2012). In low oxygen conditions, *C. auratus gibelio*'s body switches its respiratory metabolism to anaerobiosis, converting pyruvate to lactate, which increases LDH activity. Blood carries lactate to the liver, which is reconverted into glucose and glycogen to satisfy the increased energy needs under physiological stress (Teodorescu et al., 2012). Similarly to *C. auratus gibelio*, *P. assaforensis* depends on anaerobic metabolism, evidenced by increased LDH activity and lactate production, to sustain its energy needs under contaminant-induced stress. This resemblance suggests a common physiological response mechanism across these organisms when faced with high contamination levels. Moreover, increasing the anaerobic production of energy in *P. assaforensis* might also allow organisms to deal with the alkalinization of water due to contamination by ashes. The alkalinization of the water by ash can be seen in **Supplementary Table 3**, in which there is an increase in pH in the test groups compared to the control group. To counteract this phenomenon, the rise in LDH seen in the organisms, which means high levels of lactate in the fluids, triggers the production of pyruvate, NADH+, and H+, balancing the pH of the organisms' hemolymph, as occurred in a study carried out with Chinook salmon (*Oncorhynchus tshawytscha*) when exposed to ash from wildfires (Kwan et al., 2024).

Despite the previous findings for *P assaforensis*, the oxidative stress condition observed in *P. lusitanicus* was not associated with the inhibition of CAT activity, which means that ROS levels increased due to other causes. Moreover, *P. lusitanicus* could not trigger the anaerobic production of energy by activating the activity of LDH. If there is no production of lactate, there is no production of compounds that help to balance pH levels. Also, the acquisition of anaerobic energy needed to cope with the effects of ashes was not attained in exposed *P. lusitanicus,* which might explain the higher sensitivity of this species as observed with the increased levels of LPO for all the concentrations tested and despite the unchanged activities of CAT. Moreover, during the experiments, this species showed a higher mortality which was expected, as the organisms experienced extreme oxidative damage and could not cope with it. However, in *P. lusitanicus*, an extreme stress condition was impossible to determine based on increased LDH activity, following the report of Sokolova et al. (2012). The impossibility of *P. lusitanicus* to trigger the anaerobic production of energy is the leading cause of the previous fact. Therefore, the less sensitivity of *P. assaforensis* might also be linked to a more efficient acid-base regulation of the internal fluids compared to *P. lusitanicus*. The overall results and the differences observed concerning the activation of LDH suggest that oxidative stress may be associated with metals and PAHs present in the ashes and other stress factors, such as the alkalinization of the aquatic environment.

A notable difference between species was seen in their physiological responses to stress. *P. lusitanicus* responded to ash exposure by decreasing the ventilatory activity, suggesting an overall inhibition rather than an adaptive effort to acquire energy. Unlike *P. assaforensis*, which significantly increased LDH activity showing an extreme stress condition, *P. lusitanicus* did not enhance anaerobic energy production. This lack of metabolic response might be linked to an inability to trigger anaerobic energy production due to stable abiotic conditions in caves, such as temperature, dissolved oxygen, pH, limiting its ability to cope with sudden environmental changes.

P. lusitanicus decreased ventilatory activity when exposed to ashes, as an anergy-saving strategy to cope with the extreme toxicity of the exposure. Oxygen was still required for aerobic energy production to support detoxification and antioxidant defenses, but that was not verified. For that, the activity of ETS remained unchanged in exposed organisms. This decreased of ventilatory activity in *P. lusitanicus* might also explain the oxidative damage to lipids observed for all the concentrations tested due to the increased production of ROS. Moreover, this also increments the effects of ashes due to an induced pH increase. In fact, *P. lusitanicus* could not only counteract the alkalinization of water using anaerobic production of energy but tended to increase the pH of its internal fluids due to aerobic production. In addition to the regulation of pH that might play a part in the effects induced by ashes in the water, the decrease in ventilatory activity in *P. lusitanicus* does not exclude the possibility that ash particles might also interfere with the ventilation of the breathing organs.

The presence of ashes in aquatic environments and the consequent energetic demands concerning energy acquisition, allocation, detoxification, and damage repair might compromise the growth, behavior, and/or reproduction of aquatic organisms due to multiple factors (chemicals and abiotic, but also physical). However, the adaptation of subterranean species that seems to have a lower capacity to trigger anaerobic metabolism than surface ones might be a reason for their increased susceptibility to stress factors, i.e., at least PAHs, metals, pH, dissolved oxygen, and temperature.

Impacts of ashes on groundwater species

While the impacts of wildfires on surface water environments are well-documented, this study has significantly advanced our understanding of their effects on subterranean ecosystems. Like surface aquatic systems, underground aquatic organisms are vulnerable to wildfire impacts, as post-fire contaminants can leach into groundwater. This contamination may disrupt subterranean ecosystems and the vital ecosystem services they provide. (Mammola et al., 2019a). The two species used in this study, *P. lusitanicus* and *P. assaforensis*, were shown to be at risk when exposed to ash contamination. However, their sensitivity depended on the disruption of anaerobic metabolism, particularly in organisms fully adapted to groundwater environments. These species hold significant ecological importance due to their vital role in cleaning and filtering groundwater, which is essential for human consumption and other purposes (Reboleira et al., 2013b). Studying such habitats can be challenging due to the limited availability of samples and difficulties in accessing subterranean ecosystems (Castaño-Sánchez et al., 2021). Therefore, research in this field is increasingly crucial to support the development of effective conservation measures.

Although the present study contributed to the knowledge of the effects of wildfires on subterranean organisms, there is still much more to investigate. In particular, studying the baseline responsiveness of subterranean species by measuring LDH activity in a broader range of aquatic subterranean organisms and comparing it with surface species when exposed to harmful substances would provide valuable insights. Additionally, while studies on surface aquatic species often analyze the bioaccumulation of toxic elements, extending this approach to subterranean species would contribute to a more comprehensive understanding of these ecosystems. Furthermore, it is crucial to recognize that the impact of such toxic compounds extends beyond aquatic organisms to include other species, such as cave-dwelling terrestrial organisms. A key question arises: Would these terrestrial organisms respond similarly?

5 Final considerations and future research perspectives

This dissertation demonstrates that wildfire ash exerts sublethal effects on groundwater crustacean species, as evidenced by the oxidative stress observed in the two stygobiotic crustaceans studied. Biomolecular analyses revealed physiological differences between the species, highlighting distinct capacities to respond to ash exposure. From an evolutionary perspective, these differences are rooted in their levels of adaptation to subterranean habitats. *P. lusitanicus*, a species fully adapted to the underground environment, thrives in highly stable habitats with sufficient oxygen levels to survive. Consequently, it has not developed a need for anaerobic mechanisms to produce backup energy during stress, leaving it more vulnerable to adverse conditions. In contrast, *P. assaforensis* is less specialized for subterranean conditions and has retained the ability to trigger anaerobic energy production under stress. This contrast underscores the importance of investigating the sensitivity of groundwater-adapted species to various stressors—not only from an ecotoxicological perspective but also to explore the evolutionary and physiological pathways that underpin their adaptation to stable environments. Specifically, future research should examine whether natural selection has consistently compromised anaerobic metabolism as an energy-saving adaptation to stable ecosystems.

Given the fragile yet invaluable nature of subterranean ecosystems and the essential resources and services they provide, it is imperative to advance research in this domain. A deeper understanding of life in subterranean environments will enable the development of targeted conservation measures to preserve these unique and vulnerable ecosystems.

6 References

Abrantes, N., Campos, I., Ré, A., & Keizer, J. J. (2017). An assessment of the toxicity of ash-loaded runoff. In António Bento-Gonçalves (Ed.), *Wildfires: Perspectives, Issues and Challenges of 21st Century* (281–300). Nova Science Publishers.

Afonso, O. (1988). Isopoda asellidae et amphipoda de la grotte d`Assafora (Portugal)- Description de *Proasellus assaforensis* sp. n. Bol. Soc. Port. Espel. *2*, 43-50

Almeida, J. R., Gravato, C., & Guilhermino, L. (2012). Challenges in assessing the toxic effects of polycyclic aromatic hydrocarbons to marine organisms: A case study on the acute toxicity of pyrene to the European seabass (*Dicentrarchus labrax* L.). *Chemosphere*, *86*(9), 926–937. https://doi.org/10.1016/j.chemosphere.2011.10.059

Amiard-Triquet, C. & Amiard, J.-C.(2013). Introduction. In C. Amiard-Triquet , J-C. Amiard & P. S. Rainbow. *Ecological Biomarkers* (1-14). CRC Press.

Amiard-Triquet, C. (2013). Pollution Tolerance. In C. Amiard-Triquet, M. Roméo & P. S. Rainbow. *Tolerance to Environmental Contaminants* (1-23).CRC Press.

Atli, G., Alptekin, Ö., Tükel, S., & Canli, M. (2006). Response of catalase activity to Ag+, Cd2+, Cr6+, Cu2+ and Zn2+ in five tissues of freshwater fish *Oreochromis niloticus*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *143*(2), 218–224. https://doi.org/10.1016/j.cbpc.2006.02.003

Baker, M. A., Cerniglia, G. J., & Zaman, A. (1990). Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Analytical Biochemistry*, *190*(2), 360–365. https://doi.org/10.1016/0003-2697(90)90208-Q

Bian, F., Coleborn, K., Flemons, I., Baker, A., Treble, P. C., Hughes, C. E., Baker, A., Andersen, M. S., Tozer, M. G., Duan, W., Fogwill, C. J., & Fairchild, I. J. (2019). Hydrological and geochemical responses of fire in a shallow cave system. *Science of The Total Environment*, *662*, 180–191. https://doi.org/10.1016/j.scitotenv.2019.01.102

Bladon, K. D., Emelko, M. B., Silins, U., & Stone, M. (2014). Wildfire and the Future of Water Supply. *Environmental Science & Technology*, *48*(16), 8936–8943. https://doi.org/10.1021/es500130g

Bodí, M. B., Martin, D. A., Balfour, V. N., Santín, C., Doerr, S. H., Pereira, P., Cerdà, A., & Mataix-Solera, J. (2014). Wildland fire ash: Production, composition and eco-hydro-geomorphic effects. *Earth-Science Reviews*, *130*, 103–127. https://doi.org/10.1016/j.earscirev.2013.12.007

Böhlke, J.-K. (2002). Groundwater recharge and agricultural contamination. *Hydrogeology Journal*, *10*(1), 153–179. https://doi.org/10.1007/s10040-001-0183-3

Boulton, A. J. (2005). Chances and challenges in the conservation of groundwaters and their dependent ecosystems. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *15*(4), 319–323. https://doi.org/10.1002/aqc.712

Bowman, D. M. J. S., Williamson, G. J., Abatzoglou, J. T., Kolden, C. A., Cochrane, M. A., & Smith, A. M. S. (2017). Human exposure and sensitivity to globally extreme wildfire events. *Nature Ecology & Evolution*, *1*(3), 0058. https://doi.org/10.1038/s41559-016-0058

Brito, D. Q., Passos, C. J. S., Muniz, D. H. F., & Oliveira-Filho, E. C. (2017). Aquatic ecotoxicity of ashes from Brazilian savanna wildfires. *Environmental Science and Pollution Research*, *24*(24), 19671–19682. https://doi.org/10.1007/s11356-017-9578-0

Cammen, L., Corwin, S., & Christensen, J. (1990). Electron transport system (ETS) activity as a measure of benthic macrofaunal metabolism. *Marine Ecology Progress Series*, *65*, 171–182. https://doi.org/10.3354/meps065171

Campos, I., Abrantes, N., Vidal, T., Bastos, A. C., Gonçalves, F., & Keizer, J. J. (2012). Assessment of the toxicity of ash-loaded runoff from a recently burnt eucalypt plantation. *European Journal of Forest Research*, *131*(6), 1889–1903. https://doi.org/10.1007/s10342-012-0640-7

Carvalho, A., Flannigan, M. D., Logan, K. A., Gowman, L. M., Miranda, A. I., & Borrego, C. (2010). The impact of spatial resolution on area burned and fire occurrence projections in Portugal under climate change. *Climatic Change*, *98*(1–2), 177–197. https://doi.org/10.1007/s10584-009-9667-2

Carvalho, F., Pradhan, A., Abrantes, N., Campos, I., Keizer, J. J., Cássio, F., & Pascoal, C. (2019). Wildfire impacts on freshwater detrital food webs depend on runoff load, exposure time and burnt forest type. *Science of The Total Environment*, *692*, 691–700. https://doi.org/10.1016/j.scitotenv.2019.07.265

Castaño-Sánchez, A., Hose, G. C., & Reboleira, A. S. P. S. (2020a). Ecotoxicological effects of anthropogenic stressors in subterranean organisms: A review. *Chemosphere*, *244*, 125422. https://doi.org/10.1016/j.chemosphere.2019.125422

Castaño-Sánchez, A., Hose, G. C., & Reboleira, A. S. P. S. (2020b). Salinity and temperature increase impact groundwater crustaceans. *Scientific Reports*, *10*(1), 12328. https://doi.org/10.1038/s41598-020-69050-7

Castaño-Sánchez, A., Pereira, J. L., Gonçalves, F. J. M., & Reboleira, A. S. P. S. (2021). Sensitivity of a widespread groundwater copepod to different contaminants. *Chemosphere*, *274*, 129911. https://doi.org/10.1016/j.chemosphere.2021.129911

Charette, T., & Prepas, E. E. (2003). Wildfire impacts on phytoplankton communities of three small lakes on the Boreal Plain, Alberta, Canada: A paleolimnological study. *Canadian Journal of Fisheries and Aquatic Sciences*, *60*(5), 584–593. https://doi.org/10.1139/f03-049

Chen, Z., Auler, A. S., Bakalowicz, M., Drew, D., Griger, F., Hartmann, J., Jiang, G., Moosdorf, N., Richts, A., Stevanovic, Z., Veni, G., & Goldscheider, N. (2017). The World Karst Aquifer Mapping project: Concept, mapping procedure and map of Europe. *Hydrogeology Journal*, *25*(3), 771–785. https://doi.org/10.1007/s10040-016-1519-3

Cigna, A. A. (2016). Tourism and show caves. *Zeitschrift Für Geomorphologie, Supplementary Issues*, *60*(2), 217–233. https://doi.org/10.1127/zfg_suppl/2016/00305

Costa, M. R., Calvão, A. R., & Aranha, J. (2014). Linking wildfire effects on soil and water chemistry of the Marão River watershed, Portugal, and biomass changes detected from Landsat imagery. *Applied Geochemistry*, *44*, 93–102. https://doi.org/10.1016/j.apgeochem.2013.09.009

Cowell, A. L., Matthews, T. G., & Lind, P. R. (2006). Effect of fire on benthic algal assemblage structure and recolonization in intermittent streams. *Austral Ecology*, *31*(6), 696–707. https://doi.org/10.1111/j.1442-9993.2006.01595.x

Crémazy, A. Brix, K., & Wood, C. (2018). Chronic Toxicity of Binary Mixtures of Six Metals (Ag, Cd, Cu, Ni, Pb, and Zn) to the Great Pond Snail *Lymnaea stagnalis*. *Environmental Science & Technology, 52,* 5979-5988. https://doi.org/10.1021/acs.est.7b06554.s001

Culver, D. C., & Pipan, T. (2009). *The biology of caves and other subterranean habitats*. Oxford university press.

Culver, D., & Pipan, T. (2016). Shifting Paradigms of the Evolution of Cave Life. *Acta Carsologica*, *44*(3). https://doi.org/10.3986/ac.v44i3.1688

Damiens, G., Minier, C. (2013). The Multixenobiotic Transport System. In C. Amiard-Triquet, M. Roméo & P. S. Rainbow. *Tolerance to Environmental Contaminants* (228-246).CRC Press.

De Oliveira, L. F., Santos, C., Risso, W. E., & Dos Reis Martinez, C. B. (2018). Triple-mixture of Zn, Mn, and Fe increases bioaccumulation and causes oxidative stress in freshwater neotropical fish. *Environmental Toxicology and Chemistry*, *37*(6), 1749–1756. https://doi.org/10.1002/etc.4133

Deharveng, L., & Bedos, A. (2018). Diversity of Terrestrial Invertebrates in Subterranean Habitats. In O. T. Moldovan, Ľ. Kováč, & S. Halse (Eds.), *Cave Ecology* (107–172). Springer International Publishing. https://doi.org/10.1007/978-3-319-98852-8_7

Demeyer, A., Nkana, J. C. V., & Verloo, M. G. (2000). *Characteristics of wood ash and influence on soil properties and nutrient uptake: An overview*.

Di Lorenzo, T., Di Marzio, W. D., Fiasca, B., Galassi, D. M. P., Korbel, K., Iepure, S., Pereira, J. L., Reboleira, A. S. P. S., Schmidt, S. I., & Hose, G. C. (2019). Recommendations for ecotoxicity testing with stygobiotic species in the framework of groundwater environmental risk assessment. *Science of The Total Environment*, *681*, 292–304. https://doi.org/10.1016/j.scitotenv.2019.05.030

Di Lorenzo, T., & Reboleira, A. S. P. S. (2022). Thermal acclimation and metabolic scaling of a groundwater asellid in the climate change scenario. *Scientific Reports*, *12*(1), 17938. https://doi.org/10.1038/s41598-022-20891-4

Directive 2006/118/EC of the European Parliament and of the Council of 12 December 2006 on the protection of groundwater against pollution and deterioration—EU monitor. (n.d.). Retrieved

November 21, 2024, from https://www.eumonitor.eu/9353000/1/j9vvik7m1c3gyxp/vhh51naya6yx

Duarte, C., Gravato, C., Di Lorenzo, T., & Reboleira, A. S. P. S. (2023). Acetaminophen induced antioxidant and detoxification responses in a stygobitic crustacean. *Environmental Pollution*, *330*, 121749. https://doi.org/10.1016/j.envpol.2023.121749

Duruibe, J., & Egwurugwu, J. (2007). Heavy metal pollution and human biotoxic effects. *International Journal of the Physical Sciences*, 2(5), 112-118.https://doi.org/10.5897/ijps.9000289

Ermitão, T., Páscoa, P., Trigo, I., Alonso, C., & Gouveia, C. (2023). Mapping the Most Susceptible Regions to Fire in Portugal. *Fire*, *6*(7), Article 7. https://doi.org/10.3390/fire6070254

Etiégni, L., & Campbell, A. G. (1991). Physical and chemical characteristics of wood ash. *Bioresource Technology*, *37*(2), 173–178. https://doi.org/10.1016/0960-8524(91)90207-Z

Ficetola, G. F., Canedoli, C., & Stoch, F. (2019). The Racovitzan impediment and the hidden biodiversity of unexplored environments. *Conservation Biology*, *33*(1), 214–216. https://doi.org/10.1111/cobi.13179

Foster, S. S. D., & Chilton, P. J. (2003). Groundwater: The processes and global significance of aquifer degradation. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *358*(1440), 1957–1972. https://doi.org/10.1098/rstb.2003.1380

Gagné, F., & André, C. (2014). *Biochemical Ecotoxicology: Principles and Methods*. Elsevier.

Galassi, D. M. P. (2001). Groundwater copepods: Diversity patterns over ecological and evolutionary scales. In R. M. Lopes, J. W. Reid, & C. E. F. Rocha (Eds.), *Copepoda: Developments in Ecology, Biology and Systematics* (227–253). Springer Netherlands. https://doi.org/10.1007/0-306-47537-5_19

GeoCATALOGO. (n.d.). Accessed in 2024, 27th of september in https://geocatalogo.icnf.pt/catalogo_tema5.html.

Gibert, J., & Deharveng, L. (2002). Subterranean Ecosystems: A Truncated Functional Biodiversity. *BioScience*, *52*(6), 473. https://doi.org/10.1641/0006-3568(2002)052[0473:SEATFB]2.0.CO;2

Gillieson, D., Gunn, J., Auler, A., & Bolger, T. (2022). Guidelines for Cave and Karst Protection. *In IUCN books* (2nd Ed.).

Giusto, A., Salibián, A., & Ferrari, L. (2014). Biomonitoring toxicity of natural sediments using juvenile *Hyalella curvispina* (Amphipoda) as test species: Evaluation of early effect endpoints. *Ecotoxicology*, *23*(2), 293–303. https://doi.org/10.1007/s10646-013-1173-7

Goforth, B. R., Graham, R. C., Hubbert, K. R., Zanner, C. W., & Minnich, R. A. (2005). Spatial distribution and properties of ash and thermally altered soils after high-severity forest fire, southern California. *International Journal of Wildland Fire*, *14*(4), 343. https://doi.org/10.1071/WF05038

Goldscheider, N., Chen, Z., Auler, A. S., Bakalowicz, M., Broda, S., Drew, D., Hartmann, J., Jiang, G., Moosdorf, N., Stevanovic, Z., & Veni, G. (2020). Global distribution of carbonate rocks and karst water resources. *Hydrogeology Journal*, *28*(5), 1661–1677. https://doi.org/10.1007/s10040-020-02139-5

González, R. R., & Quiñones, R. A. (2000). Pyruvate Oxidoreductases Involved in Glycolytic Anaerobic Metabolism of Polychaetes from the Continental Shelf off Central-South Chile. *Estuarine, Coastal and Shelf Science*, *51*(4), 507–519. https://doi.org/10.1006/ecss.2000.0693

Gravato, C., Almeida, J. R., Silva, C., Oliveira, C., & Soares, A. M. V. M. (2014). Using a multibiomarker approach and behavioural responses to assess the effects of anthracene in *Palaemon serratus*. *Aquatic Toxicology*, *149*, 94–102. https://doi.org/10.1016/j.aquatox.2014.01.024

Gravato, C., Teles, M., Oliveira, M., & Santos, M. A. (2006). Oxidative stress, liver biotransformation and genotoxic effects induced by copper in *Anguilla anguilla L.* – the influence of pre-exposure to β-naphthoflavone. *Chemosphere*, *65*(10), 1821–1830. https://doi.org/10.1016/j.chemosphere.2006.04.005

Griebler, C., Malard, F., & Lefébure, T. (2014). Current developments in groundwater ecology—From biodiversity to ecosystem function and services. *Current Opinion in Biotechnology*, *27*, 159–167. https://doi.org/10.1016/j.copbio.2014.01.018

Halliwell, B., & Aruoma, O. I. (1991). DNA damage by oxygen-derived species Its mechanism and measurement in mammalian systems. *FEBS Letters*, *281*(1–2), 9–19. https://doi.org/10.1016/0014-5793(91)80347-6

Hartmann, A., Goldscheider, N., Wagener, T., Lange, J., & Weiler, M. (2014). Karst water resources in a changing world: Review of hydrological modeling approaches. *Reviews of Geophysics*, *52*(3), 218–242. https://doi.org/10.1002/2013RG000443

Hervant, F., Mathieu, J., Garin, D., & Freminet, A. (1996). Behavioral, Ventilatory, and Metabolic Responses of the Hypogean Amphipod *Niphargus virei* and the Epigean Isopod *Asellus aquaticus* to Severe Hypoxia and Subsequent Recovery. *Physiological Zoology*, *69*(6), 1277–1300. https://doi.org/10.1086/physzool.69.6.30164261

Hervant, F., & Renault, D. (2002). Long-term fasting and realimentation in hypogean and epigean isopods: A proposed adaptive strategy for groundwater organisms. *Journal of Experimental Biology*, *205*(14), 2079–2087. https://doi.org/10.1242/jeb.205.14.2079

Hose, G. C., Chariton, A. A., Daam, M. A., Di Lorenzo, T., Galassi, D. M. P., Halse, S. A., Reboleira, A. S. P. S., Robertson, A. L., Schmidt, S. I., & Korbel, K. L. (2022). Invertebrate traits, diversity and the vulnerability of groundwater ecosystems. *Functional Ecology*, *36*(9), 2200–2214. https://doi.org/10.1111/1365-2435.14125

Howarth, F. G., & Moldovan, O. T. (2018). The Ecological Classification of Cave Animals and Their Adaptations. In O. T. Moldovan, Ľ. Kováč, & S. Halse (Eds.), *Cave Ecology* (pp. 41–67). Springer International Publishing. https://doi.org/10.1007/978-3-319-98852-8_4

Howcroft, C. F., Gravato, C., Amorim, M. J. B., Novais, S. C., Soares, A. M. V. M., & Guilhermino, L. (2011). Biochemical characterization of cholinesterases in *Enchytraeus albidus* and assessment of in vivo and in vitro effects of different soil properties, copper and phenmedipham. *Ecotoxicology*, *20*(1), 119–130. https://doi.org/10.1007/s10646-010-0562-4

Incêndios rurais. Relatório do Estado do Ambiente. (n.d.). Accessed in 2024, 27th of September in https://rea.apambiente.pt/content/inc%C3%AAndios-rurais.

Kantor, P., Lopaschuk, G., & Opie, L. (2001). Myocardial Energy Metabolism. In N. Sperelakis, A. Terzic, Y. Kurachi, & M. V. Cohen (Eds.), Heart physiology and pathophysiology (4th Ed, Chap.32, 543-569). Academic Press.

Kováč, Ľ. (2018). Caves as Oligotrophic Ecosystems. In O. T. Moldovan, Ľ. Kováč, & S. Halse (Eds.), *Cave Ecology* (pp. 297–307). Springer International Publishing. https://doi.org/10.1007/978-3-319-98852-8_13

Kunz, T. H., Braun De Torrez, E., Bauer, D., Lobova, T., & Fleming, T. H. (2011). Ecosystem services provided by bats. *Annals of the New York Academy of Sciences*, *1223*(1), 1–38. https://doi.org/10.1111/j.1749-6632.2011.06004.x

Kwan, G. T., Sanders, T., Huang, S., Kilaghbian, K., Sam, C., Wang, J., Weihrauch, K., Wilson, R. W., & Fangue, N. A. (2024). Impacts of ash-induced environmental alkalinization on fish physiology, and their implications to wildfire-scarred watersheds. *Science of The Total Environment*, *953*, 176040. https://doi.org/10.1016/j.scitotenv.2024.176040

Lapworth, D. J., Baran, N., Stuart, M. E., & Ward, R. S. (2012). Emerging organic contaminants in groundwater: A review of sources, fate and occurrence. *Environmental Pollution*, *163*, 287–303. https://doi.org/10.1016/j.envpol.2011.12.034

Lauritzen, S.-E. (2018). Physiography of the Caves. In O. T. Moldovan, Ľ. Kováč, & S. Halse (Eds.), *Cave Ecology* (Vol. 235, pp. 7–21). Springer International Publishing. https://doi.org/10.1007/978-3-319-98852-8_2

Magniez,P. (1966). Repartition geographique et validite de l'espece troglobie Asellus lusitanicus Frade (Crustace Asellote).

Mammola, S., Cardoso, P., Culver, D. C., Deharveng, L., Ferreira, R. L., Fišer, C., Galassi, D. M. P., Griebler, C., Halse, S., Humphreys, W. F., Isaia, M., Malard, F., Martinez, A., Moldovan, O. T., Niemiller, M. L., Pavlek, M., Reboleira, A. S. P. S., Souza-Silva, M., Teeling, E. C., Zagmajster, M. (2019a). Scientists' Warning on the Conservation of Subterranean Ecosystems. *BioScience*, *69*(8), 641–650. https://doi.org/10.1093/biosci/biz064

Mammola, S., Piano, E., Cardoso, P., Vernon, P., Domínguez-Villar, D., Culver, D. C., Pipan, T., & Isaia, M. (2019b). Climate change going deep: The effects of global climatic alterations on cave ecosystems. *The Anthropocene Review*, *6*(1–2), 98–116. https://doi.org/10.1177/2053019619851594

Mann, S. L., Steidl, R. J., & Dalton, V. M. (2002). Effects of Cave Tours on Breeding Myotis velifer. *The Journal of Wildlife Management*, *66*(3), 618. https://doi.org/10.2307/3803128

Manoli, E., & Samara, C. (1999). Polycyclic aromatic hydrocarbons in natural waters: Sources, occurrence and analysis. *TrAC Trends in Analytical Chemistry*, *18*(6), 417–428. https://doi.org/10.1016/S0165-9936(99)00111-9

Mansilha, C., Carvalho, A., Guimarães, P., & Espinha Marques, J. (2014). Water Quality Concerns Due to Forest Fires: Polycyclic Aromatic Hydrocarbons (PAH) Contamination of Groundwater From Mountain Areas. *Journal of Toxicology and Environmental Health, Part A*, *77*(14–16), 806–815. https://doi.org/10.1080/15287394.2014.909301

Mansilha, C., Melo, A., Martins, Z. E., Ferreira, I. M. P. L. V. O., Pereira, A. M., & Espinha Marques, J. (2020). Wildfire Effects on Groundwater Quality from Springs Connected to Small Public Supply Systems in a Peri-Urban Forest Area (Braga Region, NW Portugal). *Water*, *12*(4), 1146. https://doi.org/10.3390/w12041146

Marschner, P. (2003). Structure and function of the soil microbial community in a long-term fertilizer experiment. *Soil Biology and Biochemistry*, *35*(3), 453–461. https://doi.org/10.1016/S0038-0717(02)00297-3

Mateus, P., & Fernandes, P. (2014). Forest Fires in Portugal: Dynamics, Causes and Policies. In F.Reboredo (Ed.), *Forest Context and Policies in Portugal, Present and Future Challenges* (Vol.19, Chap.4, 1-15). Springer

McGill, M. R., & Jaeschke, H. (2013). Oxidant Stress, Antioxidant Defense, and Liver Injury. In *Drug-Induced Liver Disease* (pp. 71–84). Elsevier. https://doi.org/10.1016/B978-0-12-387817-5.00004-2

Medina, M. J., Antić, D., Borges, P. A. V., Borko, Š., Fišer, C., Lauritzen, S.-E., Martín, J. L., Oromí, P., Pavlek, M., Premate, E., Puliafico, K. P., Sendra, A., & Reboleira, A. S. P. S. (2023). Temperature variation in caves and its significance for subterranean ecosystems. *Scientific Reports*, *13*(1), 20735. https://doi.org/10.1038/s41598-023-48014-7

Mesquita, A. F., Abrantes, N., Campos, I., Nunes, C., Coimbra, M. A., Gonçalves, F. J. M., Marques, J. C., & Gonçalves, A. M. M. (2022). Effects of wildfire ash on the growth and biochemical profiles of the aquatic macrophyte *Lemna minor*. *Aquatic Toxicology*, *250*, 106245. https://doi.org/10.1016/j.aquatox.2022.106245

Minutoli, R., & Guglielmo, L. (2009). Zooplankton respiratory Electron Transport System (ETS) activity in the Mediterranean Sea: Spatial and diel variability. *Marine Ecology-Progress Series*, *381*, 199–211. https://doi.org/10.3354/meps07862

Miranda, P., Tomé, A., Valente, M. A., & Coelho, F. (2002). 20th Century Portuguese Climate and Climate Scenarios in Climate Change in Portugal: Scenarios, Impacts and Adaptation. In F.D. Santos, K. Forbes, R. Moita, *Climate Change in Portugal: Scenarios, Impacts and Adaptation Measures* (1st Ed, pp.27-83). Gradiva.

Mohanty, D., & Samanta, L. (2016). Multivariate analysis of potential biomarkers of oxidative stress in *Notopterus notopterus* tissues from Mahanadi River as a function of concentration of heavy metals. *Chemosphere*, *155*, 28–38. https://doi.org/10.1016/j.chemosphere.2016.04.035

Moldovan, O. T. (2003). The impact of tourism in Romanian show caves: The example of the beetle populations in the Ursilor Cave of Chiscau (Transylvania, Romania).*Subterranean Biology,1*,73-78.

Moldovan, O. T., Kováč, Ľ., & Halse, S. (2018a). Preamble. In O. T. Moldovan, Ľ. Kováč, & S. Halse (Eds.), *Cave Ecology* (pp. 1–4). Springer International Publishing. https://doi.org/10.1007/978-3-319-98852-8_1

Moldovan, O. T., Kováč, Ľ., & Halse, S. (2018b). Preamble. In O. T. Moldovan, Ľ. Kováč, & S. Halse (Eds.), *Cave Ecology* (pp. 1–4). Springer International Publishing. https://doi.org/10.1007/978-3-319-98852-8_1

Mouneyrac, C., Leung, P., Leung, K. (2013). Cost of Tolerance. In C. Amiard-Triquet, M. Roméo & P. S. Rainbow. *Tolerance to Environmental Contaminants* (265-297).CRC Press.

Muñiz González, A.-B., Campos, I., Re, A., Martínez-Guitarte, J.-L., & Abrantes, N. (2023). Effects of wildfire ashes on aquatic invertebrates: First molecular approach on *Chironomus riparius* larvae. *Science of The Total Environment*, *858*, 159899. https://doi.org/10.1016/j.scitotenv.2022.159899

Nakicenovic, Alcamo, J., Davis, G., de Vries, B., Fenhann, J., Gaffin, S., Gregory, K., Grübler, A., Jung, T., Kram, T., La Rovere, E., Michaelis, L., Moris, S., Morita, T., Pepper, W., Pitcher, H., Price, L., Raihi, K., Roehrl, A., Dadi, Z. (Eds.). (2000). *Emissions scenarios: Summary for policymakers;a special report of IPCC Working Group III, Intergovernmental Panel on Climate Change*. Intergovernmental Panel on Climate Change.

Nanni, V., Piano, E., Cardoso, P., Isaia, M., & Mammola, S. (2023). An expert-based global assessment of threats and conservation measures for subterranean ecosystems. *Biological Conservation*, *283*, 110136. https://doi.org/10.1016/j.biocon.2023.110136

Newman, M. C. (2003). *Fundamentals of Ecotoxicology*. CRC Press.

Nicolosi, G., Mammola, S., Verbrugge, L., & Isaia, M. (2023). Aliens in caves: The global dimension of biological invasions in subterranean ecosystems. *Biological Reviews*, *98*(3), 849–867. https://doi.org/10.1111/brv.12933

Nriagu, J. (1989). A global assessment of natural sources of atmospheric trace metals. *Nature*, 338, 47-49. https://doi.org/10.1038/338047a0

Nunes, B., Silva, V., Campos, I., Pereira, J. L., Pereira, P., Keizer, J. J., Gonçalves, F., & Abrantes, N. (2017). Off-site impacts of wildfires on aquatic systems—Biomarker responses of the mosquitofish *Gambusia holbrooki*. *Science of The Total Environment*, *581–582*, 305–313. https://doi.org/10.1016/j.scitotenv.2016.12.129

Oliva, M., Perales, J. A., Gravato, C., Guilhermino, L., & Galindo-Riaño, M. D. (2012). Biomarkers responses in muscle of *Senegal sole* (Solea senegalensis) from a heavy metals and PAHs polluted estuary. *Marine Pollution Bulletin*, *64*(10), 2097–2108. https://doi.org/10.1016/j.marpolbul.2012.07.017

Oliveira, M., Gravato, C., & Guilhermino, L. (2012). Acute toxic effects of pyrene on *Pomatoschistus microps* (Teleostei, Gobiidae): Mortality, biomarkers and swimming performance. *Ecological Indicators*, *19*, 206–214. https://doi.org/10.1016/j.ecolind.2011.08.006

Parente, J., Pereira, M. G., & Tonini, M. (2016). Space-time clustering analysis of wildfires: The influence of dataset characteristics, fire prevention policy decisions, weather and climate. *Science of The Total Environment*, *559*, 151–165. https://doi.org/10.1016/j.scitotenv.2016.03.129

Parra, J. G., Rivero, V. C., & Lopez, T. I. (1996). Forms of Mn in soils affected by a forest fire. *Science of The Total Environment*, *181*(3), 231–236. https://doi.org/10.1016/0048-9697(95)05022-1

Pereira, M. G., Gonçalves, N., & Amraoui, M. (2024). The Influence of Wildfire Climate on Wildfire Incidence: The Case of Portugal. *Fire*, *7*(7). https://doi.org/10.3390/fire7070234

Piano, E., Nicolosi, G., Mammola, S., Balestra, V., Baroni, B., Bellopede, R., Cumino, E., Muzzulini, N., Piquet, A., & Isaia, M. (2022). A literature-based database of the natural heritage, the ecological status and tourism-related impacts in show caves worldwide. *Nature Conservation*, *50*, 159–174. https://doi.org/10.3897/natureconservation.50.80505

Pilliod, D. S., Bury, R. B., Hyde, E. J., Pearl, C. A., & Corn, P. S. (2003). Fire and amphibians in North America. *Forest Ecology and Management*, *178*(1–2), 163–181. https://doi.org/10.1016/S0378-1127(03)00060-4

Pinto, E., Sigaud-kutner, T. C. S., Leitão, M. A. S., Okamoto, O. K., Morse, D., & Colepicolo, P. (2003). Heavy metal–induced oxidative stress in algae[1]. *Journal of Phycology*, *39*(6), 1008–1018. https://doi.org/10.1111/j.0022-3646.2003.02-193.x

Pipan, T., & Culver, D. (2013). Subterranean Ecosystems. In S.A. Levin (Ed.), *Encyclopedia of Biodiversity* (Vol.7, 49-62). Academic Press.

Pohanka, M. (2013). Butyrylcholinesterase as a biochemical marker. *Bratislava Medical Journal*, *114*(12), 726–734. https://doi.org/10.4149/BLL_2013_153

Pradhan, A., Carvalho, F., Abrantes, N., Campos, I., Keizer, J. J., Cássio, F., & Pascoal, C. (2020). Biochemical and functional responses of stream invertebrate shredders to post-wildfire contamination. *Environmental Pollution*, *267*, 115433. https://doi.org/10.1016/j.envpol.2020.115433

Quina, A. S., Rodrigues, A. C. M., Soares, A. M. V. M., Da Luz Mathias, M., & Gravato, C. (2023). The use of muscle biomarkers for assessing physiological effects of heavy metal pollution in the greater white-toothed shrew (*Crocidura russula*). *Science of The Total Environment*, *888*, 164162. https://doi.org/10.1016/j.scitotenv.2023.164162

RStudio Team (2024). RStudio: Integrated Development Environment for R (4.2.3.). http://www.rstudio.com/

Rannalli, A. (2004). A Summary of the Scientific Literature on the Effects of Fire on the Concentration of Nutrients in Surface Waters. https://doi.org/10.3133/ofr20041296

Ré, A., Campos, I., Saraiva, M. J., Puga, J., Keizer, J. J., Gonçalves, F. J. M., Pereira, J. L., & Abrantes, N. (2020). Wildfire effects on two freshwater producers: Combining in-situ and laboratory bioassays. *Ecotoxicology and Environmental Safety*, *194*, 110361. https://doi.org/10.1016/j.ecoenv.2020.110361

Reboleira, A. S., Borges, P., Gonçalves, F., Serrano, A., & Oromí, P. (2011). The subterranean fauna of a biodiversity hotspot region - Portugal: An overview and its conservation. *International Journal of Speleology (Edizione Italiana)*, *40*, 23–37. https://doi.org/10.5038/1827-806X.40.1.4

Reboleira, A. S. P. S., Abrantes, N., Oromí, P., & Gonçalves, F. (2013a). Acute Toxicity of Copper Sulfate and Potassium Dichromate on Stygobiont Proasellus: General Aspects of Groundwater Ecotoxicology and Future Perspectives. *Water, Air, & Soil Pollution*, *224*(5), 1550. https://doi.org/10.1007/s11270-013-1550-0

Reboleira, A. S., Oromí, P., & Gonçalves, F. (2013b). Cave-dwelling fauna from karst areas of Portugal. In M. Filippi, P. Bosák (Ed.), 16th *International congress of Speleology* (1.,pp. 409-413). Praha: Czech Speleological Society.

Regoli, F., & Winston, G. W. (1999). Quantification of Total Oxidant Scavenging Capacity of Antioxidants for Peroxynitrite, Peroxyl Radicals, and Hydroxyl Radicals. *Toxicology and Applied Pharmacology*, *156*(2), 96–105. https://doi.org/10.1006/taap.1999.8637

Regoli, F., Benedetii, M. & Giuliani, M.(2013). Antioxidant Defenses acquisition of Tolerance to Chemical Stress. In C. Amiard-Triquet , M.Roméo & P. S. Rainbow. *Tolerance to Environmental Contaminants* (153-173).CRC Press.

Rodrigues, P., Guimarães, L., Carvalho, A., & Oliva-Teles, L. (2023). Carbamazepine, venlafaxine, tramadol, and their main metabolites: Toxicological effects on zebrafish embryos and larvae. *Journal of Hazardous Materials*, *448*, 130909. https://doi.org/10.1016/j.jhazmat.2023.130909

Rodrigo-Comino, J., & Salvati, L. (Eds.). (2024). Fire Hazards: Socio-economic and Regional Issues. *Springer International Publishing*. https://doi.org/10.1007/978-3-031-50446-4

Roméo, M. & Giambérini, L.(2013). History of Biomarkers. In C. Amiard-Triquet , J-C. Amiard & P. S. Rainbow. *Ecological Biomarkers* (15-43).CRC Press.

Saccò, M., Mammola, S., Altermatt, F., Alther, R., Bolpagni, R., Brancelj, A., Brankovits, D., Fišer, C., Gerovasileiou, V., Griebler, C., Guareschi, S., Hose, G. C., Korbel, K., Lictevout, E., Malard, F., Martínez, A., Niemiller, M. L., Robertson, A., Tanalgo, K. C., Reinecke, R. (2024). Groundwater is a hidden global keystone ecosystem. *Global Change Biology*, *30*(1), e17066. https://doi.org/10.1111/gcb.17066

Saclier, N., Duchemin, L., Konecny-Dupré, L., Grison, P., Eme, D., Martin, C., Callou, C., Lefébure, T., François, C., Issartel, C., Lewis, J. J., Stoch, F., Sket, B., Gottstein, S., Delić, T., Zagmajster, M., Grabowski, M., Weber, D., Reboleira, A. S. P. S., Malard, F. (2024). A collaborative backbone resource for comparative studies of subterranean evolution: The World Asellidae database. *Molecular Ecology Resources*, *24*(1), e13882. https://doi.org/10.1111/1755-0998.13882

Saiz-Jimenez, C., Miller, A. Z., Martin-Sanchez, P. M., & Hernandez-Marine, M. (2012). Uncovering the origin of the black stains in Lascaux Cave in France. *Environmental Microbiology*, *14*(12), 3220–3231. https://doi.org/10.1111/1462-2920.12008

Sánchez-Moral, S., Soler, V., Cañaveras, J. C., Sanz-Rubio, E., Van Grieken, R., & Gysels, K. (1999). Inorganic deterioration affecting the Altamira Cave, N Spain: Quantitative approach to wall-corrosion (solutional etching) processes induced by visitors. *Science of The Total Environment*, *243–244*, 67–84. https://doi.org/10.1016/S0048-9697(99)00348-4

Santos, D., Abrantes, N., Campos, I., Domingues, I., & Lopes, I. (2023). Effects of aqueous extracts of wildfire ashes on tadpoles of *Pelophylax perezi*: Influence of plant coverage. *Science of The Total Environment*, *854*, 158746. https://doi.org/10.1016/j.scitotenv.2022.158746

Santos, D., Félix,L., Luzio, A., Parra, S., Bellas,J., Monteiro, S. (2021). Single and combined acute and subchronic toxic effects of microplastics and copper in zebrafish (*Danio rerio*) early life stages. Chemosphere, 277, 130262. doi:10.1016/j.chemosphere.2021.130262

Šebela, S., & Turk, J. (2014). Natural and anthropogenic influences on the year-round temperature dynamics of air and water in Postojna show cave, Slovenia. *Tourism Management*, *40*, 233–243. https://doi.org/10.1016/j.tourman.2013.06.011

Shiklomanov, I. A. (1998). World water resources: A new appraisal and assessment for the 21st century.

Silva, C., Oliveira, C., Gravato, C., & Almeida, J. R. (2013). Behaviour and biomarkers as tools to assess the acute toxicity of benzo(a)pyrene in the common prawn *Palaemon serratus*. *Marine Environmental Research*, *90*, 39–46. https://doi.org/10.1016/j.marenvres.2013.05.010

Silva, V., Pereira, J. L., Campos, I., Keizer, J. J., Gonçalves, F., & Abrantes, N. (2015). Toxicity assessment of aqueous extracts of ash from forest fires. *Catena*, *135*, 401–408. https://doi.org/10.1016/j.catena.2014.06.021

Silva,C., Silva, A., Campos, D., Machado,A., Pestana, J., Gravato,C. (2021). Oxidative damage and decreased aerobic energy production due to ingestion of polyethylene microplastics by *Chrionomus riparius* (Diptera) larvae. *Journal of Hazardous Materials, 42* (123775). https://doi.org/10.1016/j.jhazmat.2020.123775

Smith, H. G., Sheridan, G. J., Lane, P. N. J., Nyman, P., & Haydon, S. (2011). Wildfire effects on water quality in forest catchments: A review with implications for water supply. *Journal of Hydrology*, *396*(1), 170–192. https://doi.org/10.1016/j.jhydrol.2010.10.043

Smith, R. (2015). Directive 2008/94/EC of the European Parliament and of the Council of 22 October 2008. In R. Smith, *Core EU Legislation* (pp. 423–426). Macmillan Education UK. https://doi.org/10.1007/978-1-137-54482-7_44

Smolders, R., Bervoets, L., De Coen, W., & Blust, R. (2004). Cellular energy allocation in zebra mussels exposed along a pollution gradient: Linking cellular effects to higher levels of biological organization. *Environmental Pollution*, *129*(1), 99–112. https://doi.org/10.1016/j.envpol.2003.09.027

Sokolova IM;Frederich M;Bagwe R;Lannig G;Sukhotin AA; (n.d.) *Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates*, *Marine environmental research*.

Stein, H., Griebler, C., Berkhoff, S., Matzke, D., Fuchs, A., & Hahn, H. J. (2012). Stygoregions – a promising approach to a bioregional classification of groundwater systems. *Scientific Reports*, *2*(1), 673. https://doi.org/10.1038/srep00673

Teodorescu, D., Munteanu, M. C., Staicu, A. C., & Dinischiotu, A. (2012). Changes in lactate dehydrogenase activity in *Carassius Auratus Gibelio (L. Pysces*) kidney, gills and intestine induced by acute exposure to copper. *Romanian Biotechonological Letters*, *17*(6), 7873-7880.

Tonini, M., Pereira, M. G., Parente, J., & Vega Orozco, C. (2017). Evolution of forest fires in Portugal: From spatio-temporal point events to smoothed density maps. *Natural Hazards*, *85*(3), 1489–1510. https://doi.org/10.1007/s11069-016-2637-x

Van Beynen, P., & Townsend, K. (2005). A Disturbance Index for Karst Environments. *Environmental Management*, *36*(1), 101–116. https://doi.org/10.1007/s00267-004-0265-9

van der Oost, R., Beyer, J., & Vermeulen, N. P. E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: A review. *Environmental Toxicology and Pharmacology*, *13*(2), 57–149. https://doi.org/10.1016/S1382-6689(02)00126-6

Vieira, L. R., Gravato, C., Soares, A. M. V. M., Morgado, F., & Guilhermino, L. (2009). Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: Linking biomarkers to behaviour. *Chemosphere*, *76*(10), 1416–1427. https://doi.org/10.1016/j.chemosphere.2009.06.005

Vutukuru, S. S., Chintada, S., Radha Madhavi, K., Venkateswara Rao, J., & Anjaneyulu, Y. (2006). Acute effects of copper on superoxide dismutase, catalase and lipid peroxidation in the freshwater teleost fish, *Esomus danricus. Fish Physiology and Biochemistry*, *32*(3), 221–229. https://doi.org/10.1007/s10695-006-9004-x

Wang, L., Feng, J., Wang, G., Guan, T., Zhu, C., Li, J., & Wang, H. (2021). Effects of cadmium on antioxidant and non-specific immunity of *Macrobrachium nipponense*. *Ecotoxicology and Environmental Safety*, *224*, 112651. https://doi.org/10.1016/j.ecoenv.2021.112651

7 Supplementary materials

Supplementary Table 1: Priority compounds that can be found in ash and their respective EQS values. NA- not available.
(1) USEPA 2017- National Recommended Criteria for Water Quality – Criteria for Aquatic Life Table EQS expressed in maximum criterion concentration (MCC);
(2) EC 2008 - DIRECTIVE 2008/105/EC EQS expressed in maximum allowable concentration;
(3) CEQG 1999 - Canadian Water Quality Guidelines for the Protection of Aquatic Life - Polycyclic Hydrocarbons (PAHs).

Substance	**Identified as a priority substance by:**		**EQS [$\mu g/L$] (Environmental Quality Standards)**
	USEPA	**EC**	
Metals			
Cr	X		$570^{(1)}$e $16^{(1)}$
Ni	X	X	$70^{(1)}$/NA $^{(2)}$
Cu	X		NA
Zn	X		$120^{(1)}$
As	X		$340^{(1)}$
Cd	X	X	$1.8^{(1)}$ /$0.45^{(2)}$
Pb	X	X	$65^{(1)}$/ NA $^{(2)}$
PAHs			
Naphthalene (NAP)	X	X	$1.1^{(3)}$
Acenaphthylene (ACY)	X		NA
Acenaphthene (ACE)	X		$5.8^{(3)}$
Fluorene (FLU)	X		$3^{(3)}$
Phenanthrene (PHE)	X		$0.4^{(3)}$
Anthracene (ANT)	X	X	$0.4^{(2)}$/$0.012^{(3)}$
Fluoranthene	X	X	$1^{(2)}$/$0.04^{(3)}$

Pyrene (PYR)	X		$0.025^{(3)}$
Benzo(a)anthracene (BaA)	X		$0.018^{(3)}$
Chrysene (CHR)	X		NA
Benzo(b)fluoranthene (BbF)	X	X	NA
Benzo(k)fluoranthene (BkF)	X	X	NA
Benzo(a)pyrene (BaP)	X	X	$0.1^{(2)}/0.015^{(3)}$
Dibenzo(a,h)anthracene (DBA)	X		NA
Benzo(g,h,i)perylene (BGP)	X	X	NA
Indeno (1,2,3-Cd) pyrene (IND)	X	X	NA

Supplementary Table 2: Values of pH and dissolved oxygen at the beginning and at the end of the essay for *P.assaforensis*.

	$\mathbf{pH\ (i)}$	$\mathbf{pH\ (f)}$	$\mathbf{O_2\ (i)}$		$\mathbf{O_2\ (f)}$	
			%	$\mathbf{mg/L}$	%	$\mathbf{mg/L}$
C	6.50	7.72	94.0	8.13	82.10	6.72
12.5%	8.12	7.6	94.4	8.14	86.30	6.89
25%	8.24	7.72	94.0	8.15	83.20	7.00
50%	8.08	7.59	94.3	8.21	82.30	6.45
75%	8.07	7.81	95.6	8.25	80.40	6.20
100%	8.18	7.31	93.4	8.24	81.30	6.49

Supplementary Table 3: Values of pH and dissolved oxygen at the beginning and at the end of the essay for *P.lusitanicus*.

	pH (i)		**pH (f)**		O_2 **(i)**				O_2 **(f)**			
	pH (i)	Average	pH (f)	Average	%	Average	mg/L	Average	%	Average	mg/L	Average
C	6.70 ± 0.03	6.72	7.72 ± 0.04	7.69	90.0 ± 2	92.00	8.23 ± 0.38	7.95	82.4 ± 2.10	80.07	6.64 ± 0.03	6.61
	6.72 ± 0.03		7.65 ± 0.04		92.0 ± 2		8.10 ± 0.38		79.5 ± 2.10		6.59 ± 0.03	
	6.75 ± 0.03		7.70 ± 0.04		94.0 ± 2		7.51 ± 0.38		78.3 ± 2.10		6.6 ± 0.03	
12.5%	8.70 ± 0.02	8.72	7.55 ± 0.03	7.52	95.1 ± 0.06	95.43	8.30 ± 0.51	8.17	88.7 ± 0.30	88.37	6.92 ± 0.18	6.80
	8.74 ± 0.02		7.52 ± 0.03		96.1 ± 0.06		7.60 ± 0.51		88.3 ± 0.30		6.58 ± 0.18	
	8.72 ± 0.02		7.50 ± 0.03		95.1 ± 0.06		8.60 ± 0.51		88.1 ± 0.30		6.89 ± 0.18	
25%	8.70 ± 0.02	8.72	7.76 ± 0.04	7.73	95.4 ± 2.05	93.30	8.38 ± 0.32	8.63	92 ± 1.91	90.43	7.25 ± 0.08	7.20
	8.72 ± 0.02		7.73 ± 0.04		93.2 ± 2.05		8.50 ± 0.32		91 ± 1.91		7.1 ± 0.08	
	8.73 ± 0.02		7.69 ± 0.04		91.3 ± 2.05		9.00 ± 0.32		88.3 ± 1.91		7.25 ± 0.08	
50%	8.63 ± 0.03	8.65	7.60 ± 0.08	7.62	95.5 ± 1.02	95.23	8.40 ± 0.11	8.33	84.5 ± 3.30	82.90	6.69 ± 0.33	6.50
	8.65 ± 0.03		7.55 ± 0.08		94.1 ± 1.02		8.20 ± 0.11		85.1 ± 3.30		6.69 ± 0.33	
	8.68 ± 0.03		7.70 ± 0.08		96.1 ± 1.02		8.40 ± 0.11		79.1 ± 3.30		6.11 ± 0.33	

	pH (i)		**pH (f)**		$\mathbf{O_2}$ **(i)**				$\mathbf{O_2}$ **(f)**			
	pH (i)	Average	pH (f)	Average	%	Average	mg/L	Average	%	Average	mg/L	Average
75%	8.59 ± 0.02	8.61	7.89 ± 0.05	7.84	95.9 ± 3.85	92.17	8.43 ± 0.10	8.32	86.1 ± 9.25	78.50	6.75 ± 0.70	5.91
	8.61 ± 0.02		7.80 ± 0.05		88.2 ± 3.85		8.31 ± 0.10		68.2 ± 9.25		5.41 ± 0.70	
	8.63 ± 0.02		7.82 ± 0.05		92.4 ± 3.85		8.22 ± 0.10		81.2 ± 9.25		6.4 ± 0.70	
100%	8.71 ± 0.02	8.73	7.25 ± 0.08	7.18	93.8 ± 2.47	91.00	8.25 ± 0.65	7.85	89.1 ± 6.22	81.93	7.15 ± 0.55	6.51
	8.73 ± 0.02		7.10 ± 0.08		90.1 ± 2.47		8.20 ± 0.65		78.7 ± 6.22		6.21 ± 0.55	
	8.75 ± 0.02		7.20 ± 0.08		89.1 ± 2.47		7.10 ± 0.65		78 ± 6.22		6.16 ± 0.55	