Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado em Biologia da Conservação

Dissertação

Assessment of the toxicity of pesticides on the terrestrial phase of development of *Pelophylax perezi*

João Vasco Ribeiro Alves Pimenta

Orientador(es) | Isabel Maria Cunha Antunes Lopes

Ana C. Sousa

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A dissertação foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

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Resumo

Avaliação da toxicidade de pesticidas na fase de desenvolvimento terrestre de *Pelophylax perezi*

Os anfíbios são o grupo de vertebrados mais ameaçado. Entre os vários fatores associados ao seu declínio, os pesticidas são um dos mais relevantes. No entanto, os dados de toxicidade sobre os efeitos dos pesticidas são limitados, nomeadamente no que respeita às fases de vida terrestres. Neste estudo, foram avaliados os efeitos da exposição dérmica ao pesticida fluazifop-p-butil e à respetiva fórmula comercial FUSILADE MAX® em juvenis de *Pelophylax perezi*. Os juvenis foram expostos através de pulverização direta ou através de solo contaminado. A viabilidade de utilização de um invertebrado como substituto de experimentação animal também foi avaliada, através da realização de ensaios de toxicidade com os mesmos compostos em *Eisenia andrei*. Os resultados obtidos revelaram que *E. andrei* parece ser mais sensível do que *P. perezi* e a formulação comercial mais tóxica que o ingrediente ativo para *E. andrei*, sendo necessário realizar mais ensaios para confirmar estes resultados.

Palavras-chave: Anfíbios; Ecotoxicologia; *Eisenia andrei*; Fluazifop-p-butyl; FUSILADE MAX®

Abstract

Assessment of pesticide toxicity in the terrestrial development stage of *Pelophylax perezi*

Amphibia is the most threatened vertebrate group. Amongst the several factors implicated in amphibian decline, pesticides are one of the most relevant. Yet, there limited toxicity data on the effects of pesticides in amphibians, particularly for terrestrial life stages. In this study, the effects of dermal exposure to the pesticide fluazifop-p-butyl and the respective commercial formula FUSILADE MAX® in juveniles of *Pelophylax perezi* were evaluated. Juveniles were exposed via direct overspray of the pesticide or via pesticide-contaminated soil. The adequacy of using an invertebrate model as a surrogate to animal experimentation, was also evaluated, conducting toxicity assays of the same compounds with the earthworm, *Eisenia andrei*. The obtained results disclosed that *E. andrei* is seemingly more sensitive than *P. perezi*, and the formulation is more toxic than the active ingredient for *E. andrei*, but further studies are necessary for confirmation of the results.

Keywords: Amphibians; Ecotoxicology; *Eisenia andrei*; Fluazifop-p-butyl; FUSILADE MAX®

INDEX

Figure Index

Table Index

Abbreviations and Acronyms

Abbreviation	Definition
a.i.	Active Ingredient
AChE	Acetylcholinesterase
ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials
CTR	Control
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
ERA	Ecological Risk Assessment
EU	European Union
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FETAX	Frog Embryo Teratogenesis Assay *Xenopus*
Folpan WDG	Folpan water dispersible granule
Glyphosate-IPA	Glyphosate isopropylammonium
GST	Glutathione S-transferase
IPCC	Intergovernmental Panel on Climate Change
IUCN	International Union for Conservation of Nature
OECD	Organization for Economic Co-operation and Development
PAN Europe	Pesticide Action Network Europe
POEA	Polyethoxylated tallow amine
PPP	Plant Protection Products
PSD	Pesticide Sampling Device
RD	Recommended Dose
USA	United States of America
US EPA	United States Environmental Protection Agency
UV-B	Ultraviolet B

1. Introduction

1.1. Global Amphibian Conservation Threat

Amphibia is the vertebrate Class group with the greatest number of threatened species (IUCN, 2022). In 2008, 32%, almost a third of species of amphibians, were threatened and 42% in decline (IUCN, 2008). In order to understand the causes of this decline, numerous studies were carried out during the last decade (*e.g.*, Cramp & Franklin, 2018; Carpio *et al.*, 2016; da Rocha *et al.*, 2020; Çiçek *et al.*, 2021; Goessens *et al.*, 2022). In 2022, 41% of amphibian species were considered threatened and 43% in decline, significantly more than in 2008 (IUCN, 2022). Several factors have been identified as responsible for the worldwide decline of amphibians, both acting solely and in synergy, including introduced species; over-exploitation**;** climate change; infectious diseases; UV-B radiation; alteration, fragmentation, and destruction of habitat; and environmental contamination (Collins & Storfer, 2003; IUCN, 2008; Collins, 2010; Beebee & Griffins, 2013; IUCN, 2022).

I) Introduced species are species which are found beyond their natural geographical range, due to accidental or intentional introductions and these are classified as invasive when they become problematic and adversely affect ecosystems (European Commission, 2023). According to the *International Union for Conservation of Nature*, IUCN (2022), these invasive species can: transmit diseases, compete with or prey upon native species, change trophic chains and even change ecosystems, possibly even leading to extinction of native species. An example of an introduced species that is identified as a threat to autochthonous amphibian species is the American Bullfrog whose consequences to native species include competition for limited resources (Bury & Whelan, 1984; Snow & Witmer, 2010), predation of eggs and descendants (Snow & Witmer, 2010) and serve as a vector for *Batrachochytrium dendrobatidis – Bd*, a fungus which affects several species of amphibians (Skerrat *et al.*, 2007).

II) Over-exploitation. Many species are targeted for exploitation for food, as pets, for education and investigation, and so forth. But if the targeted species cannot sustain considerable and regular capture of individuals, they may become threatened by overexploitation (Collins, 2010; Çiçek *et al.*, 2021). To counteract the negative effect of exploitation on many wild populations of different species, in 1973, the *Convention on International Trade in Endangered Species of Wild Fauna and Flora*, CITES, an

international accord involving currently 183 adherent voluntary countries, with the objective of making sure that the commerce of fauna and flora does not affect wild populations, was implemented (European Commission, 2022). Even so, not all populations are protected, a recent example being *Pelophylax caralitanus*, in Turkey, currently threatened due to its capture for consumption and exportation on the international market (Çiçek *et al.*, 2021).

III) Climate change. According to the United Nations (2023), climate change "refers to the long-term shifts in temperatures and weather patterns", which are connected with greenhouse gas emissions, namely global warming, and these changes affect the natural environments through disruptions or alterations in ecosystems (IPCC, 2022). There is evidence that these changes have impacts on amphibians (Li *et al.*, 2013). Changes in reproduction, for example, have been associated with climate change, with the reproductive period starting sooner than normal (Parmesan, 2007). Climate change is also responsible for the loss of climatically suitable areas which ultimately will affect the distribution and survival of amphibians (Alves-Ferreira *et al.*, 2022).

IV) Infectious Diseases. These diseases are caused by several pathogenic agents, including ranavirus and *Batrachochytrium dendrobatidis*, which are considered the most relevant ones as they affect populations of amphibians in a global scale. The most studied disease is chytridiomycosis, caused by *B. dendrobatidis*, which is thought to be the leading cause for amphibian decline (Skerrat *et al.*, 2007; Collins, 2010; Berger *et al.*, 2016). Its introduction in non-adapted species via human transport is suggested to be one of the main reasons for the recent emergence and global impact of this disease (Skerrat et. al, 2007; Picco & Collins, 2008; Krieger & Hero, 2009). Due to the aggregation of individuals during reproduction and the high mortality rate caused by the disease, it can cause abrupt and pronounced declines in populations, possibly leading to extinction (IUCN, 2008; Skerrat *et al.*, 2007).

V) Exposure to UV-B radiation is associated with landscape change, since the reduction of the vegetation cover causes an increase of solar exposure (da Rocha *et al.*, 2020). UV radiation can have a direct or indirect influence in amphibians' immunological system, and it may influence *Bd* infection (Cramp & Franklin, 2018). Some effects of UV-B radiation exposure include damage in skin integrity (*e.g.*, Flamarique *et al.*, 2000), alterations in the production of secretions with protective

functions (*e.g.*, Davidson *et al.*, 2007) and mutations in juvenile DNA, which compromises adult immunological system (Ceccato *et al.*, 2016).

VI) Alteration, fragmentation, and destruction of habitat. Forests and an heterogenous landscape are important factors for amphibians species richness (Atauri & Lucio, 2001; Hermann *et al.*, 2005). According to the IUCN, the main cause behind the amphibian decline in 2008, was habitat loss and degradation (Cordier *et al.*, 2021) Examples include, for example, the construction of roads which causes significant mortality of amphibians (Hartel *et al.* 2010; Beebee, 2013) or the destruction of riparian forests for agricultural expansion, which removes the protection from UV and agrochemical exposure (da Rocha *et al.*, 2020). The loss of heterogeneous habitats is considered a major threat to amphibian biodiversity (Atauri and Delucio 2001; Carpio *et al.*, 2016). Intensive olive tree monoculture, for example, threatens amphibian biodiversity due to its unsuitable habitat and low landscape heterogeneity that promotes generalist species (Carpio *et al.*, 2016). While mixed plots of olive groves and vineyards had a positive effect. In fact, some reports disclosed that the composition of the land in extensive land management with traditional agricultural practices, had minimum effect on local amphibian distribution (Hartel *et al.*, 2010).

VII) Contamination. Exposure of amphibians to contaminants has been documented in both environments of their biphasic life cycle (*e.g.*, Leeb *et al.*, 2020; Goessens *et al.*, 2022). These contaminants include pesticides and fertilizers (*e.g.*, Van Meter *et al.*, 2019; Goessens *et al.*, 2022). Pesticide active ingredients and formulations have been observed to cause adverse effects in different species, whether they were lethal (*e.g.*, Brühl *et al.*, 2013) or sublethal (*e.g.*, Van Meter *et al.*, 2019).

1.2 Pesticides

To protect cultures from pests and avoid losses of yield, farmers apply pesticides, which are any substance or mixture of substances of chemical or biological ingredients that are used to prevent, control, and eradicate pests or for regulation of plant growth (FAO, 2022; European Commission, 2022). Pesticides are an important and cost-effective way of protecting crops and increasing yield and thus allow to increase food production necessary to sustain the constant population growth (Oerke 2005 *in* Tudi *et al.*, 2021; Aktar & Chowdhury, 2009; Bernardes *et al.*, 2015 *in* Tudi *et al.*, 2021)

According to Akashe *et al.* (2018) and references therein, pesticides can be classified by: (i) chemical classes (organic and inorganic pesticides); (ii) pesticide function: *e.g.*, herbicide, insecticide; (iii) chemical composition (*e.g.*, carbamates, organophosphorus); (iv) toxicity (acute and chronic); (v) mode of entry (*e.g.*, systemic or contact pesticides); (vi) mode of action (*e.g.*, physical or respiratory); (vii) pesticide formulations (*e.g.*, pellets or bait); (viii) source of origin (bio-pesticides and chemical pesticides). Generally, chemical composition and pesticide function are the most used to classify the different pesticides.

According to the Food and Agriculture Organization (FAO, 2022), an average of 2.7 megatons (Mt) of active ingredients (a.i.) were used worldwide in 2020, with 1.8 Kg pesticide applied per hectare of cropland. These numbers reveal an increase of 1.2 Kg/ha when compared to the 1990's average and more than the global yearly average of 1.58 Kg/ha over the past three decades. America was the continent that used the highest quantity of pesticides throughout, averaging 1 Mt per year, 1.3 Mt in 2020 alone and with an application of 2.83 Kg/ha/year. Asia used 0.65 Mt per year with and average application of 1.17 Kg/ha/year. Europe used an average of 0.48 Mt per year, and an average application of 1.57 Kg/ha per year, with an application of 1.6 Kg/ha in 2020 alone. Oceania used an average of 62 kilotons (Kt) from 2010 to 2020 and applied 1.8 Kg/ha which contrasts with 1.4 Kg/ha applied in the 1990's. Lastly Africa used the lowest amount with 0.11 tons per year from 2010 to 2020 and 0.41 Kg/ha/year (FAO, 2022).

Using Portugal as a specific example, the consumption of pesticides for agriculture, determined by FAOSTAT (2023) using sales numbers, was of about 9716 tons in 2020, with an average of 11162 tons per year from 1990 to 2020 (Fig. 1). This value is higher than the 8998 tons in 1990. It is also higher than the lowest value during 2015-2018. Nevertheless, since 2002, an overall decreasing trend can be observed (Fig. 1).

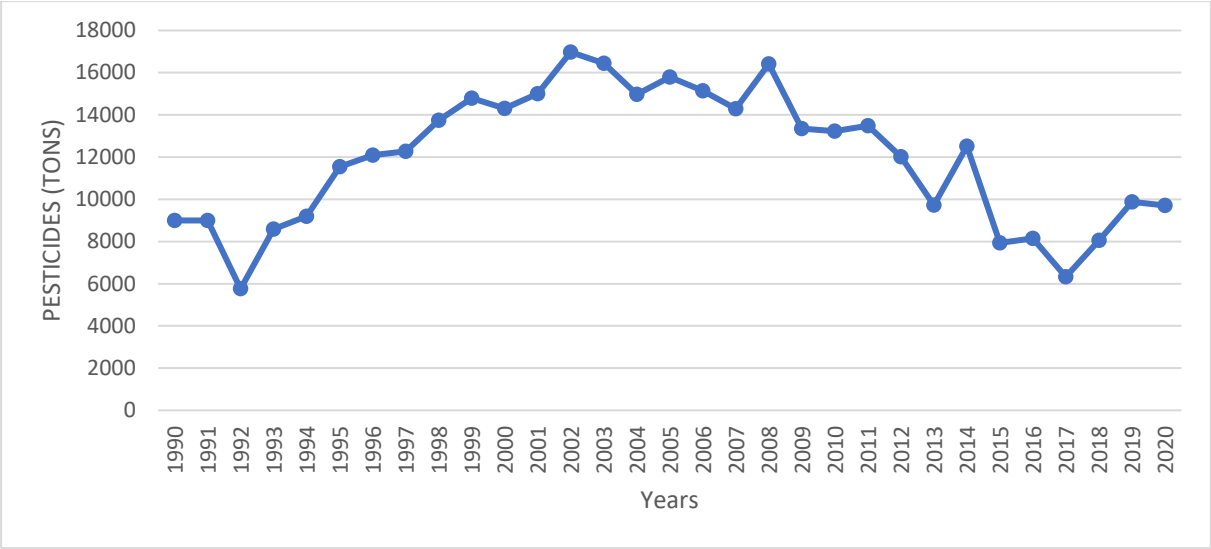

Figure 1. Pesticide consumption in Portugal (measured in terms of sales) between 1990-2020. **Source**: FAOSTAT, 2022.

When considering specific classes of pesticides, in 2020, the consumption was dominated by fungicides and bactericides, followed by herbicides, collectively accounting for 90% of all consumption (Fig. 2).

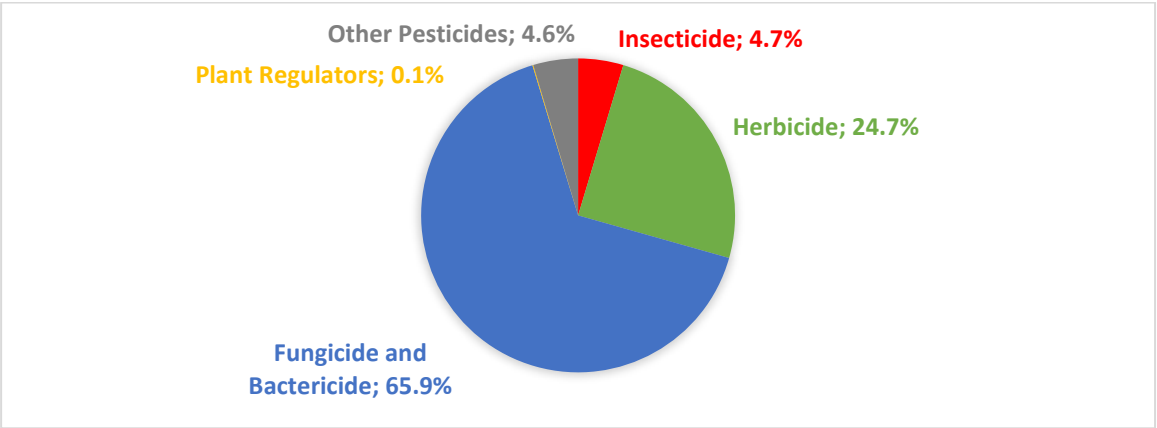

Figure 2. Relative pesticide consumption in Portugal in 2020 (measured in terms of sales) according to the type of pesticides. **Source**: FAOSTAT, 2022.

1.3 Pesticides and Amphibians

Amphibians are unique among vertebrates since they generally have a biphasic life cycle, which involves both aquatic and terrestrial environments (EFSA, 2018), as shown in figure 3. Firstly, adults lay the eggs in a breeding pond, these eggs develop

until they give rise to larvae, beginning the larval stage. This stage is spent in the same breeding ponds where individuals reproduced and where they will grow until metamorphosis which dramatically changes the anatomy and physiology of the individual, and in the case of terrestrial species, prepare them for the terrestrial environment (Gilbert, 2000).The terrestrial stage, as the name suggests, is spent in terrestrial habitats (EFSA, 2018) such as terrestrial parts of wetlands and forests (*e.g.*, Rittenhouse *et al.*, 2008).

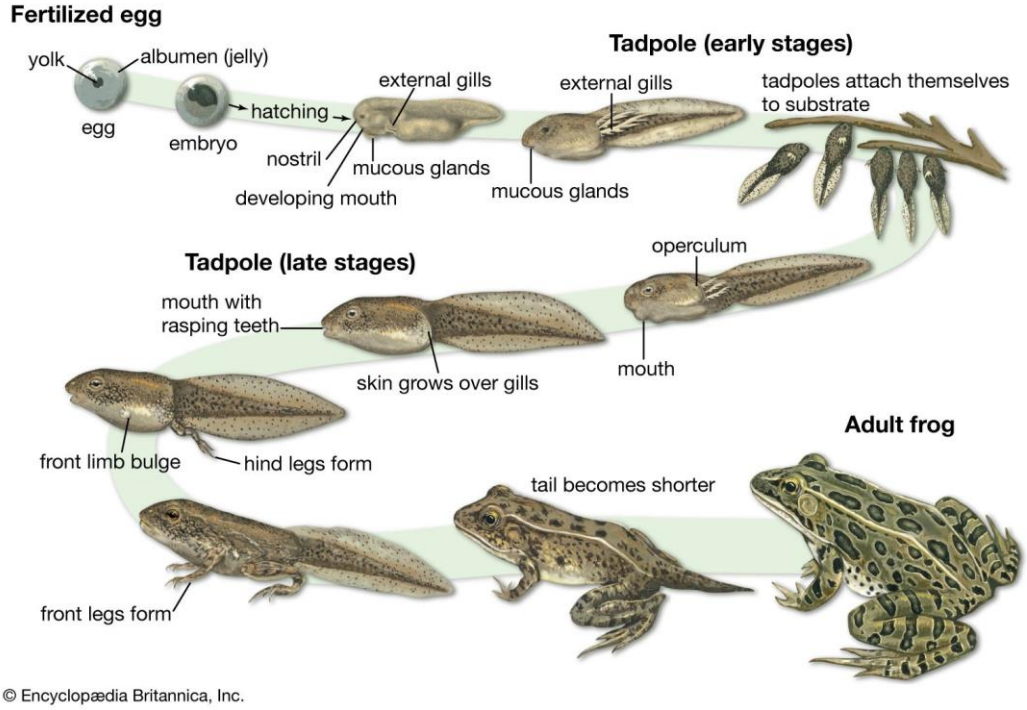

Figure 3. Visual representation of an amphibian (anuran) cycle from fertilized egg to adult frog. **Source:** *Encyclopedia Britannica*.

After metamorphosis, juveniles migrate away from the pond to terrestrial habitats where they will stay, and some species will even hibernate, indicating the beginning of their terrestrial stage (EFSA, 2018). Adults will also migrate sometime later during the breeding season, to the breeding ponds (EFSA, 2018). These migrations vary in the distance traveled (Schabetsberger, 2004). If the ponds are close to or surrounded by agricultural land, individuals may go through these agricultural fields to reach the ponds despite the problems these agricultural fields entail (Leeb *et al.*, 2020). Agricultural fields are generally unsuitable habitats for amphibians due to the chemical and mechanical practices applied (Janin *et al.*, 2012; Leeb *et al.*, 2020). But as amphibians

have shown a high degree of faithfulness to breeding habitats, it is likely that they will continue to go through agricultural land to reach the breeding pond (Reading *et al.*, 1991 *in* EFSA 2018; Vasconcelos & Calhoun, 2004 *in* EFSA 2018). Due to their biphasic life cycle, amphibians may be at a greater risk of exposure to chemical contamination (in the aquatic and adjacent terrestrial environment), this exposure may occur directly or indirectly.

Direct exposure occurs mainly for terrestrial life stages, thus, more crucial in the case of terrestrial species. It is when an individual is directly exposed to the pesticide, for example, via direct pulverization during pesticide application, which results in a higher pesticide intake (Leeb et al, 2020**;** Van Meter *et al.*, 2019) and higher mortality (Cusaac *et al.*, 2017). Direct exposure can be mitigated by crop canopy (Cusaac *et al.*, 2015), and low on-site permanence due to unsuitability of agricultural habitat (Leeb *et al.*, 2020). However, according to Berger *et al.* (2013), before sowing and stubble management, the canopy cover is low and there is a high share of bare soil, making the application of pesticides a greater risk for passing individuals. Additionally, Janin *et al.* (2012) observed a stress response and avoidance of bare soils by adult common toads (*Bufo bufo*) suggesting a lower risk to direct exposure, while they observed the opposite for juveniles, who are already at a higher risk due to their surface-to-volume ratio (Cusaac *et al.*, 2017). However, Leeb *et al.* (2020) obtained different results with a significant avoidance of soil contaminated with some formulations at maximum application rate by common toad (*Bufo bufo*) juveniles. Hence, this avoidance behavior may be dependent on the pesticides and/or their concentration.

Indirect exposure occurs when an individual is exposed to contaminated environment such as a contaminated pond or soil. These contaminations are a result of pesticides being translocated from the target location to non-target locations via different processes (Tudi *et al.* 2021). The most simple but relevant for both environments being spray drift which, according to Tudi *et al.* (2021) and references therein, is “the airborne movement of spray droplets receding from a treatment site during application”. Indirect exposure depends on many different factors that will affect the processes through which pesticides are relocated from the target area to non-target areas, such as the amount of pesticide applied, physical and chemical properties of the pesticides and soil, humidity of the soil, soil organic matter content, rainfall, etc. (Zadeh *et al.*, 2017; Glinski *et al.*, 2018; Tudi *et al.*, 2021 and references therein).

Exposure to pesticides has already been demonstrated to cause relevant negative effects in amphibians (*e.g.*, Brühl *et al.*, 2011; Baker *et al.*, 2013; Sievers *et al.*, 2019). In general, depending on the product and the species, there can be no effects, sub-lethal effects and lethal effects (Baker *et al.*, 2013; Wagner *et al.*, 2014; Sievers *et al.*, 2019).

Generally the study of pesticide exposure in amphibians is divided according to the environments associated with the life stages: the **aquatic environment**, which includes the embryonic and larval stage (*e.g.*, Agostini *et al.*,2020), adults of species such as *X. laevis* (*e.g.*, Orton *et al.*,2018; Karlsson 2021), and metamorphs (Li *et al.*, 2016; Thomson *et al.*, 2021); and the **terrestrial environment**, which includes juveniles and adults from terrestrial species (*e.g.*, Brühl *et al.*, 2013; Van Meter *et al.*, 2018). The most studied is the aquatic life stage, particularly tadpoles (*e.g.*, Pochini & Hoverman, 2017; Bach *et al.*, 2016; Agostini *et al.*, 2020; Moutinho *et al.*, 2020) and the terrestrial stage is generally neglected with less studies and data (Adams *et al.*, 2021).

1.3.1 Larval stage

Exposure of amphibian larvae or adult aquatic species to pesticides, occurs mostly indirectly through the contamination of water bodies, such as ponds or streams with pesticides (Swanson 2018**;** Glinski *et al.* 2018; Goessens *et al.* 2022). These aquatic environments can be indirectly contaminated from translocation of pesticides, as previously mentioned, or through direct contamination via application or poor practices (Tudi *et al.*, 2021 and references therein). The indirect ways of contamination include runoff and leaching of pesticides from adjacent agricultural fields. Runoff is the movement of water caused by excess amounts in the land, which can be due, for example, to heavy rainfall or over-irrigation. In this case the soil cannot absorb the excess water and the water is carried away from the target area into nearby lakes, pounds or rivers, for example (Evangelou, 1998). Leaching occurs when pesticides move through the soil into groundwater and can be influenced by many factors, such has the persistence of the pesticide in soil, pesticide solubility and soil permeability. In this case, pesticides may percolate through the soil layers with infiltrating water and

ultimately reach the groundwater. It is generally associated with pesticides that are more water soluble (Evangelou, 1998).

Studies on the effects of pesticide exposure on larval stage survival include, for example, laboratory assays with controlled exposure or field studies performed in delimited enclosures in ponds close to crops where pesticides were applied (Agostini *et al.*, 2020; Moutinho *et al.*, 2020). Agostini *et al.* (2020), for example, evaluated the effects of pesticides exposure on native tadpoles in Argentina (*Boana pulchella*, *Leptodactylus latrans*, *Rhinella fernandezae* and *Rhinella arenarum)*. The survey was conducted between 2010 to 2012 and 2016 to 2017 across 91 ponds, in field enclosures close to the crops after pesticide application. Different pesticides and formulations, either single or in mixture were evaluated across the different ponds: endosulfan (242.9 - 327.5 µg/L), glyphosate (54.5 – 315.5 µg/L) and mixtures of cypermethrin + glyphosate (102.3 – 413.9 µg/L and 67.3 – 320.7 µg/L), chlorpyrifos + glyphosate (176.9 – 256.6 µg/L and 31.2 – 155.3 µg/L), glyphosate + 2,4-D (56.8 – 330.3 µg/L and 70.4 – 209.6 µg/L), glyphosate + cypermethrin + endosulfan (<0.5, 45.6 and 230.3 µg/L). The results demonstrated a significant survival reduction after 13 of the 20 applications with a very low percentage surviving, as well as negative effect on mobility. Negative effects of pesticide exposure were also reported by Moutinho *et al.* (2020) that found a decrease in survival of *B. pardalis* tadpoles after exposure to three active ingredients: ametryn (4.00 mg/L), acetochlor (3.34 mg/L) and glyphosate (2.40 mg/L), out of the five tested. They also reported sublethal effects, including, reduced activity rate and slowed growth and development, after exposure to acetochlor and ametryn, respectively. They also observed an increase in activity of acetylcholinesterase, AChE, an important enzyme for neuronal transmission, after exposure to glyphosate, 2,4-D (1.21 mg/L), acetochlor and ametryn, as well as an increase in activity of antioxidant glutathione S-transferase, GST, a key enzyme of xenobiotic transformation after exposure to acetochlor and ametryn (Moutinho *et al.*, 2020). Pocchini & Hoverman (2017) tested commercial grade pesticides, namely Sevin® (22.5% carbaryl) and Optigard® Flex (21.6% thiamethoxam), to study the exposure dynamics in the ranavirus of *Lithobates sylvaticus*. They found that prior exposure to pesticides has implications on mortality, reducing time to death. Bach *et al.* (2016) observed sublethal effects on growth and development, including higher occurrence of abnormalities, when South American Creole frog (*Leptodactylus latrans*)

tadpoles in two distinct stages, Gosner-25 and Gosner-36, were exposed to technical-grade glyphosate (3 – 300 mg/L for both) and one of its formulations Roundup® ULTRA MAX (0.0007 – 9.62 mg a.e./L and 0.37 – 9.62 mg a.e./L respectively). For the formulation exposure a significant mortality, and an additional sublethal effect, reduction in swimming activity, at both stages was observed.

Besides the previous direct effects from exposure, pesticides can have indirect effects. Relyea & Diecks (2008) observed a trophic cascade effect caused by a continuous exposure to malathion (50 or 250 µg/L initially, and 10 µg/L per week), which reduced food availability to *Rana pipiens* tadpoles.

Additionally, the metamorphosis phase makes amphibians particularly sensitive to chemicals with endocrine disrupting properties (Ortiz-Santaliestra *et al.*, 2018), which some pesticides have been shown to have, such as a disruption of the thyroid axis (Li *et al.*, 2016; Goodier *et al.*, 2017) or of sexual differentiation (Thompson *et al.*, 2021).

1.3.2 Juvenile/adult terrestrial stage

In the terrestrial environment pesticides behave differently, there are different factors like the dynamics of pesticide and soil characteristics which differ across different types of pesticides and soil (Tudi *et al.*, 2021 and references therein).

The major process in contamination of soil is sorption, more specifically, adsorption and desorption, which is influenced by soil properties, such as pH, organic matter content, clay content and pesticide properties such as their chemical composition and solubility, that can also be influenced by soil pH (Liu *et al.*, 2010), and the pH of the compound (Liu *et al.*, 2010; Zadeh *et al.*, 2017; Bošković *et al.*, 2020; Alvarez *et al.*, 2021; Tudi *et al.*, 2021 and references therein). Of these, organic matter content in soil, is an important factor as it has been reported to be a major factor responsible for adsorption of compounds (*e.g.*, Bošković *et al.*, 2020**;** Alvarez *et al.*, 2021). Moisture is also a factor influencing adsorption since water molecules compete for binding sites (Singh, 2012 in Tudi *et al.*, 2021). While adsorption may correspond to the retention of the pesticide and possibly lower contamination in the soil, a higher desorption can mean a higher risk of soil contamination (Alvarez *et al.*, 2021).

When passing through agricultural fields during migration, amphibians are at a great risk of being exposed to pesticides particularly when there is a temporal coincidence with pesticide applications (Berger *et al.* 2011 *in* EFSA 2012; Berger *et al.* 2013; Lernhardt *et al.* 2013; Swanson *et al.*, 2018; Leeb *et al.*, 2020). Although, their exposure during this time is dependent on species and movement, amount of vegetation or canopy cover and pesticide management type (Berger *et al.* 2012; Lernhardt *et al.* 2013; Wagner *et al.*, 2014) are also important factors. Additionally, according to Wagner *et al.* (2014), the risk is especially higher for amphibians from lowland agriculture habitats since they are more likely to involve long migrations to breeding sites. But it has also been shown that amphibians move mostly through other natural corridors, like pastures (Hartel and Demeter, 2005). Besides migration, their feeding movements and habitats (Indermaur *et al.*, 2009 *in* EFSA 2018), concentration around breeding ponds (EFSA, 2018), and their proximity to agricultural land (*e.g.*, Leeb *et al.*, 2020; Goessens 2022), all entail a varying degree of potential exposure to contaminated areas or pesticide applications.

While the previous studies were mostly in field conditions, there are also studies of pesticide exposure conducted in controlled laboratorial conditions that study the ways in which their dermal exposure occurs (*e.g.*, Brühl *et al.*, 2013 e Adams *et al.*, 2021, Van Meter *et al.*, 2019), as well as the effects the studied pesticides may have (*e.g.*, Adams *et al.*, 2021 e Glinski *et al.*, 2018). One additional characteristic of exposure demonstrated in these types of studies was the influence of hydration status on pesticide uptake. Cusaac *et al.* (2017) observed an influence of dehydration on peak fungicide mortality, with a delayed mortality of dehydrated toads when compared to the hydrated ones. Glinski *et al.* (2018) observed a reduction in pesticide uptake of dehydrated toads compared to those who were hydrated, which might explain the results of Cusaac *et al.* (2017).

Generally, studies on the terrestrial stage are fewer when compared with the amount of studies on aquatic stages and include for example studies on the effects of exposure to contaminated soil (*e.g.*, Van meter *et al.*, 2018; Adams *et al.*, 2021) or field surveys evaluating exposure in agricultural land (*e.g.*, Cusaac *et al.*, 2015; Leeb *et al.*, 2020). The results ranged from direct mortality to physiological and morphological alterations, with greater emphasis in mortality since it is the easiest endpoint to assess. Juveniles were the ones who are more affected since body mass, which is lower in juveniles, has

been shown to provide protection in acute mortality, and surface-area-to-volume ratios, which are higher in juveniles, also plays an important role on exposure and mortality (Quaranta *et al.*, 2009; Cusaac *et al.*, 2017).

Regarding pesticide effects on survival, Brühl *et al.* (2013) reported elevated mortality of European common frog (*Rana temporaria*) juveniles when exposed to seven different pesticide formulations via overspray: Headline®, BAS 500 18 F (unregistered formulation), Curol B®, Captan Omya®, Dicomil®, Prosper® and Roxion®. They found that two of the tested formulations, Headline® (a.i. pyraclostrobin) and Captan Omya (a.i. Captan), reached 100% mortality at the label rate applications with Headline® causing mortality an hour after exposure, while the other pesticides had a mortality of 40-60%, at the same time. Adams *et al.* (2021) exposed the same species to two formulations of Folpan, with differing concentrations of a.i., phthalimide folpet, through contaminated soil only. They observed mortality in both formulations but only Folpan water dispersible granule (78 – 85% w/w), WDG, differed significantly from control while Folpan suspension concentrate (38 – 48% w/w) had no statistical difference from control. Cusaac *et al.* (2017) exposed *Anaxyrus cognatus* and *Anaxyrus woodhousii* adults and *A. cognatus* juveniles via direct overspray and contaminated soil to Headline® (23.6% pyraclostrobin) and Headline AMP® (13.6% pyraclostrobin and 5.14% metconazole) fungicides. These authors found low mortality of adults from direct overspray while with juveniles, Headline AMP® caused significant mortality at highest rate of application. The contaminated soil exposure with Headline AMP® caused significant mortality, but only for the highest rate applied and was dependent on the time between the treatment of the soil and the setting of individuals, although the authors hypothesize it could have been from experimental factors. Cusaac *et al.*, (2015), using enclosures on agricultural land, observed low mortality and high recovery of woodhouse toads, *A. woodhousii*, exposed to Headline AMP® fungicide (13.6% pyraclostrobin and 5.14% metconazole). The mortality occurred mostly in enclosures exposed only to the spray drift but was most likely due to dehydration since these enclosures were found to be mostly dry.

Regarding sublethal effects, Adams *et al.* (2021), observed a significant median decrease of locomotor activity in *Rana temporaria* when exposed to Folpan WDG in 4 treatments with different sums of toxic units, and non-significant median decreases in body mass, decreased locomotor activity in the other treatments. A reduction in the

consumption of *D. melanogaster* and increases in time to catch these were also observed in feeding behavior tests, 48h after exposure to both formulations. Van Meter *et al.* (2018), evaluated the worst-case scenario (pesticides at maximum application rate in bare soil) for terrestrial exposure of green frogs (*Lithobates clamitans*). There were two different studies, single exposure to each of the five different active ingredients, atrazine, metolachlor, 2,4-D, malathion and propiconazole, and exposures to mixtures of these. Exposure led to alterations in 44 metabolites in total, 12 of them being common between both studies. Many of the metabolites were amino-acids, nucleic acids, and carbohydrates critical for protein synthesis, DNA structure and replication, stress response and energy production in amphibians. In another study, Van Meter *et al.* (2019) exposed southern leopard frog (*Lithobates sphenocephala*) juveniles to contaminated bare soil for 8h, testing again for worst-case scenario. They tested the effects of single pesticide, alachlor (34.8 μg/cm^2) and atrazine (23.6 μg/cm^2) and combinations of these, also including a fertilizer, urea (2.2 mg/cm^2). They found that exposure to atrazine and a mixture of atrazine and alachlor increased AChE levels which can cause acute neurological adverse effects. Lastly, Adams *et al.* (2021) captured pairs of common toads (*Bufo bufo*) from ponds with varying agricultural surroundings in Southwest Germany, in order to assess reproductive capacity and offspring survival. They also collected and tested water samples to evaluate the amount and pesticides to which they had been exposed. They detected 22 different pesticides per pond, and when it comes to the adult toads, they observed a higher female body mass which resulted in higher fecundity, as well as a reduced fertilization rate with increasing contamination and reduced offspring survival.

1.4 Active ingredient vs. Commercial formula

There is a debate on whether formulations or their perspective active ingredient (a.i.) are more toxic, but currently there is still no consensus. Nagy *et al.* (2020), performed a systematic review and concluded that while most studies (24 of 36) found that formulations are more toxic, there were some studies who demonstrated otherwise. The most studied pesticide was glyphosate.

Cuhra *et al.* (2013) observed a difference of toxicity between acute and chronic assays of glyphosate-IPA (40% b.w. glyphosate) and the formulation Roundup Weed & Grass

Killer Concentrate Plus® (18% b.w. glyphosate) on clones of *Daphnia magna*. The active ingredient being more toxic, inducing immobility in *D. magna* in the acute assay and the formulation causing more grievous effects in the chronic assay. In an assay of toxicity of glyphosate and one of its formulations, Roundup, on Damselfly larvae (*C. pulchellum*) by Janssens *et al.* (2017) a higher toxicity of the formulation was reported, but the specific formulation and active ingredient tested were not specified. Gomes *et al.*, (2021) found that the active ingredients of two tested fungicide formulations, Prosaro® 250 EC (a.i. 12.5% prothioconazole and 12.5% tebuconazole) and Amistar® XTRA (a.i. 18.2% azoxystrobin and 7.3% cyproconazole), were more toxic than their formulations in a reproduction assay with *Enchytraeus crypticus*. While for adult survival, Prosaro was more toxic than its active ingredients.

When it comes specifically to amphibians there were only few studies who directly compared the toxicity of the active ingredient and corresponding formulation(s). Howe *et al.* (2004) exposed *R. clamitans* tadpoles to different glyphosate-based formulations: Roundup® Original, Roundup® Transorb, Roundup® Biactive, Glyfos® AU, Glyfos® BIO, Touchdown® 480 (all 360 g/L of glyphosate acid) and technical grade glyphosate (570 g/L glyphosate acid) in acute toxicity assays, and *R. pipies* tadpoles in chronic toxicity assays until metamorphosis. For the acute toxicity assays they found that only one formulation, Roundup Original® caused high mortality. Glyfos AU® caused limited mortality and the other tested compounds including technical grade glyphosate caused no mortality. In the chronic exposure assays, they observed an increase in incidence of tail damage, gonadal abnormalities, decreased snout-to-vent-length of metamorphs, decreased rate of development, alteration in levels of TRβ mRNA, a thyroid hormone receptor (Howe *et al.*, 2004), and lastly a reduction in number of animals reaching metamorphosis in individuals exposed to formulations containing POEA. Puglis *et al.* (2010) tested the toxicity of seven active ingredients, carbaryl (2.75 – 22 mg/L), malathion (1 – 8 mg/L), imidacloprid (18.75 – 150 mg/L), β-cyfluthrin (7.5 – 60 mg/L), bifenthrin (0.125 – 1 µ/L), permethrin (2.5 – 20 µ/L) and glyphosate (0.625 mg/L – 5 mg/L), and their respective formulations in green frog tadpoles (*R. clamitans*). They found that the formulations were more toxic than the technical grade active ingredient in three of the seven pesticides tested (malathion, glyphosate, imidacloprid). For bifenthrin the formulation was toxic earlier, although it was of similar toxicity later in the experiment. While for the remaining three pesticides (carbaryl, permethrin and β-

cyfluthrin), the opposite was observed. Bach *et al.* 2016 demonstrated that both technical-grade glyphosate and the glyphosate-based Roundup ULTRA MAX® caused sublethal effects on growth and development as well as abnormalities (edema and oral abnormalities, see more in Bach *et al.*, 2016) in South American Creole frog (*Leptodactylus latrans*) tadpoles. However, Roundup ULTRA MAX® was five orders of magnitude more toxic than glyphosate, resulting in a significant mortality, unlike glyphosate and caused an additional sublethal effect (reduction in swimming activity). Although the majority of studies focused on glyphosate and glyphosate-based herbicides as seen during reading of literature (*e.g.*, Howe *et al.*, 2004; Bach *et al.*, 2016) and observed by Nagy *et al.* (2020).

Formulations are a cocktail of chemicals which include the active ingredient and other ingredients, called “adjuvants” or “co-formulants” (Nagy *et al.*, 2020). According to PAN Europe these co-formulants are used to enhance product efficiency and usability (PAN Europe, 2023). They act as solvents, surfactants or preservatives among many other functions (U.S. EPA, 2002 *in* Cox and Surgan, 2006; U.S. EPA, 2005 *in* Cox and Surgan, 2006). Surfactants, a common type of co-formulants, are added to increase solubility of the a.i. and protect it from degradation (Mesnage *et al.*, 2018).

While some studies focus on the toxicity of active ingredients (*e.g.*, Daam *et al.*, 2019) when it comes to the toxicity of formulations, there are more factors influencing it than active ingredients, particularly, the aforementioned co-formulants (Mesnage *et al.*, 2018). Depending on their physicochemical properties and that of the active ingredient, co-formulants can have different effects on toxicity, possibly even acting synergistically or antagonistically (Cox and Surgan 2006; Nagy *et al.*, 2020 and references therein; Gomes *et al.*, 2021). These co-formulants can vary between formulations of the same active ingredient (Mesnage *et al.*, 2018; Nagy *et al.*, 2020), having even more possible variability of effects. Niedobóva (2022) studied the effects on the Wolf-spider (genus *Pardosa*) and showed that different formulations of glyphosate-based herbicides had different effects on the same species or genus. The terrestrial exposure study of European common frogs (*Rana temporaria*) by Adams *et al.* (2021) observed a difference in toxicity between two distinct formulations, Folpan® 80 water dispersible granule (WDG) and Folpan® 500 suspension concentrate with the same amount of active ingredient and differing additives. Also, co-formulants alone can have toxic effects independently of the active ingredient, as seen in the example of the surfactant

polyethoxylated tallow amine, POEA, in glyphosate-based formulations which was banned from being used in these types of formulations in 2016 (European Commission, 2023) after studies demonstrating its toxic effects to organisms throughout the years (*e.g.*, Howe *et al.*, 2004, Relyea 2005, Moore *et al.*, 2012). Additionally, Brühl *et al.* (2013) observed higher mortality due to additives, when exposing European common frogs (*Rana temporaria*) juveniles via overspray. The authors found that the formulation with the highest percentage of the additive (naphta), had a mortality of 100% while the formulation with lowest additive content had the lowest mortality of just 20%. However, as specified by Nagy *et al.* (2020) and the other studies involving adjuvants, components of formulations are not required to be disclosed by law, unless they are hazardous to the environment or human health causing difficulty for the evaluation of the toxicity of these compounds.

Additionally, the different types of active ingredient and formulation tested, if not disclosed properly can cause confusion on which type causes the effects as brought to attention by Cuhra *et al.* (2013). The authors reported studies using the common name glyphosate without distinction of the two possible active ingredients, technical grade glyphosate which has low solubility and glyphosate-IPA salt, which is water soluble.

Considering these factors and the large amount of possible and current formulations, studying these formulations to ascertain whether they are more toxic is much harder and strenuous than active ingredients alone.

1.5 Considerations for risk assessment and pesticide approval

In order to prevent health risks on the population and environmental damage caused by pesticides, there are risk assessments frameworks, laws and regulations based on scientific research conducted by several regulatory agencies. In the EU, there is a long process for licensing and approval of pesticides from companies, including risk assessment and risk management (Siviter *et al.*, 2023). Risk assessment involves, in the Analysis phase, experiments conducted or commissioned by the company proceeded by evaluation by the member state, who then carries out an Environmental Risk Assessment, ERA, and which, in the end, will be reviewed by the *European Food*

Safety Authority, EFSA (Siviter *et al.*, 2023; EFSA, 2023). The risk management is the legislative and regulatory part of the process (see Siviter *et al.*, 2023).

Currently in risk assessment there is a lack of amphibian toxicological data. Surrogates are used, including fish for the aquatic stage, birds, and mammals for the terrestrial stage (Crane *et al.*, 2016). While surrogates used in aquatic stages for acute toxicity, for example, Rainbow trout (*Oncorhynchus mykiss*), have been found to be protective of amphibians (Ortiz-Santaliestra *et al.*, 2018; Glaberman *et al.*, 2019; Adams *et al.*, 2021), when it comes to chronic toxicity in aquatic stages and acute and chronic toxicity in terrestrial stages the surrogates employed are not sufficient (Ortiz-Santaliestra *et al.*, 2018). Amphibians hold physiological characteristics (Quaranta *et al.*, 2009), including metamorphosis, that are unique among terrestrial vertebrates (Ortiz-Santaliestra *et al.*, 2018), which influence exposure dynamics and effects, and are not considered when using these groups as surrogates. There is a need for reevaluation of current or new surrogates which are compliant with the EU directive 63/2010/EC, but there is a lack of toxicological data from terrestrial exposure for comparison (Adams *et al.*, 2021).

There has been an increasing literature on the toxicity data for amphibians, particularly for the aquatic and early stages of development due to easier logistics and ethical approval compared to juveniles and adults (Sievers *et al.*, 2019). However, the focus on these stages causes neglect of potential terrestrial exposure (Van *et al.*, 2014), which has been less documented and is extremely relevant (Sievers *et al.*, 2019). It is therefore of paramount importance to have more toxicity data for amphibians across all stages.

1.5. Study species

Pelophylax perezi, commonly known as the Perez's frog or Iberian water frog, is an Anuran of the Ranidae family, it is endemic to the Iberian Peninsula, where it has an ample distribution. This species also occurs in southwest France, and that has been introduced into the whole archipelago of Azores (except S. Jorge, Graciosa and Corvo), the Baleares, the Canary islands and lastly the United Kingdom (Feio & Ferreira, 2019; Sousa, 2021; IUCN, 2022). It has a high abundance and ample distribution throughout Portugal (Feio & Ferreira, 2019; Sousa, 2021), having great ecological plasticity, occupying several different aquatic and terrestrial habitats, such as streams, ponds, agricultural fields, pastures, etc. (Feio & Ferreira, 2019; Sousa, 2021).

Figure 4. Adult of *Pelophylax perezi* (López-Seoane, 1885). **Source**: *Museu Virtual da Biodiversidade*, 2023.

This species is active thought the year, they breed most of the year, from March to October, the only exception being cold season during which they bury themselves in mud or aquatic vegetation (Feio & Ferreira, 2019; Sousa, 2021).

They have a larval stage period of a few months, during which they inhabit the bottom of the water feeding on periphyton, and individuals may spend a whole year in this stage until spring, when they will complete their metamorphosis (Feio & Ferreira, 2019; Sousa, 2021). After metamorphosis, they will reach sexual maturity after 1 to 3 years

(Sousa, 2021). In the adult stage, they rarely leave the vicinity of water bodies and feed mostly on flying insects, but they also feed on other organisms like snails and crustaceans, and even eggs and larvae of fish and other amphibians (Sousa, 2021).

Based on their abundance and ample distribution, this species currently has a Least Concern, LC, conservation status, despite its population trending towards a decline (IUCN, 2022). The threats that affect this species, according to the IUCN (2022) are agriculture, via changes in habitat; invasive species, through predation by the introduced fish *L. gibbosus*; and the iridovirus which can be deadly to amphibians.

1.6 Study objectives

Considering the importance of amphibians to ecosystems and their distinctive characteristics, it is important to generate toxicity data for this important and threatened group of organisms. This study aimed at assessing the effects of dermal exposure to fluazifop-p-butyl and the respective commercial formula FUSILADE MAX® in juveniles of the anuran species *P. perezi*. For this, juveniles were exposed both via direct overspray of the pesticide or via pesticide-contaminated soil, allowing also to estimate which route was the most relevant for dermal exposure. Additionally, it was also aimed to assess the adequacy of using an invertebrate model as a surrogate to animal experimentation, to assess the dermal effects of pesticides in amphibians. For this, toxicity assays with the same compounds were performed with the species of earthworm, *Eisenia andrei*.

2. Methodology

2.1. Chemicals

The toxicity of the aryloxyphenoxypropionate herbicide fluazifop-p-butyl was studied in form the of active ingredient (a.i.) and in the form of the commercial formulation FUSILADE MAX®. Fluazifop-p-butyl (CAS NO. 79241-46-6, purity of 97.2%, molecular weight of 383.36) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) as a liquid. The commercial formulation FUSILADE MAX®, composed of 12.5% p/v (125 g/l) or 13% (p/p) of the a.i. fluazifop-p-butyl, was purchased from Nufarm (Barcelona, Spain). To prepare the test concentrations, the a.i. fluazifop-p-butyl was directly dissolved in a solution of 100 ml of FETAX medium (Dawson & Bantle, 1987) with 1% analytical grade acetone (CAS NO. 67-64-1, molecular weight of 58.08), which was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). To prepare the test concentrations of FUSILADE MAX®, it was directly dissolved in FETAX medium, without acetone.

2.2. Model species

Eisenia andrei, a species of Oligochaeta, was chosen as a possible non-animal surrogate species (according to the definition of animal in EU Directive 63/2010) to replace the use of amphibian life stages when assessing the risks of dermal exposure to plant protection products in this class of vertebrates. The reasons for selecting this species were: (i) respiration in *E. andrei* occurs through the bare and highly permeable skin (Edwards & Bohlen, 1996); (ii) they are terrestrial, being a candidate for substitution in the terrestrial component (Dominguez & Edwards, 2011); (iii) easiness to maintain cultures and to manipulate the organisms (Dominguez & Edwards, 2011); (iv) *Eisenia* sp. have been used for many terrestrial ecotoxicological studies, being recommended as a model species by several standard guidelines (*e.g.,* OECD, 1984; Babić *et al.*, 2016; Uwizeyimana *et al.*, 2017; Ramires *et al.*, 2020).

The Anura species *P. perezi* was selected to perform this study because: (i) it is a very abundant species with ample geographical distribution in the Iberian Peninsula and Southern France (Feio & Ferreira, 2019; IUCN, 2022); (ii) its endemic of the Iberian Peninsula (Feio & Ferreira, 2019; Sousa, 2021; IUCN, 2022), (iii) it is a species of

amphibian with IUCN conservation status of LC - Least Concern (IUCN, 2022); (iv) several natural populations are known to inhabit aquatic and terrestrial ecosystems adjacent to/or within agricultural fields (*e.g.*, EFSA, 2018; Feio & Ferreira, 2019; Goessens *et al.*, 2021).

2.3. Acquisition and maintenance of model species

Adults of *E. andrei* were obtained from a certified culture grown in the lab, originated with individuals purchased from CloverSTrategy Lda. (Coimbra, Portugal) and identified through barcoding. The cultures of this species were maintained in a room with controlled temperature (23 ± 1ºC), inside plastic containers filled with a 1:1 mixture of *Sphagnum* peat and cow manure, covered on the top with a black plastic so they would not be exposed to light. The manure was provided by Escola Superior Agrária de Coimbra, being of biological production, meaning it did not contain any chemicals (pharmaceuticals and Plant Protection Products, PPPs) that could influence the results obtained in the experiment. The manure passed by a process of two cycles of freezing (at -20 ºC) and thawing to eliminate microfauna/macrofauna. The substrate used in the cultures was kept with 70-80% humidity and covered with black plastic bags to prevent penetration of light and evaporation of the water. These cultures were renewed every two weeks, substituting the humus leftover with new manure for feeding.

Egg masses of *P. perezi*, at Gosner stages 10-11 (Gosner, 1960) were collected between 25th March 2022 and 10th April 2022, from reference areas, Quinta da Boavista (40°35'37.644"N 8°41'47.976"W) and Jardim da Baixa de Santo António (40°38'17.0"N 8°39'18.0"W), and promptly transported to the laboratory where they were grown and maintained. Upon arrival to the laboratory, viable eggs were selected and transferred to rectangular plastic containers, filled with a mixture (*ca* 50:50) of water from the sampling site and of the standard medium FETAX (recommended by ASTM and OECD guidelines to perform toxicity assays with amphibians (Dawson & Bantle, 1987). The proportion of FETAX in which the animals are maintained was gradually increased until reaching 100%, allowing for a gradual acclimatization of the organisms to this standard medium. Organisms were maintained in these conditions, in a room with a controlled temperature (23 ± 1ºC) and photoperiod (14:10 h light:dark) until they reached development stage G25 (Gosner, 1960). At this stage, larvae were

transferred to 25 L glass aquaria containing 10 to 14 L of FETAX medium, in densities of 4 individuals per liter, and with constant aeration. The culture media was renewed every Monday, Wednesday and Friday, and the aquaria were routinely washed, to maintain the quality of the water. Tadpoles were fed daily with a mixture of the microalgae *Raphidocelis subcapitata* and Tetramin®, in *ad libitum* quantities. Upon reaching the metamorphic stage, where forelimbs emerged and reabsorption of the tail initiates (G42), organisms were transferred to plastic containers with a lid, sizing 15 cm length x 7 cm height x 12.5 cm width, with approximately 100 ml FETAX. These containers were placed inclined in a bench, so that part of it was immersed in FETAX medium while the other part was emersed, allowing the animals to exhibit their natural behavior and select which type of environment they prefer, and also preventing them to die due to drowning. After the total reabsorption of the tail, the organisms were transferred to new similar plastic containers filled with approximately 100 g of BIO SIRO®, biologic germination substrate (Siro Agro 1 [Pine bark humus - RAL certified], sphagnum blonde peat, coconut peat and organic fertilizer of animal origin, SIRO, Portugal), where they were kept for 8 days prior to their use for toxicity assays. During this period, the juveniles were fed *ad libitum* with fruit flies (*Drosophila melanogaster* or *D. hydei*) and micro-crickets (*Acheta domesticus*), in a daily basis, enriched with REPTI PLANET® Multivitamin (fine calcium powder and multivitamin supplement with vitamin D3, Plaček Pet Products, s.r.o., Czech Republic). To keep the soil humidity at ~30%, and avoid desiccation of the juveniles, it was oversprayed with FETAX medium daily.

2.4. Experimental design

The effects of dermal exposure to the active ingredient fluazifop-p-butyl and to its commercial formulation FUSILADE MAX® were studied on adults of *E. andrei* and on juveniles of *P. perezi*.

Both species were pulse exposed to the following concentrations of the pesticides (measured as a.i.): 62.5 mg/L, 625 mg/L, 6250 mg/L, which correspond to 0.1x, 1x and 10x of the recommended dose (RD) to be applied in cereal crops, respectively. The highest concentration (10x RD) was only tested for FUSILADE MAX®, due to the low solubility of the a.i. in water, even in the presence of acetone. Organisms were also

exposed to a negative control (CTR: consisting of FETAX medium) and to a solvent control (CTR Sol: consisting of FETAX medium with 1% acetone), this later treatment was necessary when testing the a.i. solely. *Eisenia andrei* exposure scenarios included only dermal overspray to the mentioned concentrations of fluazifop-p-butyl and FUSILADE MAX®, and to the negative control and solvent control. As for the exposure of juveniles of *P. perezi* to the a.i. fluazifop-p-butyl and FUSILADE MAX® those same exposure scenarios were performed. However, regarding the exposure of *P. perezi* juveniles to fluazifop-p-butyl the following additional dermal exposure scenarios were tested: exposure through direct contact of the skin with oversprayed soil, and simultaneous exposure through dermal overspray and oversprayed soil, to mimic real scenarios that may occur in the field. For each of these scenarios, organisms were exposed to 1x RD and to the negative control.

2.5. Overspray system

A spraying system was used to control the spray volume of organisms and the soil, aiming to mimicking a scenario coherent to what happens in the field. The system consisted of a pump (of 12 Volt) with a solution-aspiration system and an outlet valve connected with a digital temporizer that allowed to control the time and volume of overspray. (Fig. 5). The pump was fixed in a wooden plank and set on top of a fixed iron support so that there was no variation on its position, with a plastic container beneath the spray nozzle where the replicas are set, as well as a plastic bag beneath it to prevent contamination of the ground (Fig. 5).

Figure 5. Photo of the setup of the pump used for performing the overspraying.

Before spraying, the pump was cleaned with FETAX with 50% acetone to remove remaining residue from previous sprays and followed by a last clean only with FETAX medium. After cleaning, the timer is set to 2 seconds of spray and 40-45 seconds between sprays, giving enough time to switch replicas. The replicas were placed beneath the spray nozzle and sprayed with the each of the test solutions. Control overspray was done with FETAX medium, while solvent control overspray was done with FETAX with 1% analytical acetone. The volume of each solution sprayed by the pump was of 200 µl, corresponding to an application rate of 250 g/ha, and a volume sprayed of 40 ml/m^2.

2.6. *Eisenia andrei* assay performance

Firstly, an unset *E. andrei* with clitellum were collected at random, from the laboratory culture and left in a separate recipient until the guts were emptied. To perform the assays, only earthworms weighting between 300 and 600 mg were selected (OECD, 1984). Before weighing, the organisms were washed with FETAX medium to remove all the dirt or soil particles; any excess water was removed as well. The earthworms that were within the required weight were selected and set aside randomly in a Petri dish corresponding to their treatment. Each treatment had a total of 5 replicas, each

replicate with one organism, totaling 25 earthworms required for the assay with the active ingredient and 20 in the case of the commercial formula (since the control solvent was not carried out in the latter case) (Figure 6). The initial average (± standard deviation), weight of organisms used to start the assay with FUSILADEMAX® was, in milligrams (mg): 442.50 ± 11.14 (CTR), 356.44 ± 22.51 (0.1xRD), 338.06 ± 13.99 (RD) and 339.68 ± 14.26 (10xRD). And when it comes to Fluazifop-p-butyl: 334.62 ± 18.49 (CTR), 360.56 ± 44.39 (CTR Sol), 346.30 ± 23.94 (0.1xRD) and 353.16 ± 12.84 (RD).

For spraying, the 5 replicas of each treatment were sprayed at the same time. While in the Petri dish they clumped together so before spraying they were pulled from each other using tweezers, in the Petri dish, so that all organisms were exposed to an equal amount of pesticide. After exposure they were individually placed in opaque small circular plastic containers, Ø 7.5cm and 4.5cm height, containing filter paper (Whatman type 1) with 1mL of FETAX to preserve humidity. They were placed and maintained in a climatized room with a temperature of 23±1ºC and covered to prevent light exposure.

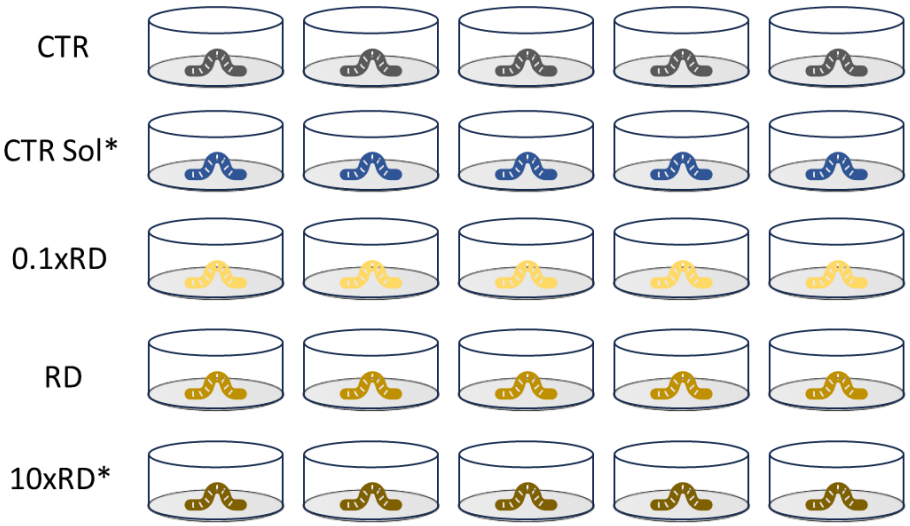

Figure 6. Scheme of the experimental design for the assays performed with Eisenia andrei. Black color: sprayed with FETAX; Blue: sprayed with 1% acetone; from yellow to brown: sprayed with 0.1x, 1x and 10x the recommended dose (RD) of each compound, respectively. *CTR Sol being only for the assay performed with the active ingredient fluazifop-p-butyl and 10xRD being only tested in the assay with FUSILADE MAX®.

This assay had a total duration of 72 h, and the survival of the individuals was checked every 24 h, registering any visual abnormalities in the skin or mortality, and humidifying the paper with 100-200 µl of FETAX in case it was necessary. At the end of assay the organisms were weighed, photographed both in the clitellum region and in another part of the body or where an abnormality was visualized, using the Dino Eye equipment and program. Three replicas were put in 15 mL FALCON® tubes and stored in a chamber at -80ºC, for ulterior chemical analysis. The other 2 replicas for histological analysis were euthanized by being submerged in Ethanol at 50% and put in equal 15 mL FALCON® tubes with Bouin for 24h, substituted by alcohol after that period.

2.7 *Pelophylax perezi* assays performance

Before starting the assays, juveniles of *P. perezi*, were weighted in an AND® analytical balance (sd=0.1 mg) and photographed with graph paper (1 mm) in the background for subsequent morphometric measurement using ImageJ. The juveniles used in the FUSILADEMAX® assay, weighted, in milligrams (mg), an average 362.21 ± 97.84 (CTR), 303.54 ± 66.11 (0.1x RD), 345.64 ± 57.81 (RD), 387.02 ± 34.21 (10x RD). The averages for morphometric parameters are presented in table 1.

Table 1. Average (±standard deviation) of morphometric parameters measured (mm) in juveniles of *Pelophylax perezi*, at the start of the assays with FUSILADE MAX®.

Morphometry (mm)/ Treatment	CTR	0.1xRD	RD	10xRD
Interorbital distance	1.886 ± 0.207	1.615 ± 0.136	1.779 ± 0.244	1.827 ± 0.222
Head width	6.451 ± 0.664	6.283 ± 0.416	6.304 ± 0.457	6.417 ± 0.192
Right leg length	11.464 ± 1.228	11.195 ± 1.243	11.569 ± 0.983	11.843 ± 0.740
Snout-to-vent length	15.703 ± 1.508	15.259 ± 1.214	15.834 ± 0.988	16.027 ± 0.559

Regarding the juveniles used for the assay with fluazifop-p-butyl the averages of initial weights were, in milligrams (mg), 354.75 ± 105.82 (CTR), 337.81 ± 49.00 (CTR Sol), 366.51 ± 59.35 (0.1xRD), 321.14 ± 57.57 (RD), 356.78 ± 60.58 (Soil Overspray, SP+F), 345.09 ± 63.28 (Direct + Soil Overspray, SP+FP). The averages of morphometric parameters are presented in table 2.

Table 2. Morphometry initial values (mm) for each parameter and treatment for Fluazifop-p-butyl.

Morphometry (mm)/ Treatment	CTR	CTR Sol	0.1xRD	RD	SP+F	SP+FP
Interorbital distance	1.867 ± 0.211	1.754 ± 0.219	1.596 ± 0.150	1.728 ± 0.209	1.687 ± 0.174	1.642 ± 0.200
Head width	6.542 ± 0.674	6.412 ± 0.389	6.641 ± 0.367	6.272 ± 0.421	6.417 ± 0.317	6.393 ± 0.419
Right leg length	12.234 ± 1.255	12.021 ± 1.127	12.216 ± 1.050	12.009 ± 0.924	12.885 ± 1.266	12.334 ± 1.187
Snout-to-vent length	16.119 ± 1.506	15.865 ± 0.848	16.554 ± 1.031	15.571 ± 0.958	16.546 ± 1.250	16.077 ± 1.113

There was also an additional parameter, body condition, which was only calculated at the end of each time period, by dividing the final weight with the measured snout-to-vent-length.

Afterwards they were arranged in pairs (each pair comprising one replica) and temporarily put in small, labeled, plastic containers Ø 7.5cm and 4.5cm height for overspray. Once they were sprayed, the pairs were put in plastic containers (15 cm length x 7 cm height x 12.5 cm width described in sub-section *Origin and maintenance*), with 100 mg standardized Lufa 2.2. soil, humidified with 10 ml of FETAX and stored in a room with controlled temperature (23±1ºC) and photoperiod (14h:10h light:dark) (Figure 7). During the assays, juveniles were fed every Mondays, Wednesdays, and Fridays, with fruit flies (see Table 3) and the amount remaining was always registered, before feeding again. They were monitored for mortality every day. At the 7th, one individual of each replica was removed (if none of the two before, otherwise, the remaining individual was maintained until the end of the assay), weighed and photographed for measurement. After this, they were euthanized through exposition to

an overdose of anesthetic, MS-222 (CAS NO. 886-86-2,purchased from Sigma-Aldrich, St. Louis, Missouri, USA) at a concentration of 6 gm/L, mixed with double the concentration of NaHCO3 to get a neutral pH (~7.2). Their carcasses were divided crosswise, the upper half of the body being preserved in a chamber at -80ºC for chemical analyses, and the bottom half for histological analyses, preserved in Bouin for 24h, substituted by alcohol after that period. At the end of the assay, at the 21st day, the previous procedure was repeated.

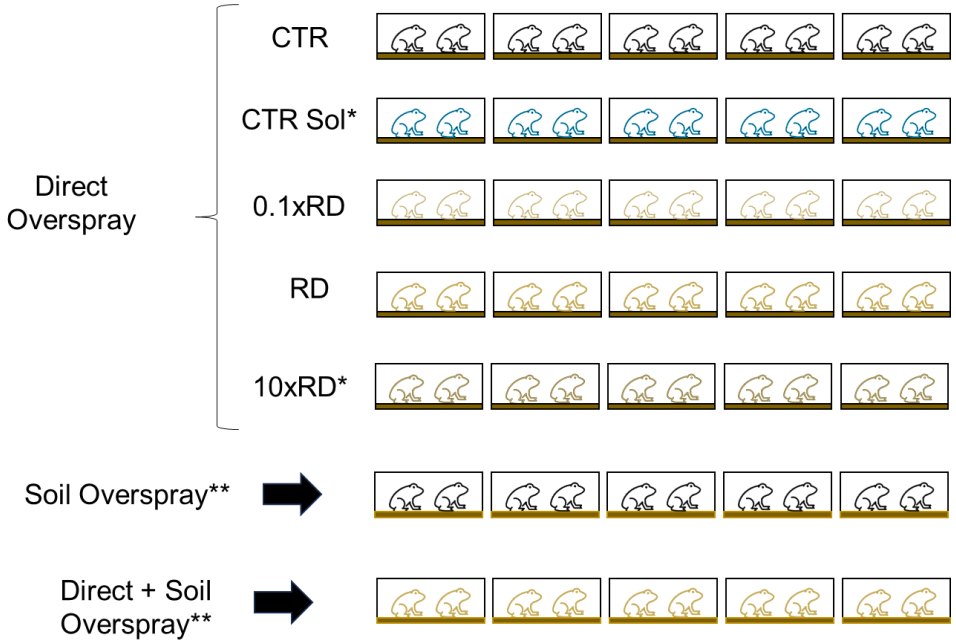

Figure 7. Scheme of the experimental design used to perform the assays with juveniles of *Pelophylax perezi*. Black color = sprayed with FETAX; Lighter blue for solvent); Yellowish Brown = sprayed with pesticide (intensity according to gradient, lighter being lower concentration and darker a higher concentration). *CTR Sol being only for the a.i. assay and 10xRD being only in the case of FUSILADEMAX® exposure, as stated before. **Soil exposure was for the a.i. exposure only as well.

Table 3. Quantity of feed each day of the experiment.

Time period	**Number of animals introduced for feeding**
1st – 7th day	20 flies
8th until the 21st day	12 flies

Relatively to soil monitoring, on the day of spraying, the day after, and at the 7th day since spraying, a small sample of soil is also taken and stored at -20ºC. The endpoints analyzed throughout the experiment were: survival, weight, feeding rate and morphometric parameters (snout-to-vent length, head (eardrum to eardrum), inter-orbital length and the right hind limb (heel to tip of longest finger)). Individuals that died in the experimental period were removed to avoid proliferation of microorganisms which could compromise the viability of other individuals in the same replica. Measurements were taken using the program ImageJ, using a graph paper as background in the photos, for scaling. All assays were conducted according to European Union Directive 63/2010 and the 3 R's policy regarding animal welfare.

2.8. Data Analysis

Statistical analysis and graphical display were conducted using the R statistics program version 4.1.1. To test for significant effects of the two compounds on final body weight and morphometric measurements, a One-Way ANOVA test was performed, followed by the multicomparison Dunnett's Test to identify differences between the control and the tested concentrations of each compound. The assumptions of normal distribution and homogeneity of variances were tested for using Shapiro-Wilk and Bartlett's tests respectively. For comparison of juvenile frog survival between all treatments, Kaplan-Meier survival analysis was used. All statistical analysis for *P. perezi* were divided between the two time periods, first seven days and from eight to 21 days, and for both species the significance levels were set at p-value ≤ 0.05.

3. Results

3.1. *Eisenia andrei*

No significant mortality was observed in *E. andrei* oversprayed with the commercial formulation FUSILADEMAX® or with the active ingredient fluazifop-p-butyl. However, significant effects were observed in the body weight of the earthworms over-sprayed with RD and 10xRD of FUSILADEMAX®, which weighted significantly less than those from the control (p = 0.007 and p = 0.008, respectively; Fig. 8). The active ingredient fluazifop-p-butyl induced no effects on the weight of *E. andrei* (p = 0.689, Fig. 8).

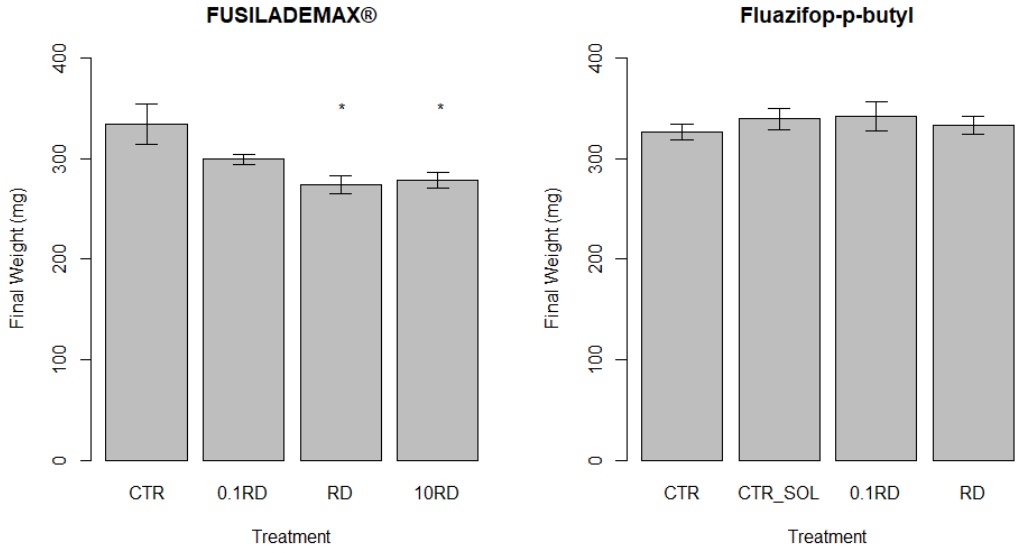

Figure 8. Average body weight of adults of *Eisenia andrei*, 72 h after being exposed to a pulsed overspray of 0.1x, 1x and 10x of the recommended dose (0.1RD, RD, 10RD, respectively) of FUSILADE MAX® (concentrations are relative to the active ingredient fluazifop-p-butyl), of 0.1 and 1x RD of fluazifop-p-butyl, and of the negative (CTR) and solvent control (CTR_SOL) solutions. Error bars represent the standard deviation. *Indicate significant differences relatively to the control (p < 0.05).

Visual inspection of the skin of earthworms did not allow to identify gross skin lesions in any of the exposed organisms (*e.g.*, Fig. 9). Except for the recommended dose of Fluazifop-p-butyl, were alterations on the skin, like vesicles, were observed on one replica (Fig. 9). For future work it is predicted to further analyze the skin for any histopathologies.

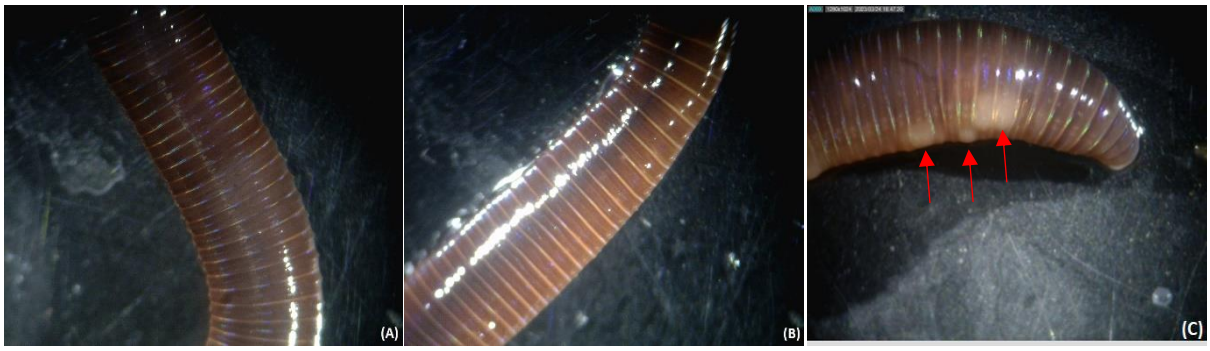

Figure 9. Pictures illustrating the skin of *Eisenia andrei* exposed to a pulsed overspray of control medium (A), and of 0.1RD (B), RD of fluazifop-p-butyl (red arrows in the image indicates skin lesions) (C).

3.2. *Pelophylax perezi*

Effects on survival

The survival of juveniles of *P. perezi* was not affected (0% mortality) 7 days after being oversprayed and/or being in contact with over-sprayed soil with the different doses of FUSILADEMAX®.

For the fluazifop-p-butyl assay, some mortality was registered during the first 7 days on the treatment of soil oversprayed with the RD of this active ingredient (SP+F), though, it was less than 20% (14%, Fig. 10).

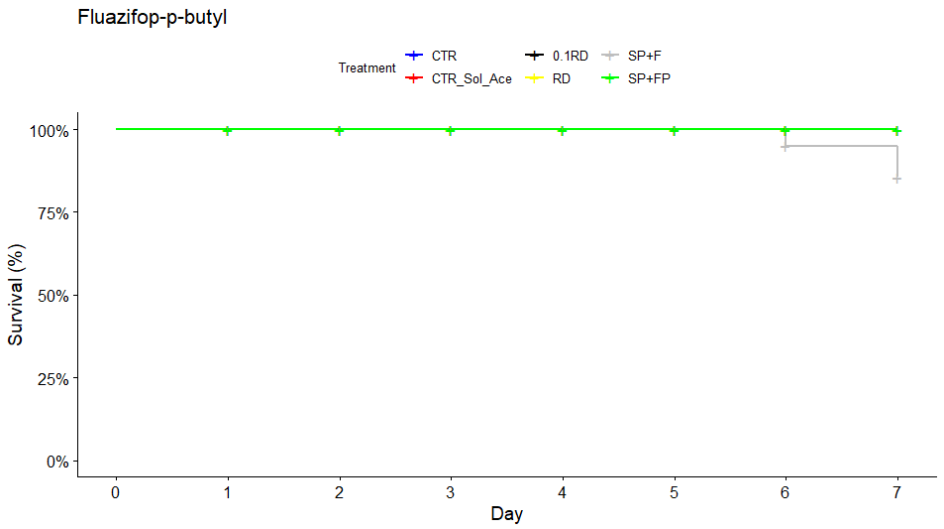

Figure 10. Survival curves for juveniles of *P. perezi*, during the first seven days of exposure, after a pulse exposure overspray with different doses of fluazifop-p-butyl (0.1RD, and DR), soil oversprayed with the recommended dose of fluazifop-p-butyl (SP+F), overspray of both soil and the juvenile with the recommended dose of fluazifop-p-butyl (SP+FP).

During the prolonged exposure period (from 8 to 21 days), following the overspraying procedures, no significant mortality was registered (Fig. 11). Though some mortality was observed in the control, in the assay with FUSILADE MAX®, it was also below 20% (14%; Fig. 11).

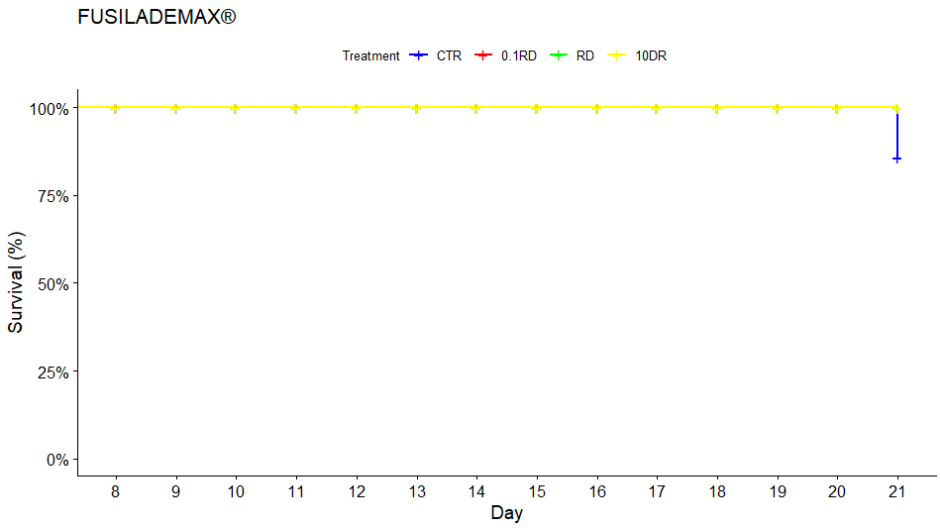

Figure 11. Survival curves of juveniles of *P. perezi*, during the period of 8 to 21 days of exposure, succeeding a pulse exposure by overspray, to three doses of FUSILADEMAX® (corresponding to 0.1x, 1x and 10x the recommended dose-RD of the active ingredient fluazifop-p-butyl) and a negative control (CTR).

During the same exposure period (8 to 21 days), and for the assay with fluazifop-p-butyl, no significant mortality was registered (Fig. 12).

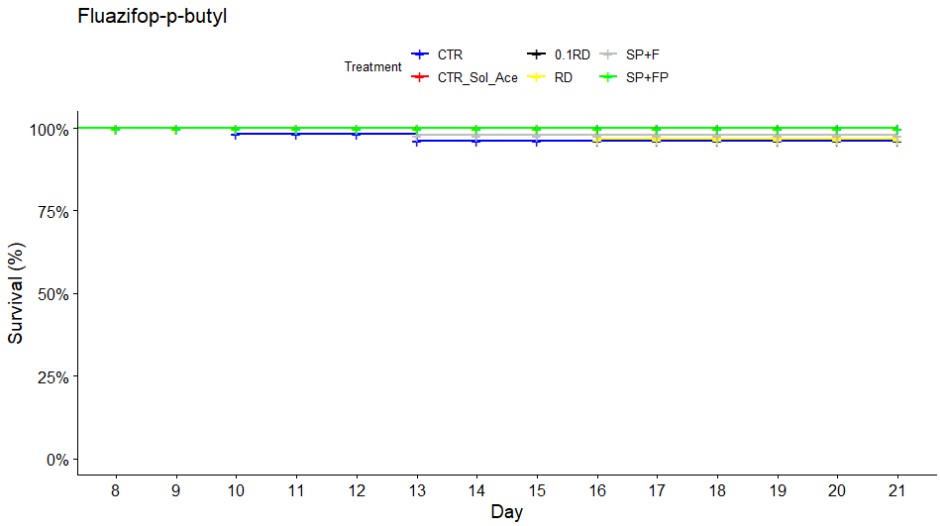

Figure 12. Survival curves for juveniles of *P. perezi*, during the period of 8 to 21 days of exposure, after a pulse exposure overspray with different doses of fluazifop-p-butyl (0.1RD, and DR), soil oversprayed with the recommended dose of fluazifop-p-butyl (SP+F), overspray of both soil and the juvenile with the recommended dose of fluazifop-p-butyl (SP+FP).

Effects on feeding, body lengths and weight

During the overall exposure period of juveniles of *P. perezi* to pulses of FUSILADEMAX® and fluazifop-p-butyl no significant changes were observed in their feeding behaviour, when compared to control organisms (See Table A1, A2, A3, A4).

Similarly, the exposure of *P. perezi* juveniles to the different treatments with FUSILADEMAX® and fluazifop-p-butyl caused no significant alterations on their body weight, when compared with organisms from control treatments ($p > 0.05$; Fig. 13).

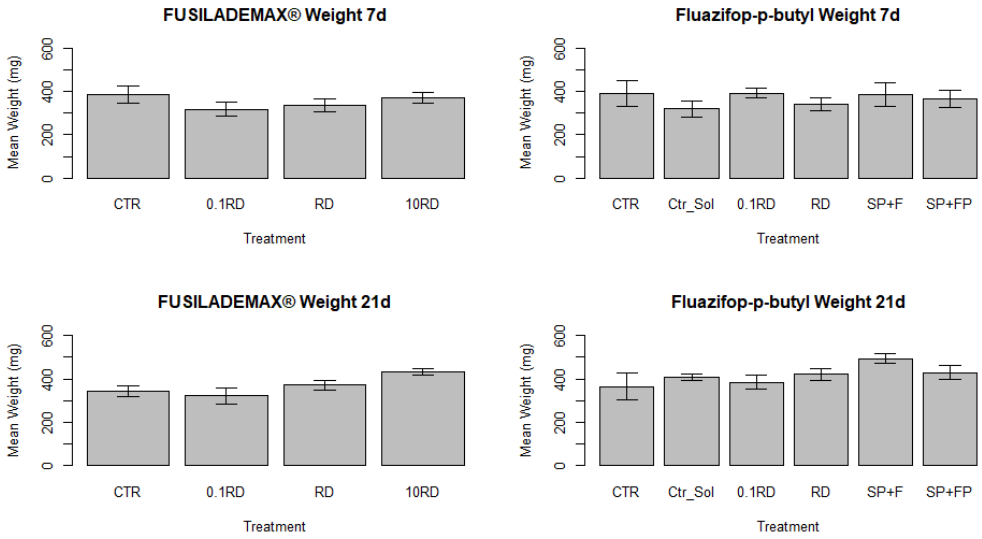

Figure 13. Average total body weight of juveniles of *P. perezi* 7 and 21 days after being exposed to: overspray with different doses of fluazifop-p-butyl (0.1RD, and DR) and FUSILADEMAX® (0.1RD, RD and 10RD); soil oversprayed with the recommended dose of fluazifop-p-butyl (SP+F), overspray of both soil and the juvenile with the recommended dose of fluazifop-p-butyl (SP+FP). “Ctr_sol” being the solvent control treatment.

Regarding the morphometric parameters, exposure to FUSILADEMAX® and fluazifop-p-butyl caused no significant effects in juveniles of *P. perezi* ($p > 0.05$; Figs. 15, 16, 17, 18). One exception occurred with organisms exposed to treatment SP+F, 21 d after the application of the pulse of the RD of fluazifop-p-butyl (Fig. 14). In this treatment, the length of the right hindlimb of the juveniles was higher than that of the juveniles exposed in control conditions ($p = 0.003$; Fig. 13).

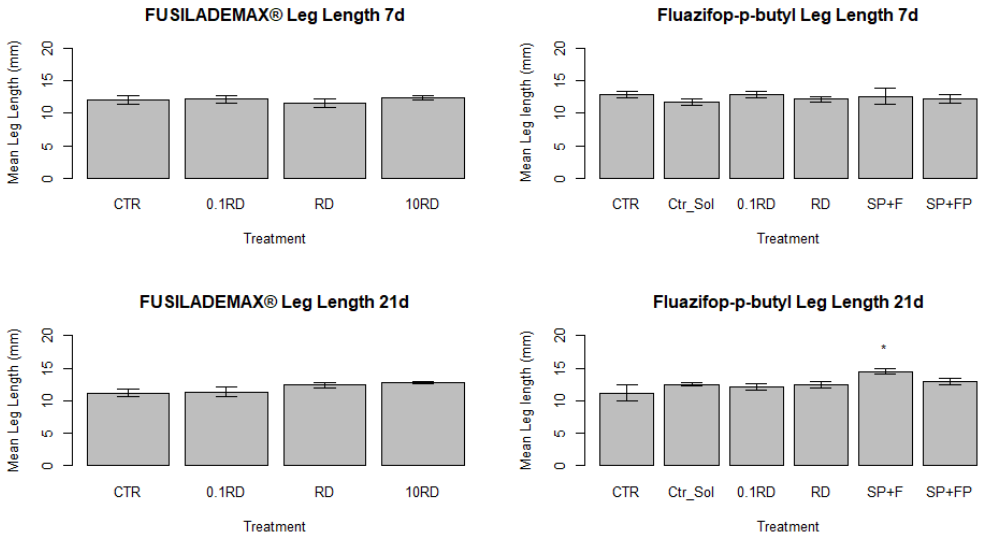

Figure 14. Average of the length of the right hindlimb of juveniles of *P. perezi*, 7 and 21 days after being exposed to: overspray with different doses of fluazifop-p-butyl (0.1RD, and DR) and FUSILADEMAX® (0.1RD, RD and 10RD); soil oversprayed with the recommended dose of fluazifop-p-butyl (SP+F), overspray of both soil and the juvenile with the recommended dose of fluazifop-p-butyl (SP+FP). “Ctr_sol” being the solvent control treatment.

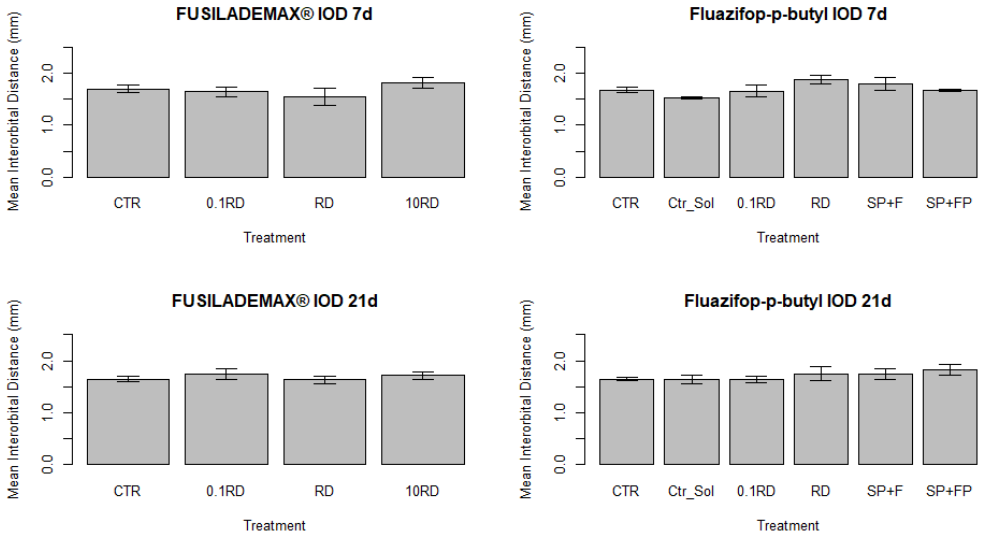

Figure 15. Average interorbital distance of juveniles of *P. perezi*, 7 and 21 days after being exposed to: overspray with different doses of fluazifop-p-butyl (0.1RD, and DR) and FUSILADEMAX® (0.1RD, RD and 10RD); soil oversprayed with the recommended dose of fluazifop-p-butyl (SP+F), overspray of both soil and the juvenile with the recommended dose of fluazifop-p-butyl (SP+FP). “Ctr_sol” being the solvent control treatment.

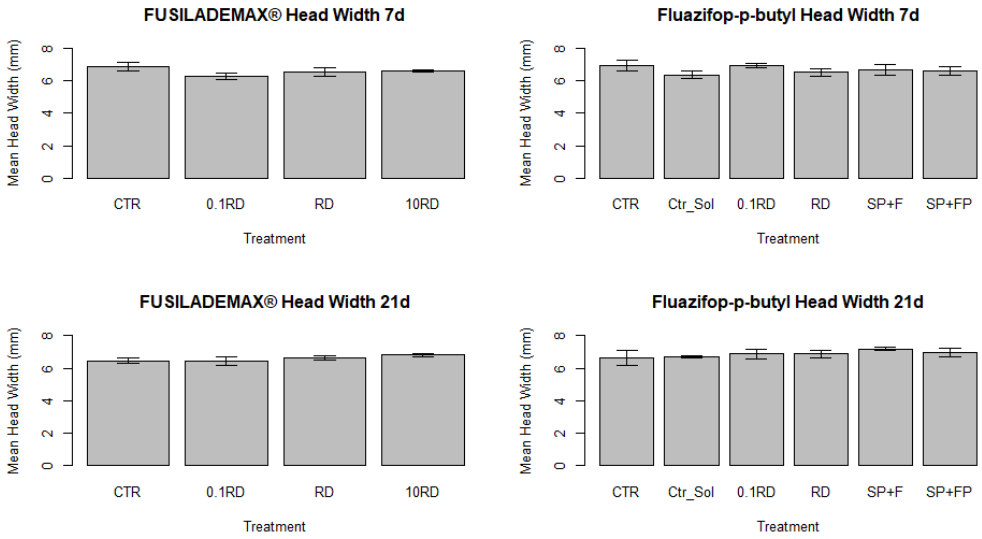

Figure 16. Average head width of juveniles of *P. perezi*, 7 and 21 days after being exposed to: overspray with different doses of fluazifop-p-butyl (0.1RD, and DR) and FUSILADEMAX® (0.1RD, RD and 10RD); soil oversprayed with the recommended dose of fluazifop-p-butyl (SP+F), overspray of both soil and the juvenile with the recommended dose of fluazifop-p-butyl (SP+FP). “Ctr_sol” being the solvent control treatment.

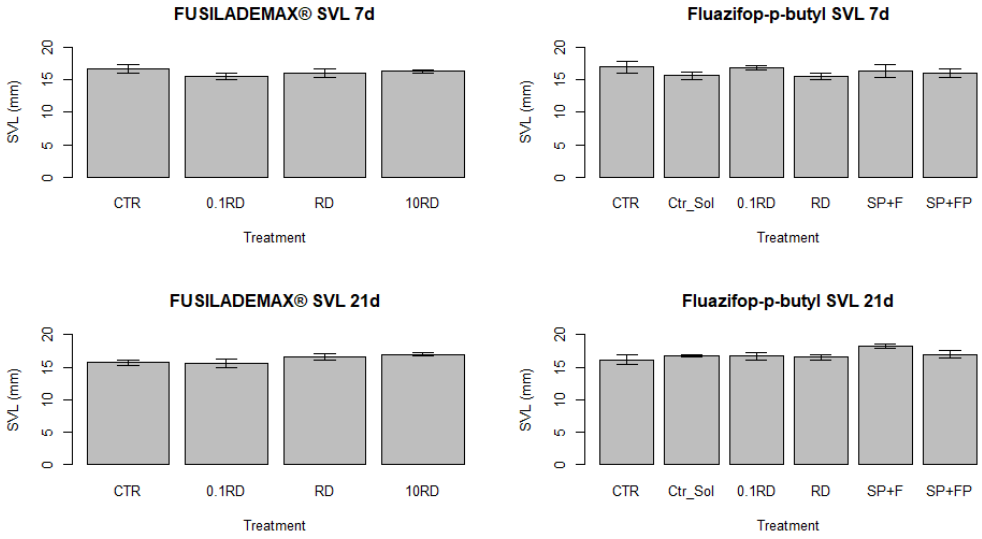

Figure 17. Average of snout-to-vent length of juveniles of *P. perezi*, 7 and 21 days after being exposed to: overspray with different doses of fluazifop-p-butyl (0.1RD, and DR) and FUSILADEMAX® (0.1RD, RD and 10RD); soil oversprayed with the recommended dose of fluazifop-p-butyl (SP+F), overspray of both soil and the juvenile with the recommended dose of fluazifop-p-butyl (SP+FP). “Ctr_sol” being the solvent control treatment.

The integration of snout-to-vent length and body weight enabled the calculation of the body condition of the organisms. For this latter parameter, no significant differences were observed between control organisms and any of the FUSILADEMAX® and fluazifop-p-butyl treatments ($p > 0.05$; Fig. 18).

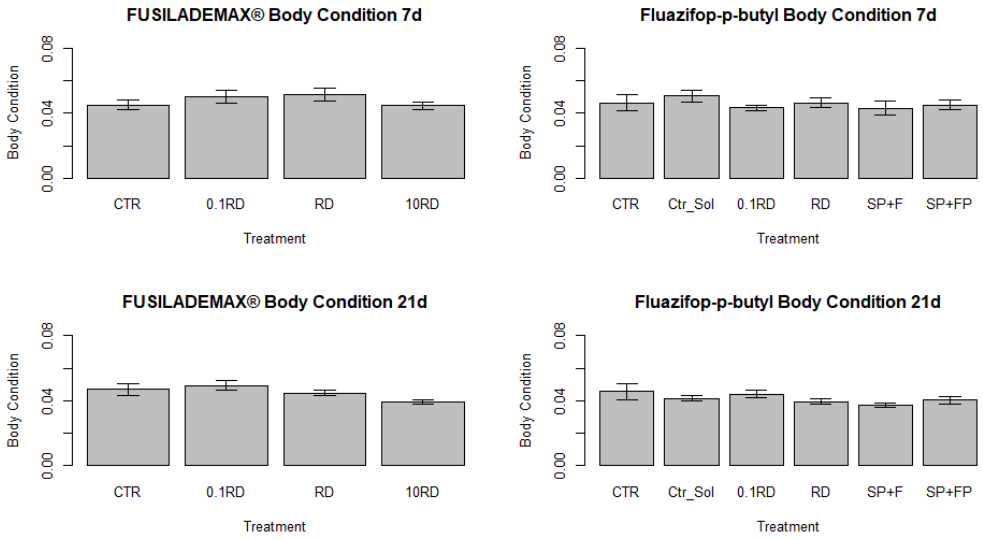

Figure 18. Average of body condition values, for juveniles of *P. perezi*, 7 and 21 days after being exposed to: overspray with different doses of fluazifop-p-butyl (0.1RD, and DR) and FUSILADEMAX® (0.1RD, RD and 10RD); soil oversprayed with the recommended dose of fluazifop-p-butyl (SP+F), overspray of both soil and the juvenile with the recommended dose of fluazifop-p-butyl (SP+FP). “Ctr_sol” being the solvent control treatment.

4. Discussion

Amphibia is the most threatened vertebrate group with 47% of species globally threatened (IUCN, 2022). There are several factors behind their decline, one of them being contaminants such as pesticides. In this study, *P. perezi* juveniles were exposed to the active ingredient fluazifop-p-butyl and its commercial formulation FUSILADEMAX®. Fluazifop-p-butyl is an acetylcholinesterase inhibitor, with an half-life of one to two weeks, used to destroy cell membranes by inhibiting lipid synthesis (Tu *et al.*, 2001). To the author's knowledge, this was the first study of terrestrial exposure to these compounds.

Two distinct exposure pathways were used: overspray and contaminated soil, to estimate which route was the most relevant for dermal exposure. Furthermore, to verify the adequacy of using an invertebrate model as a surrogate to animal experimentation, to assess the dermal effects of pesticides in amphibians, toxicity assays with the same compounds were performed with the earthworm, *E. andrei* via the paper filter method.

4.1. Effects on *E. andrei* and *P. perezi*, and replacement viability

Overall, no significant lethal effects were observed for *E. andrei* after pulse exposure of either compounds which is within expectations since according to EFSA (2012), fluazifop-p-butyl has low toxicity for soil dwelling organisms. However, there were significant sublethal effects observed in weight after exposure to the two highest concentrations of FUSILADEMAX®, contrasting with the Fluazifop-p-butyl treatment, that had no significant effects (Figure 8). These results suggest that the formulation has a higher toxicity than the active ingredient, as previously reported for other pesticides (Nagy *et al.*, 2020). This higher toxicity of the formulation is possibly due to one of its co-formulants (Mesnage *et al.*, 2018). Though it is to highlight that this pattern of results must be further confirmed with additional assays since the body weight of *E. andrei* exposed to the concentrations of Fusilade, at the start of the assays, was as lower than initial body size of earthworms in the control.

When it comes to *P. perezi*, there was non-significant mortality after pulse exposure to Fluazifop-p-butyl across the two time periods, being similar to controls (Figures 10, 12). FUSILADEMAX® had almost zero mortality with only one control dying in the last

day (Figure 11). This indicates that there are no significant lethal effects from pulse exposure to Fluazifop-p-butyl or its formulation FUSILADE MAX® on *P. perezi* juveniles.

Sublethal effects were not significant or very limited for the endpoints tested, and were exclusive to the second time period, eight to 21 days after pulse exposure (Figures 14,15,16,17). While feeding had some reductions, these were not significant in both amount and time (Tables A1,A2,A3,A4) and most likely were due to another reason and not an effect of the pesticide exposure. Similarly, there were also no detectable effects of pulse exposure on the morphometry endpoints studied, the only exception being the significant reduction of right leg length of individuals exposed via contaminated soil, solely in the second time period of the Fluazifop-p-butyl assay (Figure 14).

Overall, earthworms, *E. andrei* had similar or higher sensitivity to FUSILADE MAX® than *P. perezi*, with no significant effects on mortality and a significant reduction on earthworms weight after FUSILADEMAX® exposure. There was no significant contrast between species exposed, although *P. perezi* had an observed mortality in the Fluazifop-P-butyl assay, it was non-significant and likely not caused by exposure to the active ingredient. To the author's best knowledge this study is the first to compare both species. The fact that *E. andrei* exhibited similar or higher sensitivity to the test substances demonstrates that *E. andrei* might be a potential viable replacement however further research is necessary for more evidence, especially when it comes to other amphibian species since sensitivity may vary between species.

4.2. Overspray and Contaminated Soil

According to the results of this study there are no significant differences between exposure via contaminated soil or direct overspray, apart from right leg length. There was higher mortality in soil treatments compared to overspray, although it was not significant. These results contrast with the ones reported in the literature for other species and/or pesticides. Exposure of adults and juveniles of *Anaxyrus cognatus* and adults of *Anaxyrus woodhousii* to Headline (23.6% pyraclostrobin) and Headline AMP (13.6% pyraclostrobin and 5.14% metconazole) resulted in higher mortality when juveniles were exposed via direct overspray (Cusaac *et al.*, 2017). The same authors

also found that mortality was higher when individuals were exposed to contaminated soil immediately following contamination, compared to those who were exposed to the soil sometime after contamination (6 or 12h after).

Since exposure of all individuals and soil was done at the same time and amphibian pairs were only exposed to contaminated soil at least 30 min after contamination, the results for soil exposure are in concordance with what was expected. However, this study and the one by Cusaac *et al.* (2017) cannot be directly compared as they used a different compound, a fungicide, Headline AMP, which has already been shown to be toxic to amphibians (*e.g.*, Brühl *et al.*, 2013; Cusaac *et al.*, 2015; Cusaac *et al.*, 2017), while the tested compounds in this study were herbicides which were not found to have been previously tested in amphibians. Furthermore, there may be differences in soil dynamics or exposure dynamics between Headline AMP and Fluazifop-P-butyl and its formulations. Chemical analysis should ideally be performed. For *P.* perezi, for example, the analyses of whole-body concentrations in individuals, would allow to accurately answer the question of whether direct overspray or contaminated soil was the main pathway for pesticide exposure. Furthermore, these analyses could identify possible physiological effects (Van Meter *et al.*, 2019) or experimental problems that explain the results with more detail.

The water-octanol partition coefficient (LogKow), and more so the soil adsorption coefficient (LogKoc), have been shown to be predictors of soil contamination and have an important role in exposure dynamics (Van Meter *et al.*, 2014). These values for the studied compounds were not disclosed in the product datasheet (Supelco®, 2023) and it was not possible to determine them in this study. These values could further improve understanding of the exposure dynamics in contaminated soil with this compound (Van meter *et al.*, 2014). Therefore, in future studies where these values are unknown it is recommended that they are determined.

Histological analyses also provide important data useful to explain the results obtained, however due to lack of time, they were not performed. For example, in earthworms, although only observed in one replica, there were vesicles in the skin of one individual in the RD treatment of the active ingredient, suggesting possible effects that will only be known after these analyses.

4.3. A.I. vs Commercial formulation

Generally, formulations seem to be more toxic when compared to its corresponding active ingredients (Mesnage *et al.*, 2018; Nagy *et al.*, 2020), and this higher toxicity is thought to be due to the dynamics of the active ingredient and the other ingredients in the formulation (Mesnage *et al.*, 2018). These other ingredients or co-formulants in formulations can have antagonistic or synergistic effects with pesticides, or even have toxicity of their own (Howe *et al.*, 2004; Mesnage *et al.*, 2018; Nagy *et al.*, 2020 and references therein; Adams *et al.*, 2021; Gomes *et al.*, 2021). In this study, FUSILADEMAX® had a significant effect on *E. andrei* weight, at the recommended dose (RD) and 10xRD, compared to controls, while the active ingredient Fluazifop-p-butyl showed no adverse effects on *E. andrei*, indicating a probable effect of either co-formulants on toxicity or a synergistic effect that elevates toxicity. However, there was no significant differences in toxicity between the active ingredient Fluazifop-p-butyl and its formulation FUSILADEMAX® on *P. perezi*, with exception of one treatment of one of the studied endpoints. These results suggest a higher sensitivity of *E. andrei* to the formulation FUSILADEMAX®, probably due to its co-formulants. Still, as mentioned above, further studies are needed to confirm this pattern of results.

A similar experiment carried out by Lackmann *et al.* (2018), for the same species, and the same a.i., based with another formulation, FUSILADE FORTE®, which had a much higher concentration of active ingredient, 150 g/L (Syngenta®, 2023), found significant mortality to those exposed, assessed from LC_{50} (5.070 mg/L) they stated it was extremely toxic. They used a formulation with much higher concentration, did not study the toxicity of the active ingredient alone, and used a method of continuous exposure which lead to the higher toxicity. For comparison, the highest concentration of active ingredient tested in this study was of 6250 mg/L (10xRD) in the FUSILADEMAX® formulation which did not show significant mortality on *E. andrei*, only sublethal effects. Additionally, the lowest concentration where sublethal effects were observed was 625 mg, much higher than the LC_{50} observed. This was most likely due to the method of continuous exposure, compared to the single pulse exposure of this study. It could also be due to a synergistic effect of the compound with the co-formulants, or even toxicity of the co-formulant alone, but currently that is not possible to ascertain since co-formulants are unknown and are not required to be disclosed unless proven to be hazardous (Mesnage., 2018; Nagy *et al.*, 2020).

4.4. Implications for real scenario exposures

Although laboratory assays allow for an estimation of exposure and evaluation of possible effects these pesticides may have in amphibians, they do not realistically represent the field environment. It is very difficult to estimate amphibian field exposure since there are many factors that modulate exposure, as for example field proximity to sources of contamination (*e.g.*, Cusaac 2015; Goessens *et al.*, 2021), which also affects spray drift (Cusaac *et al.*, 2015), animals' permanence in the fields (Leeb *et al.*, 2020) and difficulty of tracking or estimating realistic amphibian movement (Lernhardt *et al.*, 2014; Leeb *et al.*, 2020). Some of these vary between types of exposure, direct and indirect, which in this study was via overspray and contaminated soil.

In terrestrial environments, exposure via direct overspray is generally simpler. The factors that affect this type of exposure include the wind, temporal coincidence (*e.g.*, Leeb *et al.*, 2020) and vegetation buffers, such as canopy cover (Cusaac *et al.*, 2015).

Indirect exposure on the other hand, is much more complex. Different soil types and pesticides have different properties, which will impact the processes of pesticide translocation governing indirect exposure (Kah *et al.*, 2007; Zadeh *et al.*, 2017; Neuwirthóva *et al.*, 2019). The numerous possible combinations of pesticides and different soil types incur a great variation in the soil-pesticide dynamics, further complicating indirect exposure assessment and field reality (Kah *et al.*, 2007; Zadeh *et al.*, 2017). Sorption-desorption processes (see section 1.3.2) are also important factors for pesticide availability and contamination in soil. Pesticide sorption depends on properties of both soil and pesticides (Zadeh *et al.*, 2017; Tudi *et al.*, 2021 and references therein). The chemical composition of the pesticide, such as nature of functional and substituting groups (Khan, 1980 *in* Zadeh 2017); pesticide and soil molecular charge, whether they are cationic, basic, anionic or neutral (Khan, 1980 *in* Zadeh 2017); soil amendment with organic matter, are important factors for pesticide sorption. Biochar, for example, has been shown to significantly increase pesticide retention and is a contender for application to reduce contamination (*e.g.*, Liu *et al.*, 2018; Khalid *et al.*, 2020).

In field conditions, the reduction on the concentrations of pesticides in soils is generally lower than in laboratory conditions. Their DT50 values will vary across different

chemical groups and individual pesticides (Neuwirthóva *et al.*, 2019). Additionally, a considerable time after application of the pesticide, “aging”, the sorption of the pesticide continues to increase, which translates into less availability for degradation (Regitano & Koskinen, 2008).

Degradation involves rupture of organic pesticides into inorganic constituents, and it can be both abiotic and biotic (Verma 2014; Zadeh 2017). Abiotic degradation is through non-living entities and may be catalyzed in different ways, for example, clay content or organic matter, hydrolysis and oxidation, these last two being the most common (Kah *et al.*, 2007; Zadeh 2017). Biotic degradation is mediated through soil microorganisms, specifically fungi and bacteria, that are responsible for the degradation of various xenobiotic compounds (Siddique 2003; Verma 2014). This type of degradation is dependent on microbial activity which is influenced by similar factors as other processes like sorption, namely, soil and pesticide physico-chemical properties, as well as being dependent on sorption itself (Kah *et al.*, 2007; Zadeh 2017). Photodecomposition, where pesticides suffer chemical transformations resulting in unique structures or ones identical to products of degradation is also an important mechanism (Zadeh 2017). Although it occurs only on the surface and close to it, due to limited sunlight penetration in the ground, it may still be a relevant process affecting pesticides (Zadeh 2017).

Another factor to take into consideration when it comes to representation of field exposures, is the curious finding of Swanson *et al.* (2018), who found a discrepancy of the pesticide concentrations found in amphibians and those found in deployed field Passive Sampling Devices, PSD’s, of corresponding habitat types. Some pesticides detected in PSD’s were not detected in amphibians, and vice-versa. This means that the pesticides to which amphibians could be exposed to, may not be exclusively those used in close agricultural fields, probably due to either translocation of those pesticides (Tudi *et al.*, 2021) or maybe migration through other fields employing different pesticides. They stated three distinct reasons, “physico-chemical properties of the pesticides, metabolism, and limited time in the habitat”, but an additional unmentioned one may be the hydration status of individuals. Glinski *et al.* (2018) and Cusaac *et al.*, (2017) demonstrated an influence of hydration on pesticide uptake and mortality, respectively, namely a decrease in uptake proportional with the decrease in hydration. In this study since initial hydration of containers where amphibians were held was

uniform it is likely that hydration had no effect on the overall results, especially when considering that the influence observed in both studies was within the first 12h. But in the field, since conditions are not controlled, hydration could have an effect on exposure, possibly increasing or decreasing depending on the hydration status of individuals during exposure.

In this study and other similar ones, it was only evaluated the effects after a single pulse exposure, which may not be realistic to field exposures where there are several pesticide applications throughout the year (*e.g.*, Berger *et al.*, 2013; Berger *et al.*, 2011 *in* Lernhardt *et al.*, 2014; Leeb *et al.*, 2020; Agostini *et al.*, 2020). Furthermore, there could be effects which are only observable when there is a continuous exposure to pesticides, as per the previously mentioned example of Lackmann et al., 2018. In another example, Relyea & Diecks (2008) exposed outdoor mesocosms with *Rana pipiens* and *R. sylvatica* to malathion (10 µg/L) every week for seven weeks and observed a trophic cascade effect in the aquatic environment. This significantly affected periphyton, a food source of *R. pipiens* tadpoles, and consequently their growth and development. These effects were only detectable after a continuous exposure assessment, which they argued was the most realistic.

P. perezi inhabits many different habitats, both aquatic and terrestrial, with the latter including agricultural and urban areas (Feio & Ferreira, 2021), which may be targets of pesticides. Additionally, individuals of these species also tend to stick close to water bodies (Feio & Ferreira, 2021), unlike other species such as *Bufo bufo* that are more terrestrial.

Considering these and all factors previously mentioned governing field exposure, it is necessary to study exposure dynamics of this and other species, in a case-by-case basis for a realistic estimation.

5. Conclusion

This work intended to provide new toxicity data on the effects of the active ingredient fluazifop-p-butyl and its commercial formulation FUSILADEMAX® on *P. perezi* juveniles by using two exposure pathways, namely overspray and contaminated soil. It further aimed to evaluate if the invertebrate model *Eisenia andrei* could potentially be as a surrogate to assess the dermal effects of pesticides in amphibians.

The obtained results disclosed no significant differences in lethal and sublethal endpoints between the different application pathways. No significant effects on mortality were observed for the active ingredient and its commercial formulation for both animals. Nevertheless, sublethal endpoints revealed that *E. andrei* is more sensitive than *P. perezi*. Further studies are necessary to validate the obtained results and the potential use of *E. andrei* to assess the dermal effects of pesticides in amphibians as a surrogate to animal experimentation.

Considering that there are many factors influencing pesticide exposure dynamics in field compared to the controlled conditions further studies under more realistic scenarios are fundamental to understand the real impact of pesticides on amphibian decline.

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Appendix 1

Table A1. Number of food items ingested each two days, during the first 7 days of exposure, following the application of the fluazifop-p-butyl treatments.

1st Time Period						
Treatment/day	Day 1	Day 3		Day 5		Day 8
	Available	Ingested	Available	Ingested	Available	Ingested
CTR R1	20	20	20	20	20	20
CTR R1	20	20	20	20	20	20
CTR R2	20	20	20	20	20	20
CTR R2	20	20	20	20	20	20
CTR R3	20	20	20	20	20	20
CTR R3	20	20	20	20	20	20
CTR R4	20	20	20	20	20	20
CTR R4	20	20	20	20	20	20
CTR R5	20	20	20	20	20	20
CTR R5	20	20	20	20	20	20
CTR_Sol R1	20	20	20	20	20	20
CTR_Sol R1	20	20	20	20	20	20
CTR_Sol R2	20	20	20	20	20	20
CTR_Sol R2	20	20	20	20	20	20
CTR_Sol R3	20	20	20	20	20	20
CTR_Sol R3	20	20	20	20	20	20
CTR_Sol R4	20	20	20	20	20	20
CTR_Sol R4	20	20	20	20	20	20
CTR_Sol R5	20	20	20	20	20	20
CTR_Sol R5	20	20	20	20	20	20
0.1RD R1	20	20	20	20	20	20
0.1RD R1	20	20	20	20	20	20
0.1RD R2	20	20	20	20	20	20
0.1RD R2	20	20	20	20	20	20
0.1RD R3	20	20	20	20	20	20
0.1RD R3	20	20	20	20	20	20
0.1RD R4	20	20	20	20	20	20
0.1RD R4	20	20	20	20	20	20
0.1RD R5	20	20	20	20	20	20
0.1RD R5	20	20	20	20	20	20
RD R1	20	20	20	20	20	20
RD R1	20	20	20	20	20	20
RD R2	20	20	20	20	20	20
RD R2	20	20	20	20	20	20
RD R3	20	20	20	20	20	20
RD R3	20	20	20	20	20	20
RD R4	20	20	20	20	20	20
RD R4	20	20	20	20	20	20
RD R5	20	20	20	20	20	20
RD R5	20	20	20	20	20	20

Table A1. Number of food items ingested each two days, during the first 7 days of exposure, following the application of the fluazifop-p-butyl treatments.

SP+F R1	20	20	20	20	Died	
SP+F R1	20	20	20	20	20	20
SP+F R2	20	20	20	20	20	20
SP+F R2	20	20	20	20	20	20
SP+F R3	20	20	20	20	20	20
SP+F R3	20	20	20	20	20	20
SP+F R4	20	20	20	20	20	20
SP+F R4	20	20	20	20	20	20
SP+F R5	20	20	20	20	20	20
SP+F R5	20	20	20	20	20	20
SP+FP R1	20	20	20	20	20	20
SP+FP R1	20	20	20	20	20	20
SP+FP R2	20	20	20	20	20	20
SP+FP R2	20	20	20	20	20	20
SP+FP R3	20	20	20	20	20	20
SP+FP R3	20	20	20	20	20	20
SP+FP R4	20	20	20	20	20	20
SP+FP R4	20	20	20	20	20	20
SP+FP R5	20	20	20	20	20	20
SP+FP R5	20	20	20	20	20	20

Table A2. Number of food items ingested each two days, during the exposure period from 8 to 21 days, following the application of the fluazifop-p-butyl treatments.

2nd Time Period												
Treat/day	Day 8	Day 10		Day 12		Day 15		Day 17		Day 19		21
	Avail.	Ing.	Avail.	Ing.	Avail.	Ing.	Avail.	Ing.	Avail.	Ing.	Avail.	Ing.
CTR R1	12	12	12	12	12	12	12	12	12	12	12	12
CTR R1	Removed											
CTR R2	12	12	12	12	12	12	12	12	12	12	12	12
CTR R2	Removed											
CTR R3	*15	Died										
CTR R3	Removed											
CTR R4	12	12	12	12	12	12	12	12	12	12	12	12
CTR R4	Removed											
CTR R5	12	10	14	Died								
CTR R5	Removed											
CTR_Sol R1	12	12	12	12	12	12	12	12	12	12	12	12
CTR_Sol R1	Removed											
CTR_Sol R2	12	12	12	12	12	12	12	12	12	12	12	12
CTR_Sol R2	Removed											
CTR_Sol R3	12	12	12	12	12	12	12	12	12	12	12	12
CTR_Sol R3	Removed											
CTR_Sol R4	12	12	12	12	12	12	12	12	12	12	12	12
CTR_Sol R4	Removed											
CTR_Sol R5	12	12	12	12	12	12	12	12	12	12	12	12
CTR_Sol R5	Removed											
0.1RD R1	12	12	12	12	12	12	12	12	12	12	12	12
0.1RD R1	Removed											
0.1RD R2	12	12	12	12	12	12	12	12	12	12	12	12
0.1RD R2	Removed											
0.1RD R3	12	12	12	12	12	12	12	12	12	12	12	12
0.1RD R3	Removed											
0.1RD R4	12	12	12	12	12	12	12	12	12	12	12	12
0.1RD R4	Removed											
0.1RD R5	12	12	12	12	12	12	12	12	12	12	12	12
0.1RD R5	Removed											
RD R1	12	12	12	12	12	12	12	12	12	12	12	12
RD R1	Removed											
RD R2	12	12	12	12	12	12	12	12	12	12	12	12
RD R2	Removed											
RD R3	12	12	12	12	12	12	12	12	12	12	12	12

Table A2. Number of food items ingested each two days, during the exposure period from 8 to 21 days, following the application of the fluazifop-p-butyl treatments.

RD R3	Removed											
RD R4	12	8	16	12	16	6	18	Died				
RD R4	Removed											
RD R5	12	12	12	12	12	12	12	12	12	12	12	12
RD R5	Removed											
SP+F R1	Died											
SP+F R1	12	12	12	12	12	12	12	12	12	12	12	12
SP+F R2	12	10	14	Died								
SP+F R2	Removed											
SP+F R3	12	12	12	12	12	12	12	12	12	12	12	12
SP+F R3	Removed											
SP+F R4	12	12	12	12	12	12	12	12	12	12	12	12
SP+F R4	Removed											
SP+F R5	12	12	12	12	12	12	12	12	12	12	12	12
SP+F R5	Removed											
SP+FP R1	12	12	12	12	12	12	12	12	12	12	12	12
SP+FP R1	Removed											
SP+FP R2	12	12	12	12	12	12	12	12	12	12	12	12
SP+FP R2	Removed											
SP+FP R3	12	12	12	12	12	12	12	12	12	12	12	12
SP+FP R3	Removed											
SP+FP R4	12	12	12	12	12	12	12	12	12	12	12	12
SP+FP R4	Removed											
SP+FP R5	12	12	12	12	12	12	12	12	12	12	12	12
SP+FP R5	Removed											

*Due to an experimental lapse, there was a delay in removal of specimens with this treatment (CTR R3) being the only exception in which there was a reduction in feed prior.

Table A3. Number of food items ingested each two days, during the first 7 days of exposure, following the application of the FUSILADEMAX® treatments.

1st Time Period						
Treatment/day	Day 1	Day 3		Day 5		Day 8
	Available	Ingested	Available	Ingested	Available	Ingested
CTR R1	20	20	20	20	20	20
CTR R1	20	20	20	20	20	20
CTR R2	20	20	20	20	20	20
CTR R2	20	20	20	20	20	20
CTR R3	20	20	20	20	20	20
CTR R3	20	20	20	20	20	20
CTR R4	20	20	20	20	20	20
CTR R4	20	20	20	20	20	20
CTR R5	20	20	20	20	20	20
CTR R5	20	20	20	20	20	20
CTR_Sol R1	20	20	20	20	20	20
CTR_Sol R1	20	20	20	20	20	20
CTR_Sol R2	20	20	20	20	20	20
CTR_Sol R2	20	20	20	20	20	20
CTR_Sol R3	20	20	20	20	20	20
CTR_Sol R3	20	20	20	20	20	20
CTR_Sol R4	20	20	20	20	20	20
CTR_Sol R4	20	20	20	20	20	20
CTR_Sol R5	20	20	20	20	20	20
CTR_Sol R5	20	20	20	20	20	20
0.1RD R1	20	20	20	20	20	20
0.1RD R1	20	20	20	20	20	20
0.1RD R2	20	20	20	20	20	20
0.1RD R2	20	20	20	20	20	20
0.1RD R3	20	20	20	20	20	20
0.1RD R3	20	20	20	20	20	20
0.1RD R4	20	20	20	20	20	20
0.1RD R4	20	20	20	20	20	20
0.1RD R5	20	20	20	20	20	20
0.1RD R5	20	20	20	20	20	20
RD R1	20	20	20	20	20	20
RD R1	20	20	20	20	20	20
RD R2	20	20	20	20	20	20
RD R2	20	20	20	20	20	20
RD R3	20	20	20	20	20	20
RD R3	20	20	20	20	20	20
RD R4	20	20	20	20	20	20
RD R4	20	20	20	20	20	20
RD R5	20	20	20	20	20	20
RD R5	20	20	20	20	20	20
10RD R1	20	20	20	20	20	20

Table A3. Number of food items ingested each two days, during the first 7 days of exposure, following the application of the FUSILADEMAX® treatments.

10RD R1	20	20	20	20	20	20
10RD R2	20	20	20	20	20	20
10RD R2	20	20	20	20	20	20
10RD R3	20	20	20	20	20	20
10RD R3	20	20	20	20	20	20
10RD R4	20	20	20	20	20	20
10RD R4	20	20	20	20	20	20
10RD R5	20	20	20	20	20	20
10RD R5	20	20	20	20	20	20

Table A4. Number of food items ingested each two days, during the exposure period from 8 to 21 days, following the application of the FUSILADEMAX® treatments.

2nd Time Period												
Treat/day	Day 8	Day 10		Day 12		Day 15		Day 17		Day 19		21
	Avail.	Ing.	Avail.	Ing.	Avail.	Ing.	Avail.	Ing.	Avail.	Ing.	Avail.	Ing.
CTR R1	12	12	12	12	12	12	12	12	12	12	12	12
CTR R1	Removed											
CTR R2	12	12	12	12	12	12	12	12	12	12	12	12
CTR R2	Removed											
CTR R3	12	12	12	12	12	12	12	12	12	12	12	12
CTR R3	Removed											
CTR R4	12	12	12	12	12	12	12	12	12	12	12	12
CTR R4	Removed											
CTR R5	12	12	12	12	12	12	12	12	12	12	12	12
CTR R5	Removed											
CTR R6	12	12	12	12	12	12	12	10	14	14	12	12
CTR R6	Removed											
CTR R7	12	12	12	12	12	12	12	12	12	12	12	12
CTR R7	Removed											
CTR_Sol R1	12	12	12	12	12	12	12	12	12	12	12	12
CTR_Sol R1	Removed											
CTR_Sol R2	12	12	12	12	12	12	12	12	12	12	12	12
CTR_Sol R2	Removed											
CTR_Sol R3	12	12	12	12	12	12	12	12	12	12	12	12
CTR_Sol R3	Removed											
CTR_Sol R4	12	12	12	12	12	12	12	12	12	12	12	12
CTR_Sol R4	Removed											

Table A4. Number of food items ingested each two days, during the exposure period from 8 to 21 days, following the application of the FUSILADEMAX® treatments.

CTR_Sol R5	12	12	12	12	12	12	12	12	12	12	12	12
CTR_Sol R5	Removed											
0.1RD R1	12	12	12	12	12	12	12	12	12	12	12	12
0.1RD R1	Removed											
0.1RD R2	12	12	12	12	12	12	12	12	12	12	12	12
0.1RD R2	Removed											
0.1RD R3	12	12	12	12	12	12	12	12	12	12	12	12
0.1RD R3	Removed											
0.1RD R4	12	12	12	12	12	12	12	12	12	12	12	12
0.1RD R4	Removed											
0.1RD R5	12	12	12	12	12	12	12	12	12	12	12	12
0.1RD R5	Removed											
RD R1	12	12	12	12	12	12	12	12	12	12	12	12
RD R1	Removed											
RD R2	12	12	12	12	12	12	12	12	12	12	12	12
RD R2	Removed											
RD R3	12	12	12	12	12	12	12	12	12	12	12	12
RD R3	Removed											
RD R4	12	12	12	12	12	12	12	12	12	12	12	12
RD R4	Removed											
RD R5	12	12	12	12	12	12	12	12	12	12	12	12
RD R5	Removed											
10RD R1	12	12	12	12	12	12	12	12	12	12	12	12
10RD R1	Removed											
10RD R2	12	12	12	12	12	12	12	12	12	12	12	12
10RD R2	Removed											
10RD R3	12	12	12	12	12	12	12	12	12	12	12	12
10RD R3	Removed											
10RD R4	12	12	12	12	12	12	12	12	12	12	12	12
10RD R4	Removed											
10RD R5	12	12	12	12	12	12	12	12	12	12	12	12
10RD R5	Removed											