Deliverable 10 - Chances of inter-taxonomical surrogacy and potential to use replacement methods for exposure and toxicity assessment in amphibians and reptiles in pesticide ERA

CA18221 – PERIAMAR

PEsticide RIsk AssessMent for Amphibians and Reptiles

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Table of content

Summary
This document constitutes the deliverable # 6 of PERIAMAR. It reports the major conclusions of the short term scientific missions (STSM) that were performed within the topic "explore chances of inter-taxonomical surrogacy and potential to use replacement methods for exposure and toxicity assessment in amphibians and reptiles in pesticide ERA". This deliverable is related with the activities developed within WP3 and directly supports achieving *RCO 3: To propose innovative strategies for implementing ERA schemes for amphibians and reptiles based on increasing representativeness and ecological relevance of the assessment while minimising animal use*.

A total of 6 STSMs were carried out in this topic involving 5 young researchers (3 female and 3 male reserachers) from diverse nationalities, as listed below:

- Title: *In vitro* testing applied to herpetofauna studies.
  Grantee name: Gledjan Caka (M)
  Country: Albania

- Title: Can *in vitro* testing predict toxicity *in vivo*?
  Grantee name: Sebastian Topliceanu (M)
  Country: Croatia

- Title: Review on the endpoints used to assess the ecotoxicity of pesticides to reptiles needed to improve animal welfare when making risk assessment
  Grantee name: Bárbara Santos (F)
  Country: Portugal

- Title: Potential of biologically based models to assess combined effects of pesticides and pathogens on amphibians.
  Grantee name: Blagovesta Dimitrova (F)
  Country: Bulgaria

- Title: Amphibian *vs* fish: comparison of their chronic toxicity patterns (still ongoing)
  Grantee name: Sebastian Topliceanu (M)
  Country: Croatia

- Title: Update the database on amphibian ecotoxicological literature and review information on chronic endpoints indicative of reproductive effects, including quality assessment.
  Grantee name: Frances Orton (F)
  Country: UK

Introduction
Chemical pollution is recognized as a major cause of worldwide critical decline of amphibians and reptiles. Among the existing types and sources of chemical pollution that are known to affect amphibians, pesticides that are usually applied in large quantities in agroecosystems constitute a major concern. Specifically in Europe, such a concern relates with the fact that many amphibian and reptile species were shown to be present in agricultural lanscape (Băncilă et al., 2023). As for amphibians, at least 43% of European amphibian species are acknowledge to be present arable lands (IUCN, 2020). Considering the protection status of these taxa (41% and 21% of extant species of amphibians and reptiles, respectively, are considering as threatened of extinction), the characterization of the toxicity of pesticides for herpetofauna is crucial for their protection. Though increasing numbers of works already addressed pesticide effects on amphibians and reptiles (for the latter taxa very few studies on

the effects of pesticides still exists; e.g. Freitas et al., 2020) an alarming number has still to be studied (e.g., Ortiz-Santaliestra et al., 2018; Freitas et al., 2020). One way to potentiate this would be to include amphibians in legislation for pesticide risk assessment. Actually, some regulations in EU already address this, acknowledging the need for specific data on the effects that pesticides may cause for terrestrial life stages of amphibians (e.g. EU regulations 283/2013, 284/2013). However, some constrains arise for conducting such amphibian and reptiles risk assessment, namely related with ethical issues, since standard guidelines available to run toxicity assays with amphibians involve *in vivo* exposure (while for reptiles no standard toxicity gudidelines exist) and the number of pesticides to be tested is enormous. At present, many international regulatory frameworks target the 3 Rs policy (reduce, refine, replace), discouraging the use of animal experimentation, e.g. in EU: (i) Directive EC/63/2010 recommends implementation of replacement protocols to reduce animal experimentation; (ii) REACH2018 challenges industry/scientific community to develop surrogate methods for animal experimentation; (iii) EFSA just released a scientific opinion on the state of science of pesticide risk assessment for amphibians and reptiles, where strongly recommends to focus scientific research on developing *in vitro* assays to serve first tier in RA for amphibians.

Currently, the environmental risk assessment of pesticides involves the exposure and effect characterization for several aquatic and terrestrial species that are then used as surrogates for other taxa. The endpoints that are for such surrogate species are used to estimate the toxicity on other non-target species that are not tested. This approach may constitue a way to reduce the above mentioned requirements of animal testing for amphbiians and reptiles, by enabling inter-taxonomical surrogacy. *Id est*, the identification of possible indicators in the array of endpoints that are delivered by surrogates to flag a risk to amphibians and reptiles may facilitate screening substances with high risks to these taxa. Whereas full predictability of risks to a non-assessed group is difficult to achieve, there are chances to make predictions for the effects of certain groups of substances or modes of action on amphibians and reptiles. Another approach to consider for reducing and replacing the use o animal experimentation, while performing risk assessment of pesticides or amphibians and reptiles, involves the use of *in vitro* and *in silico* methodologies, to improve predictability of effects before proposing specific tests *in vivo*. Though these tools have been widely explored, for example, for fish and mammals (for which standard *in vitro* assays already exist, e.g., OECD 243, 2016; OECD 249, 2021), limitided research has been dedicated on this topic to amphibians and reptiles. These two approaches have been addressed within PERIAMAR cost action, namely, within 4 dedicated STSMs.

STSM major findings

Inter-taxonomical surrogacy

A review on the endpoints used to assess the ecotoxicity of pesticides to reptiles needed to improve animal welfare when making risk assessment was maede by Bárbara Santos during the STSM that was granted to her. During this period, a literature review on “reptiles and pesticides” was done on Web of Science and Scopus, framed within February 2016 and July 2022, from which 138 publicatyions were analysed and included in a database that was merged with the one previously done by Ortiz-Santaliestra et al. (2017). Afterwards, the endpoints monitored in each study were retrieved and analysed. In a first analysis assessment it was clear that lethal endpoints were the ones most widely used and that non-lethal parameters are still poorly applied. It was also perceived that most studies focused on field surveys, mainly on the evaluation of pesticide bioaccumulation in tissues from lizards, crocodiles and turtles. The group of organisms mostly used were lizards, and within these, males are the ones more used in the studies impairing an accurate assessment of toxic effects at the population level, since females may respond differently. Due to time-constrains, to date only this preliminary analysis has been done to the retrieved data, though, a follow-up with a more elaborated statistical analysis of the data complied is foreseen. Among the topics still to be addressed in more detail, it is important to compare the sensitivity of different species of reptiles in order to define the most

sensitive ones that could be used as representative of the taxonomic group and allow extrapolations. A lack of studies in snakes and crocodiles exists, two groups that have lately been study using mostly bioaccumulation data but that could profit from the application of non-invasive and non-lethal methods to address pesticide toxicity due to their biological and ecological restrictions. By using these sub-lethal and non-invasive endpoints, it will allow to minimize animal use, by enabling extrapolations. Further studies should compare in the same species, the use of lethal non-lethal and non-invasive parameters to assess the possibility of using the latter as representative and ecological relevant in ecotoxicology assays.

The potential of biologically based models to assess combined effects of pesticides and pathogens on amphibians was addressed in the STSM attributed to Blagovesta Dimitrova. High quality amphibian toxicity data was retrieved to feed toxicokinetics/toxicodynamics (TKTD) models. These models, simulate the processes influencing the toxicity, at organismal level, over time, allowing to quantity it and providing a conceptual framework to better understand the causes for variability in different species' sensitivity to the same compound, as well as causes for different toxicity of different compounds to the same species. Therefore, the availability of a high quality large dataset may enable these models to accurately predict pesticide toxicity using an inter-taxa surrogacy approach. During the STSM, 4099 papers were reviewed, of this total only data from 123 papers (3% of the analysed ones) was selected for the model. This exercise, showed that only a small percentage of the available data can be used and incorporated into the TKTD model, but it is nonetheless not impossible and there are studies and papers available, which can be considered high quality sources for such an analysis.

Frances Orton performed a review on the data available for tadpole morphometry from the AMA test, to assess the sensitivity of snout-to-vent, body mass and condition index to chemicals exposure. Further, she aimed at comparing such effect data with that collected as part of the Early Life Stage fish assay, in order to determine whether effects on fish can be considered protective for amphibians, thus enabling inter-taxonomical surrogacy. Overall, the morphological endpoints measured in the AMA did not provide a more sensitive indication of adversity compared to similar endpoints measured in sheepshead minnow (*Cyprinodon variegatus*) or rainbow trout (*Oncorhynchus mykiss*). On the contrary, the effects recorded on fish species as part of the ELS test guideline were consistently more sensitive than those observed in amphibians tested using the AMA.

Adding to the STSMs of Bárbara, Blagovesta, and Frances in a still ongoing STSM, Sebastian Topliceanu is focusing on retrieving chronic toxicity data of pesticides for amphibian and reptiles aiming at comparing toxicity patterns. It is foreseen that this STSM will be finished until the 20th March 2024. A significant positive correlation has been previously found on the acute toxicity of chemicals for fish and aquantic life stages of amphibians, suggesting that risk estimation for qautic life stages of amphbiians may be estimated from fish data. A similar approach is intended to be done with the chronic toxicity data that is currently being retrieved by Sebastian.

Replacement methods

Two STSM were dedicated at developing and optimising amphibian *in vitro* tools, to contribute for the reduction and replacement of amphibian animal experimentation while carring out risk assessment of pesticides. The first STSM was attributed to Gledjan, and aimed to assess and compare the effects of different groups of chemicals (metals and pesticides) on aquatic life stages (embryos and tadpoles) and cell lines [kidney epithelial cells (A6) and fibroblast cells (XTC-2)] of the anuran model species *Xenopus laevis*. Three compounds, with different modes of action, were selected to be tested potassium chloride (KCl), copper sulphate ($CuSO_4$) and the commercial formulation of the insecticide Epik® (a.i. acetamiprid). Several assays were performed with embryos and tadpoles of X, laevis, but no NOEC, LOEC or ECx parameters could be estimated due to the lack of effects. As for the *in vitro* assays, cultures of A6 and XTC cell lines were put to grow for two weeks in order to run *in vitro* cytotoxicity assays with the same chemicals tested in the ecotoxicity assays with embryos and tadpoles, but though two attempts were made to grow the cultures they turn out to be contaminated and for that reason it was not possible to run the planned experiments. After the end of the STSM, this work was followed

up for other metals and for Epik®. Some ECx could be computed for embryos and tadpoles and cell lines (Fig. 1). Regarding the cell lines ECx could also be computed for some metas, but as for Epik® copncentrations as high as 200mg/L did not significantly affected the viability of the two tested cell lines.

In a second STSM, Sebastian Topliceanu, further generated *in vitro* toxicity data for six variants of the surfactant sodium lauryl ether sulphate (SLES), each variant holding a different number of ethylene oxide groups, regarding the two *X. laevis* cell lines (A6 and XTC-2) (this study is already published: Topliceanu et al., 2023) . This data jointly with that generated following the STSM of Gledjan, was used to compare the sensitivity of *in vivo* and *in vitro* methodologies. No significant correlations were found ($p < 0.05$; Fig. 1) between LC_{50} computed from *in vitro* and *in vivo* assays (the later with embryos and tadpoles of two anuran species). Though, it must be highlighted that these correlations were performed with a very limited number of data points (n≈7).

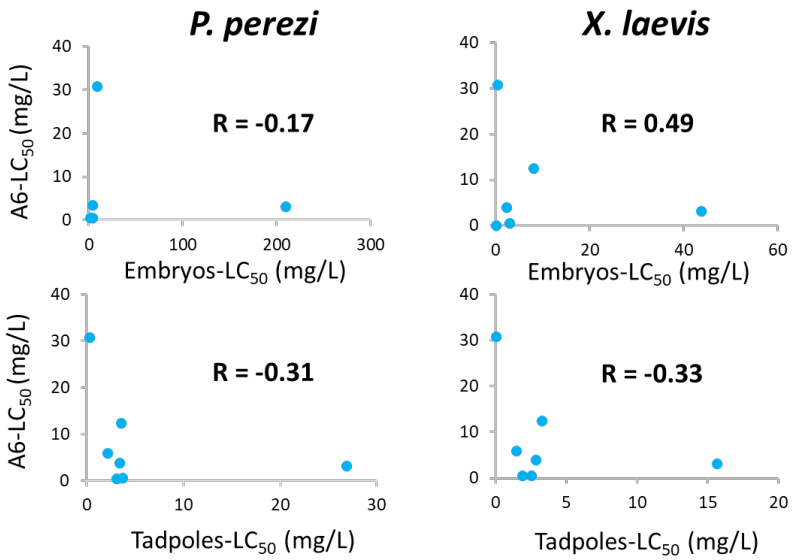

Figure 1 – Relationship between median lethal concentrations computed for several chemical, for embryos and tadpoles of *Xenopus laevis* and *Pelophylax perezi*, and the median lethal concentrations obtained for the same chemicals in *X. laevis* cell line A6.

Within these two STSM, attemps to establish primary cell lines from different tissues of *Xenopus laevis* (skin, muscle, lungs, liver) were also performed. However, none primary cell line was successfully established due to problems with bacteria contaminations and as well to the reduced reproductive rate of the isolated cells.

Concluding remarks

The data gathered within the 5 STSM suggest the need to generate more ecotoxicity data on reptiles to allow establish correlation with existing data for other terrestrial vertebrates and then elaborate models that may enable inter-taxa surrogacy for risk estimation. From the data retrieved in the STSM on "Review on the endpoints used to assess the ecotoxicity of pesticides to reptiles needed to improve animal welfare when making risk assessment", it is acknowledge that currently the available toxicity data for reptiles is very scarce and mainly focused on fields surveys, which does not allow determining an accurate relationship between toxicity os pesticides to reptiles and other terrestrial vertebrates species. Meaning that further knowledge is still needed to be generated. Nevertheless, the database gathered in this STSM adds to existing databases, contributing to improve comparisions between existing data for reptiles and surrogate terrestrial vertebrates. Regarding amphibians, available

knowledge already suggests that acute toxicity of pesticides to aquatic early life stages of amphibians may be extrapolated from acute data available for fish. In the ongoing STSM on "Amphibians vs fish: comparison of their chronic toxicity patterns" a similar exercise is to be done by comparing chronic toxicity for amphibians and reptiles and infer on the possibility to use fish chronic data to estimate corresponding chronic risk for amphibians. Nevertheless, a preliminary comparison of AMA and ELS data, suggests no evidence that amphibians are more sensitive than fish for detecting effects of chemicals on morphometric endpoints.

The use of models may help as well in reducing animal experimentation during the pesticide risk assessment framework for amphibians, given that high quality toxicity data is vailable to feed modles such as TDTK. The STSM performed on this topic, showed that only a small percentage of such type of data, in published scientific literature, is available. This was retrieved and is going to be used to feed TDTK model in a follow-up study.

Regarding replacement methods to animal experimentation, the obtained data in the two STSM revealed the technical difficulties in establishing new primary cell lines of different tissues *X. laevis*. This work is being followed up at the University of Aveiro, and new attemps with improved protocols are being made to establish primary cell lines of the skin, muscle, lunfs and liver of *X. laevis*. The *in vitro* data obtained with cell lines A6 and XTC-2 revealed no association with available

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