Deliverable 1 – Assessment of exposure routes of amphibians and reptiles to pesticides and extrapolation of pesticide toxicity from sub-organismal level to apical effects

CA18221 – PERIAMAR

PEsticide RIsk AssessMent for Amphibians and Reptiles

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Summary

This document constitutes the deliverable # 1 of PERIAMAR. It reports the major conclusions of the short-term scientific missions (STSM) that were performed within the topics on “assess exposure routes of amphibians and reptiles to pesticides” and to “extrapolate pesticides toxicity from sub-organismal level to apical effects”. This deliverable is related to the activities developed within WP1 (namely task 1.1. and 1.2) and directly supports achieving RCO 2: To continuously update and analyse the available information potentially useful to fill the gaps relative to the characterisation of pesticide exposure and effects on amphibians and reptiles.

A total of 12 STSM were carried out involving 11 researchers from 9 nationalities.

- Title: Sub organismal endpoints for reproductive toxicity: histological analysis of anuran gonads, nuptial pads and other organs.
  Grantee name: Daniele Marini (M)
  Country: Italy

- Title: Evaluation of the influence of widely used pesticides on *Bufo viridis* tadpoles.
  Grantee name: Bruno Bekič (M)
  Country: Italy

- Title: Telemetry study on lizards *Lacerta agilis*.
  Grantee name: Giulia Simbula (F)
  Country: Italy

- Title: Extended review of the potential use of ecological mesocosms in herpetofauna studies and their application to ERA of pesticides.
  Grantee name: Enerit Saçdanaku (M)
  Country: Albania

- Title: Predicting maternal transfer of pesticides in reptiles based on pollutant molecular structure.
  Grantee name: Cynthia Munoz (F)
  Country: Norway

- Title: Proof-of-concept model regarding exposure and accumulation of currently used pesticides in sand lizards.
  Grantee name: Peter Vermeinen (M)
  Country: Germany

- Title: Identify endpoints potentially useful for the extrapolation and calculation of assessment factors.
  Grantee name: John Howieson (M)
  Country: UK

- Title: Extrapolate long-term, breeding output effects from developmental testing in amphibians. Virtual Mobility Grant (VMG)
  Grantee name: Frances Orton (F)

Country: UK

- Title: Evaluating the Risk of Pesticide Exposure for Amphibians Using Residue Data from Water Sources in an Extensive Agrarian Region.
  Grantee name: Samuel González López (M)
  Country: Spain

- Title: Define trigger values for maternal transfer as an exposure route of reptile embryos. VMG
  Grantee name: Cynthia Munoz (F)
  Country: Norway

- Title: A review of literature dealing with amphibian ecology/biology in agricultural environments. VMG
  Grantee name: Bogdan Jovanović (M)
  Country: Serbia

- Title: A literature review to support modelling of amphibian oral uptake of pesticides. VMG
  Grantee name: Maria Keroglidou (F)
  Country: Greece

Introduction

A large number of extant species of amphibian and reptiles, respectively, 41% and 21%, are currently considered as threatened of extinction (IUCN, 2020). Diverse environmental perturbations are acknowledged as drivers for such a decline, with habitat loss and chemical contamination among the major ones. Agricultural ecosystems merge these two types of perturbations due to the alterations made in the landscape (related to soil preparation, e.g. plowing, for receiving the crops) and due to the application of pesticides for pest management. In Europe, it has been reported that at least 43% and 44% of the amphibian and reptile species, respectively, are present in arable lands (IUCN, 2018), some of which are considered as threatened with extinction in the IUCN Red List of Threatened Species (e.g., Mingo et al. 2016). Given the multiple stressors to which they may be exposed to in these agroecosystems, it is comprehensible that the populations of these two taxa inhabiting agricultural landscapes seem to be declining (Arntzen et al. 2017). In particular, it is acknowledged that exposure to pesticides may lead to severe impacts on amphibians (both for aquatic and terrestrial life stages) and reptiles (e.g., Mingo et al., 2016, 2017; van Meter et al., 2019). Considering the diversity of life histories and life cycles that occur in these two taxa, exposure to pesticides may occur through different routes, either in the aquatic or terrestrial environment, including dermal (e.g., water-soilborne, overspray), foodborne, and/or inhalation. At present, the framework for pesticide risk assessment does not integrate those different routes to estimate exposure levels, since it usually assumes that exposure mainly occurs through a single route (e.g., waterborne for fish and foodborne for birds or mammals). This approach neglects some of the most relevant exposure routes, namely for amphibians, where dermal exposure is expected to be of much relevance (e.g., van Meter et al., 2015; Purucker et al., 2023). Therefore, it becomes essential to deeply understand the relative importance of different exposure routes for the integrated toxicity outcome of pesticides to amphibians and reptiles, including ingestion or dermal in the water, surface of sediments or other contaminated substrates, consumption of contaminated prey, overspray and inhalation. Adding to this, it must be taken into consideration that pesticides toxicity assessment, in risk assessment frameworks, is mostly based on apical effects assessments that have direct influence on individual and population growth, development, reproduction and survival. Amphibians and reptiles comprise a wide variety of organisms and life histories often involving dramatic physiological modifications during their lifetime. Depending on species, sex, age, or life stage, some molecular or physiological responses may be especially sensitive to certain substances and hence can be used as early indicators of apical effects. For example, the process of metamorphosis in amphibians is susceptible to the exposure to exogenous chemicals with thyroid activity (Mann et al., 2009). Even though the specific mechanism involved in the alterations induced at the thyroid axis remains unclear, it has been suggested to be associated with increased expression of TH receptors, which could be suggested as an early indicator for possible effects in the timing to metamorphosis, if deep knowledge on the cascade of signaling events existed. Also, exposure at a certain time of the life cycle or generational exposure can result in delayed effects appearing after exposure termination. As an example, Karlsson et al. (2021) reported that the exposure of parent males of *Xenopus laevis* to linuron resulted in several adverse effects (e.g., reproductive and endocrine impairments) in the F1 and F2 adult males, confirming multi and transgenerational effects of the parents' exposure to the pesticide. Considering the above, within WG 1, PERIAMAR intended to: (i) identify the relevant exposure routes of amphibians and reptiles to pesticides and (ii) extrapolate pesticide toxicity in amphibians and reptiles from sub-organismal level to apical effects.

STSM major findings

Exposure routes of amphibians and reptiles to pesticides

In brief, the five STSMs accomplished within this topic pursued the collection of information from the scientific literature regarding parameters that may influence the different routes of exposure of amphibian and reptiles to pesticides. This data served as support to develop models capable of predicting exposure and uptake of pesticide by these taxa and feed the phase of characterization of exposure in the risk assessment frameworks.

Aiming to bring deeper knowledge on the way amphibians explore agroecosystems, which may influence their exposure to pesticides, Bogdan Jovanović performed a literature review in google scholar (within 1900 to 2024) retrieving information on the biology and ecology of amphibians in the agricultural environment. The collected information targeted diverse parameters, namely: type of agricultural environment, type of pesticide, taxonomic group, life stage, time spent in the field, diet composition, food regime, daily activity/patters, among others. In general, most of the papers that were analyzed involved studies with amphibian adult life stages. Some of the major patterns highlighted from this review point out that European amphibian species may use various types of agricultural landscapes (e.g., maize fields, vineyards, orchards) to accomplish their annual migration routes, which may foresee an exposure to a diverse range of pesticides. Furthermore, the assessed papers indicate that ponds adjacent to crop fields can be used as reproductive grounds and larval stages habitats, namely during the pesticide application season, which suggests exposure to those chemicals from early life to juveniles and/or adult stages. As for the group of reptiles, attempting a deeper understanding on how *Lacerta agilis* explore specifically vineyards landscape, Giulia Simbula aimed to assess the possibility of using telemetry data for estimating the portion of diet from pesticide treated area for lizards foraging on agricultural fields (by assuming that the time spent in the treated area is equivalent to the portion of diet taken from that area). For this, lizards from two vineyards landscapes were marked with microchips and a radio transmitter and were measured for conventional endpoints (weight and snout to vent length). The obtained experimental results confirmed that the sand lizards used the vineyards (mainly habitats located at their boarders) as foraging habitats, being mostly covered by vegetation or other shelter during 'active phases' in vineyards and being open exposed to the sun only for thermoregulation.

The VMG of Maria Keroglidou specifically targeted the foodborne exposure of amphibians to pesticides and aimed to parameterize a model for understanding amphibian oral uptake of pesticides. For this, a comprehensive literature review (on Scopus, Web of Knowledge, among others) was carried out regarding diet composition of the European amphibian species, their food intake rates and as well the analysis of digestive assimilation efficiencies. A categorization of the food items was performed based on the food item groups outlined by EFSA in the updated Risk Assessment for Birds and Mammals. As a result of this VMG, all the retrieved information generated a data set that is organized in an excel file that will serve as a support for the construction of the model on amphibian oral uptake of pesticides.

With the aim of studying maternal exposure to pesticides in reptiles, Cynthia Muñoz performed a STSM where she created a database containing information on the maternal transfer of organic pollutants (i.e., with measured concentrations of pollutants in paired mother-egg tissue samples), which served as the basis to develop a Gaussian orthogonal regression model. In the model, the concentration of pollutants in the eggs and in females are associated to random variables that are linked to the observed concentrations via a Gaussian probability distribution. It is implemented in R code and allows to predict concentrations of organic pollutants in eggs or females via females or eggs, respectively. As a follow-up, Cynthia performed a VMG that aimed to investigate the properties influencing the magnitude to which a chemical is maternally transferred in reptiles, and to determine a trigger value to consider this maternal transfer route of exposure in the risk assessment frameworks. To achieve this goal, a data base with more than 17 000 datapoints regarding reptile's species and chemical compounds was developed, which served to construct a model structure that was afterwards extended, via the inclusion of Kow, to assess the relation between persistence and maternal transfer rates. Peter Vermeinen used a different approach to develop a predictive model regarding the exposure and accumulation of pesticides by the sand lizard, which is considered of great potential to act as a focal species for reptiles given its wide geographic distribution across Europe. This model was established after the analogue model developed for American alligators and integrates information on ecology, physiology and ecotoxicology that is available for sand lizards. To deliver the conceptual model, firstly the mechanistic modelling of key life processes (e.g., reproduction, pollutants accumulation) and the validation of the outcome of the model (e.g., life stage duration, size, reproductive output) were achieved to support developing a lifecycle sketch. Secondly, an environmental scenario of exposure, based on a vineyard landscape, was developed by using quantitative data on the temperature and diet exposure under diverse environments (namely, the nests, hibernacula, vineyards field), that were integrated into the model with a daily resolution. Following these steps, the model was calibrated by employing Add-my-Pet parameters for bioenergetics driven development of individual lizards, data and models on activity behaviours, preys' caloric content, pollutants concentrations, and the model of maternal transfer mentioned above. Finally, model validation was accomplished against data obtained from the scientific literature regarding growth, reproduction, and activity patterns of sand lizards. Since a lack of data exists on pollutants accumulation, the toxicokinetic of the model was assessed with expert judgement.

Extrapolate pesticide toxicity from sub-organismal level to apical effects

Nine short-term scientific missions were performed with the main goal of generating new knowledge and compile existing one in the scientific literature to support extrapolation of pesticide toxicity across different levels of biological organization for amphibians and reptiles. Aiming at identifying sub-organismal endpoints that could serve as early indicators of effects in reproduction, Daniele Marini performed a histological analysis of the gonads, nuptial pads, and other organs of anuran species. Such an analysis was supported on information, retrieved from the published scientific literature, concerning the anatomy, histology and physiology of amphibians and the effects caused by the exposure to endocrine disrupters chemicals (EDCs) on reproductive related parameters (including, primary and secondary reproductive organs including nuptial pads) of anuran species. Within this STSM, the optimization and development of protocols was also accomplished to perform histological analysis of diverse amphibian tissues related with reproductive related organs (e.g., gonads, nuptial pads). Regarding the histological endpoints used to assess EDC effects on reproductive disruptions, histopathologies on primary sexual characteristics are firstly evaluated in metamorphs (stage NF66), while histopathologies on secondary sexual characteristics are assessed in adult stages. Among the most widely assessed reproductive-related histological endpoints were the: number of spermatogonia/cell nests/germ cells per seminiferous tubule, seminiferous tubule diameter, presence of ovotestis, number of Sertoli cells, presence/absence of diplotenic oocytes or of Müllerian duct regression, among others. From the information gathered in the literature, it is to highlight that the scoring of descriptive results is of much relevance to promote the harmonization and interpretation of data. When results are not harmonized or standardized its sensitivity and quality decreases, whatever the quality of the acquired data. In line with this, it was identified that endpoints such as seminiferous tubule diameter, epithelium size and height of nuptial pads, number and volume of breeding glands of nuptial pads, number and keratinization of papillae of nuptial pads, and number and size of dilator larynges muscle fiber are easily calculable features. Also, countable endpoints, like presence/absence of diplotenic oocytes or of Müllerian duct regression, should also be considered as easy endpoints to identify and monitor, though may require a high expertise on their identification. The darkness intensity of nuptial pads could also be a simple endpoint to evaluate, though it is not easily standardized as many variables may influence it. Finally, endpoints that are too descriptive (e.g., cytopathological alterations) are considered with lower sensitivity and quality, regardless of the quality of data that was obtained.

Also focused on effects at sub-individual level, Bruno Bekič assessed the influence of alpha cypermethrin and difenoconazole on stress biomarkers (activity of catalase, content on protein carbonyl, and estimated maximum capacity for metabolism-ETS) of tadpoles of *Bufo viridis*. The measurements of catalase activity, after exposure to alpha cypermethrin, indicated that the lowest tested concentrations induced an increase on its activity, suggesting and effective mitigation of oxidative stress by breaking down hydrogen peroxide. This response pattern was not observed at higher pesticide concentrations, where catalase could not degrade the excess hydrogen peroxide, resulting in elevated oxidative stress. Carbonyl measurements revealed higher values across all tested concentrations compared to the control, indicating protein damage. The exposure to difenoconazole induced no alterations in the ETS. However, catalase activity decreased even at low concentrations of difenoconazole, highlighting the significant harmfulness of this pesticide. Additionally, carbonyl values were higher than the control at the lowest tested concentrations, suggesting that difenoconazole is more detrimental than alpha cypermethrin and causes protein damage at very low concentrations. This generated new knowledge may afterwards be used for comparative assessments with adverse effects that have been assessed at the individual level (mortality and behaviour) and, thus, enabling to identify relationships between the two levels of biological organization. New ecotoxicity data was also generated within the STSM performed by Samuel López, who assessed the effects of the insecticide thiametoxam on the metamorphosis of larvae (stage G27) of *Rhinella arenarum* and *Hypsiboas pulchellus*. The obtained data will also contribute to extend the toxicity dataset available for non-standard amphibian species and enable comparisons with available data at for sub-individual endpoints, potentiating the identification of associations among the different levels of biological organization.

In the STSM performed by John Howieson a systematic literature review on the effects of contaminants on the behaviour of amphibians was carried out, to identify behavioural endpoints that are sensitive, observable, more humane, and that could be used as indicators of stress across a wide range of pesticides and species. From the retrieved information it was possible to identify 12 behavioural endpoints as indicators of larval stress. Some types of behaviours, such as those related with equilibrium, were more commonly monitored in species of Urodela than of Anura. When comparing the data obtained in the analyzed papers, activity emerged as a particularly sensitive endpoint. The retrieved data was compiled in a structured way in an excel file being available to make comparison with similar dataset available for other endpoints or other taxa, allowing to establish relationships among different levels of biological organization and/or among other taxa. Additionally, John also performed a bioassay with early life stages of *Xenopus laevis*, exposed to NaCl, and monitored endpoints at both sub-individual (gene expression) and individual (e.g., behaviour, morphometry) to identify signaling pathways, at the molecular level, of effects observed at the individual level (this data is still being processed). As a follow up of the revision made by John, Frances Orton performed a literature review to evaluate the possibility of extrapolating long-term, breeding output effects from developmental testing in amphibians. Furthermore, it was intended to identify endpoints with potential to indicate adversity in laboratory exposed amphibians and revise the existing test guidelines and identify if any improvements or amendments could be made in order to better incorporate adversity in the assessment of the impacts of chemicals on amphibians. Shortly, the revised literature highlighted that larva exposed to water from polluted sites showed inadequate fat reserves, which are needed to complete metamorphosis, resulting in under-sized metamorphs. Since size at metamorphosis is known to be of much relevance to determine survival of juveniles until reaching sexual maturity, it is estimated that this type of effect on metamorphs size may impact population stability. Additionally, a smaller size at metamorphosis may have other indirect impacts on fitness, by influencing the successful prey acquisition, the jumping performance and subsequent escape from predators, age at sexual maturity, which may influence reproductive success. This knowledge suggests that body mass at metamorphosis is, at present, the endpoint with the most evidence to monitor the occurrence of adversity, this type of information has rarely been assessed. Furthermore, changes in the time to reach metamorphosis can also have significant impacts at the population level, but the potential outcomes are varied and not well understood. For instance, accelerated metamorphosis can lead to lower weight and size, reducing individual fitness and potentially affecting survival in natural conditions. On the other hand, delayed or failed metamorphosis might result in increased mortality or even the complete loss of a tadpole population due to their inability to adapt to environmental changes such as pond drying or freezing.

Finally, at a higher level of biological organization, Enerit Saçdanaku compiled and structured available knowledge on experiments performed, under mesocosms or other semi-natural conditions, with amphibians and reptiles, aiming at better understanding the indirect effects of pesticides in a community framework. Mesocosm experiments provide a powerful tool to link between *in situ* and field studies with small-scale far from natural laboratory experiments including a single or a few species only. Most of the papers that were published on this topic focused on amphibians (93.5%), while only 6.5% studied reptiles. In general, the reviewed literature confirmed the expected negative influence of pesticides in both amphibians and reptiles, with most of the analyzed papers reporting a detrimental impact of pesticides on various life stages of these animals. However, a few studies noted that at low concentrations, pesticides might have positive indirect effect by reducing the population of predators or parasites. For instance, Boone and Semlitsch (2003) examined the interactive effects of the insecticide carbaryl and predators on the body size, development, survival, and activity of bullfrog (*Rana catesbeiana*) tadpoles. Their study found that carbaryl negatively affected predator survival by eliminating crayfish from all ponds and bluegill sunfish from ponds with the highest carbaryl concentration. With crayfish eliminated by carbaryl, bullfrogs experienced reduced predation pressure, resulting in survival rates similar to those in predator-free controls at low carbaryl concentrations. This study demonstrates that contaminants can alter community dynamics by releasing prey from predator pressure under certain exposure scenarios. All this data is still being analysed and interpreted to identify indirect effects of pesticides in herpetofauna.

Concluding remarks

The data gathered and newly generated within the performed STSM, revealed the need for developing further research to generate knowledge on the routes of exposure and uptake of pesticides on amphibians and, more urgently, on reptiles for which very scarce information still exists. Namely, knowledge on the potential food sources existing on agricultural fields for amphibians and reptiles is essential. Also, additional information on the behaviour of herpetofauna species (e.g., in reptiles would be relevant to understand escape behaviour during activities related with pesticides application and re-entry time) is required to predict species requirements for spending time in agroecosystems. This knowledge will allow a more accurate characterization of exposure and refinement of risk assessment frameworks; as well it will enable identifying management routines to reduce the effects of pesticides on amphibians and reptiles, like for example applying pesticides in periods where the presence of these taxa is less probable in the agricultural fields. Even though the identified knowledge gaps, progress in developing predictive models of exposure was achieved for amphibians and reptiles. Namely, for reptiles an orthogonal regression model to predict concentrations of organic pollutants in eggs from females or in females from eggs is developed and described, including its relevant extensions. Additionally, a proof-of-concept model to estimate exposure and accumulation of pesticides in sand lizards was developed. However, further development of this model is still ongoing as it relates with diet preferences, associated pollutants uptake and refinements and extensions of its calibration. Also, the current feasibility of the model in terms of data needs and model technical abilities are still under discussion to provide accurate recommendations for its further development. Though not being developed within STSMs, it must be highlighted that within Periamar mechanistic effect models were explored and adapted for use in amphibians, some of these models involve estimation of internal concentrations of pesticides on this taxon (please see deliverable 11 for further details).

Regarding the extrapolation of pesticide toxicity from sub-organismal level to apical levels, some advances were also achieved within the STSM, though knowledge gaps were also identified. Specifically, to establish and accurate relationship between histological endpoints to reproductive related endpoints at the individual level) in amphibians, it was identified the need to uniformize the classification/scoring of the former endpoints and of stages of reproductive germ cells to allow any comparison of results obtained within different studies. It was also acknowledged that the most relevant organs to be evaluated at the sub-organismal histological endpoints for reproductive toxicity were the gonads, namely because their impairment would gradually end on adverse effects in the reproductive outputs. Though, it must be highlighted that other primary and secondary reproductive organs should not be disregarded since at a long-term their impairment may result in impacts on behavioral and fitness characteristics. Furthermore, at the individual level some endpoints were highlighted as good predictors for long-term reproductive toxicity. However, there are few data on reproductive toxicity on amphibians, namely because only the standard LAGDA test evaluates such endpoints, and it is rarely performed; as well, a limited number of studies on the impacts of pesticides in the life cycle of amphibians were carried out. Based on these drawbacks, it is recommended that body mass at metamorphosis completion and time to metamorphosis should be monitored in parallel to other endpoints that are conventionally measured at stage NF62. In addition, optimization of measurements related with behavioural endpoints (e.g., swimming activity, feeding rates) should be further assessed for possible inclusion in the test guidelines. In order to fill the gaps on reproductive toxicity data, it is suggested that such type of data could be obtained by performing assays with the amphibian species *Silurana tropicalis*, which has a shorter life-cycle. In such assays, endpoints like gonadal histopathology or gonado-somatic-index should be assessed to infer on their accuracy/applicability as predictors of the observed reproductive toxicity endpoints (such as fertility, offspring fitness) measured in sexually mature adults. To conclude, given the data gaps that were identified, it has been suggested that the development of population modelling may be a reasonable approach to estimate the probability of population effects in the absence of definitive data. But, for this, a deeper understanding of fundamental biology of a wide range of amphibian species is required.

Finally, when considering higher than organismal levels of biological organization (e.g., community level), very scarce information exists, namely for reptiles. Thus, substantial knowledge should be considered in the future to include more amphibian and reptile species when performing mesososms, in situ, semi-field, etc., studies aiming to better understand the direct and indirect effects of pesticides at higher levels of biological organization for these taxonomical groups.

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