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Final report

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Abstract: Designing a strategy based on toxicity evaluation to improve pesticide risk assessment for terrestrial amphibians (TerAmphiTox)

Anthropogenic pollution is recognized as one of the major factors threatening amphibian populations. Plant Protection Products (PPP) are among pollutants of major concern, despite of which environmental risk assessment (ERA) conducted as part of the PPP authorization process does not routinely consider risks to amphibians. Evidence has grown pointing that current ERA protocols do not provide adequate protection to amphibians, in part because of the high susceptibility of amphibians to dermal exposure to PPP. This is related to the high permeability of amphibian tegument to diffusion of chemicals and to the variety of physiological functions played by amphibian skin that could be affected because of direct exposure to PPP. To improve the protectiveness of pesticide ERA to amphibians without requesting for in vivo testing, it is necessary to consider effects of dermal exposures through a strategy supported by current knowledge. This project aimed at increasing the knowledgebase of pesticide toxicity on amphibian terrestrial stages to support the definition of a strategy for characterization of PPP toxicity to amphibians exposed via dermal routes. We reviewed the information on the physico-chemical properties of active substances that influence their toxicity to amphibians when exposure happens through dermal contact. Then, we tested the toxicity on juvenile of an amphibian frog model, *Pelophylax perezi*, via overspray and/or contact with treated soil of 16 PPP active substances, six formulations and two co-formulants. Significant mortality was caused by three of the active substances (isoxaben, pirimicarb and lambda-cyhalothrin), as well as by formulations containing tebuconazole and alpha-cypermethrin, which resulted more toxic than their active ingredients alone. Histological analysis of skins revealed an epidermal thickening caused by exposure to seven active substances, which in the case of alpha-cypermethrin formulation was dose-dependent, and hyperplasia caused by an azoxystrobin-based formulation. Amphibian sensitivity was compared to that shown by earthworms *Eisenia andrei*, which were experimentally exposed to the same substances and concentrations as amphibians. A correlation between sensitivity of amphibians and earthworms was found. The analysis of potential coverage provided by current ERA schemes on amphibians was completed with the evaluation of toxicological and physico-chemical properties explaining sensitivity shown by amphibians tested in the present study. Whereas acute toxicity of PPP to terrestrial organisms (mammals and bees) explained better the accumulation of pesticides in frog livers and skins, amphibian toxicological sensitivity was associated with acute effects of PPP on *Daphnia*. Regarding physico-chemical properties, more lipophilic substances showed higher potential to compromise juvenile frog survival and growth. The results of the present project can support regulatory decisions to prevent PPP impacts on amphibians because of dermal exposures and contribute to the development of a test-free ERA for these animals. In particular, the combined consideration of the results obtained here could serve to identify substances of high concern, on which a special look regarding their risks to amphibians should be placed.

Kurzbeschreibung: Entwicklung einer auf Toxizitätsbewertungen basierenden Strategie zur Verbesserung der Risikobewertung von Pestiziden für terrestrische Amphibien

Anthropogene Umweltverschmutzung gilt als einer der Hauptfaktoren, die Amphibienpopulationen bedrohen. Pflanzenschutzmittel (PSM) gelten als besonders besorgniserregende Schadstoffe. Dennoch berücksichtigt die im Rahmen des PSM-Zulassungsprozesses durchgeführte Umweltrisikobewertung (ERA) die Risiken für Amphibien nicht routinemäßig. Es gibt jedoch immer mehr Hinweise darauf, dass die aktuellen Risikobewertungsleitlinien Amphibien nicht ausreichend schützen. was auch an der hohen

Anfälligkeit von Amphibien für eine dermale Exposition gegenüber PSM liegt. Die Amphibienhaut weist eine hohe Durchlässigkeit für Chemikalien durch Diffusion auf und hat gleichzeitig verschiedene physiologische Funktionen. Diese können durch eine direkte Exposition gegenüber PPP beeinträchtigt werden. Damit Amphibien durch die Risikobewertung für Pestizide wirksam geschützt werden können, ohne *in vivo*-Tests zu verlangen, müssen die Auswirkungen dermaler Exposition mithilfe einer von aktuellem Wissen gestützten Strategie berücksichtigt werden können. Ziel dieses Projekts war es, die Wissensbasis zur Toxizität von Pestiziden gegenüber terrestrischen Lebensstadien von Amphibien zu erweitern, um zur Entwicklung einer Strategie zur Charakterisierung der Toxizität von PSM für Amphibien bei Exposition über die Haut beizutragen. Dazu stellten wir Informationen zu physikochemischen Eigenschaften von PSM-Wirkstoffen zusammen, die deren Toxizität für Amphibien bei Hautkontakt beeinflussen können. Anschließend testeten wir die Toxizität von 16 Wirkstoffen, sechs PSM-Formulierungen und zwei Beistoffen an Jungtieren der Modellspezies *Pelophylax perezi* durch Übersprühen und/oder Kontakt mit behandeltem Erdsubstrat. Drei der Wirkstoffe (Isoxaben, Pirimicarb und Lambda-Cyhalothrin) verursachten eine signifikante Mortalität, ebenso die getesteten Formulierungen mit den Wirkstoffen Tebuconazol und Alpha-Cypermethrin, die toxischer waren als ihre jeweiligen Wirkstoffe allein. Die histologische Untersuchung der Haut zeigte Verdickungen der Epidermis durch die Einwirkung von sieben der Wirkstoffe, die im Fall der Alpha-Cypermethrin-Formulierung dosisabhängig waren, sowie eine Hyperplasie hervorgerufen durch eine Azoxystrobin-haltige Formulierung. Die Empfindlichkeit der Amphibien wurde mit der von Regenwürmern der Art *Eisenia andrei* verglichen, die experimentell den gleichen Substanzen und Konzentrationen wie Amphibien ausgesetzt wurden. Es wurde eine Korrelation hinsichtlich der Empfindlichkeit zwischen den beiden Taxa festgestellt. Die Analyse der Surrogat-Eignung verschiedener Organismengruppen wurde durch eine Bewertung toxikologischer Eigenschaften ergänzt, die mit der Empfindlichkeit von Amphibien korrelieren können. Während die akute Toxizität von PSM für terrestrische Organismen (Säugetiere und Bienen) die Anreicherung von Pestiziden in Froschlebern und -häuten besser abbildete, korrelierte die toxikologische Empfindlichkeit der Amphibien mit den akuten Auswirkungen von PSM-Wirkstoffen auf Daphnien. Hinsichtlich der physikochemischen Eigenschaften zeigten lipophilere Substanzen ein höheres Potenzial, das Überleben und Wachstum juveniler Frösche zu beeinträchtigen. Die Ergebnisse des vorliegenden Projekts können regulatorische Entscheidungen zur Vermeidung schädlicher Auswirkungen von PSM auf Amphibien durch dermale Exposition unterstützen und zur Entwicklung einer Risikobewertung für diese Tiere ohne zusätzliche Tierversuche beitragen. Insbesondere könnte die Gesamtschau der hier erzielten Ergebnisse dazu dienen, hinsichtlich ihrer Risiken für Amphibien besonders besorgniserregende Stoffe zu identifizieren.

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Abbreviation	Explanation
ADI	Acceptable daily intake
ANOVA	Analysis of the variance
AOEL	Acceptable operator exposure level
AR	Application rate
ATP	Adenosine triphosphate
BCF	Bioconcentration factor
bw	Body weight
CAS	Chemical Abstracts Service
d.f.	Degrees of freedom
DT_{50}	Median degradation time
DT_{90}	Time to degrade 90% of the initial substance amount
EC_{50}	Median effect concentration
ECHA	European Chemical Agency
ERA	Environmental risk assessment
FIR	Food intake rate
GzLM	Generalized linear model
K_{oc}	Organic carbon-water partition coefficient (linear)
K_{foc}	Freundlich organic carbon-water partition coefficient
K_{ow}	Octanol/water partition coefficient
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
MMC	Melanomacrophage centres
MSDS	Material Safety Data Sheet
PC	Principal Component
PCA	Principal Component Analysis
pKa	Acid dissociation constant
POEA	Polyethoxylated tallow amine
PPP	Plant Protection Product
PSM	Pflanzenschutzmittel
RL_{50}	Median residual level
ROI	Region of interest

Abbreviation	Explanation
RSD	Residual standard deviation
SVL	Snout to vent length

Summary

Amphibians are the most endangered group of vertebrates, being anthropogenic pollution recognized as one of the major factors threatening their populations. Among all types and sources of environmental pollution affecting amphibians, Plant Protection Products (PPP) are of major concern. Amphibians are recognized as being particularly sensitive to PPP because these substances are directly released into large extensions of the habitats where they live, which, given the low dispersal of amphibians, limits the ability of populations to avoid pesticide impacts. In addition, amphibians combine aquatic and terrestrial phases in their life cycles, thereby becoming affected by pesticide present either in water bodies or in terrestrial habitats. A last characteristic that accounts for the special susceptibility of amphibians to pesticides and other environmental chemicals is their naked skin. Whereas other vertebrates possess tegumentary structures that may limit the uptake of pesticides from the environment, amphibian skin properties favour this uptake, as this organ is designed to stimulate water balance and is therefore very permeable to the diffusion of substances from the external medium.

The high sensitivity of amphibians to pesticides raises concern not only because of the generally poor status of conservation of these animals, but also because current risk assessment procedures conducted as part of the regulatory process for PPP authorization do not consider, in general, the risk to amphibians. This risk has been traditionally considered to be addressed throughout the assessment conducted on other groups, including some vertebrates like fish, birds or mammals. However, evidence has grown indicating that the assessments conducted on those groups is not sufficiently covering the potential risks posed by PPP to amphibians. Among the several factors because of which current risk assessment protocols are not considered protective enough to amphibians is precisely the impact that dermal exposures may have on terrestrial amphibian stages. Besides its high permeability to diffusion of chemicals, amphibian skin plays diverse physiological functions including breathing, immunology or intraspecific communication. The direct contact of the skin with pesticides may result in a quick absorption because of the high permeability, but this could come along with a more direct series of effects if skin is disrupted by direct contact and some of their physiological functions are affected.

To improve the protectiveness of environmental risk assessment of PPP, it is necessary to consider effects of dermal exposures on amphibian terrestrial stages. However, as vertebrate animals, it is discouraged to include new data generated from amphibians in risk assessment practices. Therefore, a strategy to consider dermal toxicity to terrestrial amphibians without involving in vivo testing needs to be elaborated, which needs to be supported by current knowledge on substance properties determining this type of toxicity.

This project aimed at increasing the knowledgebase of pesticide toxicity on amphibian terrestrial stages to support the definition of a strategy for characterization of pesticide toxicity to amphibians exposed via dermal routes. To achieve this general goal, the following specific objectives were defined:

- To review the physico-chemical properties of PPP that potentially influence their toxicity to amphibians when exposure happens through dermal contact.

- To investigate the toxicity of different pesticides, including active ingredients and formulations, on amphibian terrestrial stages and establish patterns of toxicity based on the properties of the substances.
- To determine effects on skin because of direct exposure.
- To establish criteria for identification of those types of pesticides whose toxicity to amphibians can be extrapolated from surrogate taxa, and those pesticides for which there could be a need of specific toxicity testing on amphibians.

For the first objective, we conducted a review of available literature on pesticide properties determining dermal toxicity. In general, given the scarcity of information on pesticide exposure data for terrestrial amphibians, there are limited possibilities to get conclusions in this context. The octanol/water partition coefficient is typically contemplated as a determinant factor for pesticide diffusion through the skin, but the few available data from signal to either an important role of this factor (higher absorption of substances with higher coefficient) or to no influence at all. Several studies have found that the organic carbon-water partition coefficient and the water solubility would be better predictors of body burdens and skin absorption in amphibians than octanol/water partition coefficient. Apart from physico-chemical properties, skin characteristics could be also very important in determining chemical diffusion, and such characteristics are highly variable not only across species but also among different body regions, with ventral pelvic region being comparatively more permeable than other regions of the body surface. We concluded from this review that the collection of data about absorption of active substances covering a wide range of chemical properties would be needed to elucidate the contribution of these properties to the absorption of pesticides through terrestrial amphibian skin.

After this review, we designed a strategy for testing different active substances, formulations and co-formulants on terrestrial amphibians. Substances to be tested were selected to cover a wide range of different physico-chemical and toxicological properties, with the aim of correlating the responses shown by amphibians with these properties. The selected substances included 16 active ingredients, classified as five fungicides (metrafenone, oxathiapiprolin, benzovindiflupyr, azoxystrobin and tebuconazole), six herbicides (MCPA, fluazifop-p-butyl, isoxaben, pendimethalin, metsulfuron-methyl and mesotrione), and five insecticides (pirimicarb, acetamiprid, flupyradifurone, alpha-cypermethrin and lambda-cyhalothrin); six formulations (Vivando, Quadris, Folicur 25 EW, Fusilade Max, Sivanto Prime and Fasthrin 10 EC), and two co-formulants (naphtha and N,N'-dimethyldecanamid). This strategy resulted in the implementation of the second specific objective defined above. Experimental exposure was conducted on juvenile Iberian waterfrogs (*Pelophylax perezi*). Individuals were exposed to the different substances in a simulated overspray scenario, involving the recommended rate for spray application of the substances, as well as, for some of the substances, an increased and a reduced application rate to explore possible dose-dependent effects. In addition, half of the substances were also applied on standard LUFA soil substrates, to test their effects on frogs that would be exposed because of contact with these treated substrates; in these cases, an additional scenario combining exposure via overspray and continuous contact with treated soil was also evaluated. Frogs were monitored for 21 days following exposure, with an interim evaluation on day 7 post-exposure.

The majority of the tested substances did not cause lethal effects to juvenile *P. perezi* under the experimental conditions considered in this study. Among active ingredients, significant

mortality was found only in three out of the 16 tested substances: isoxaben, pirimicarb and lambda-cyhalothrin. It is noteworthy that a herbicide like isoxaben has caused lethal effects at a higher level than some of the expectedly more toxic insecticides like flupyradifurone, acetamiprid, or especially the pyrethroid alpha-cypermethrin. Formulations were clearly more toxic than active ingredients alone; this was especially noted for the formulations containing alpha-cypermethrin and tebuconazole. However, we failed to find significant effects of two of the co-formulants that were present in those formulations. It must be assumed that it is the combination of the active ingredients and co-formulants what causes the increased toxicity.

To establish patterns of toxicity based on the properties of the substances, we assigned a toxicity index depending on the apical effects observed for each tested active ingredient and correlated them with the physico-chemical and toxicological properties. Among the former, our results showed that lipophilic substances would have higher potential to compromise juvenile frog survival and growth, which could be related with the higher potential of these substances to diffuse through body membranes. Likewise, substances with higher sorption coefficients to soils appeared to be more toxic to amphibians than those with the opposite characteristic. Among toxicological properties, sensitivity shown by the *P. perezi* juveniles related mostly to that shown by aquatic species, including acute or chronic toxicity to fish, and acute toxicity to daphnids. The latter was the parameter better explaining sensitivity of juvenile frogs to pesticide overspray; substances with a 48h-EC_{50} value for Daphnia below 0.02 mg/l tended to be associated with the occurrence of lethal effects on juvenile frogs.

From the residue analysis of up to 11 of the active substances referred above, which was performed in livers and skins of *P. perezi* exposed via overspray or contact with treated soils, we can affirm that accumulation of pesticides associates better with the sensitivity of terrestrial organisms to these pesticides than with any physico-chemical property. This is presumably linked to certain characteristics of the substances leading simultaneously to an easier absorption and/or accumulation and to stronger effects on terrestrial organisms, especially acute effects. Therefore, we can suggest that substances posing special risks to amphibians in terms of dermal uptake would be those leading to a higher acute toxicity to bees or mammals. Interestingly, those associations were not observed with sensitivity of aquatic organisms, despite the abovementioned association between sensitivity of these organisms and apical effects reported in amphibians. Moreover, our results do not allow for determining any influence of octanol/water partition coefficient, the organic carbon-water partition coefficient or the solubility in water in the likelihood of absorption of the tested substances via amphibian skin. In general, there does not seem to be a correlation between the factors driving accumulation of pesticides on amphibians and those causing toxic effects following dermal exposures.

Apart from apical responses on frog survival and growth, the effects on skins, constituting the third specific objective above, were evaluated through the examination of histological effects of animals from overspray or control treatments after 7 days of experiment. The clearest response to pesticides at the histological level was an increase in the epidermal index 2 (EI2), which is indicative of morphological changes that alter the epidermal structure. This EI2 increase reflected a thickening of the epidermal tissue and was observed for seven out of the 16 analysed substances. Increased skin thickness as a response to dermal contact with pesticides could be interpreted as a protective response to reduce absorption via the skin. In frogs exposed to the azoxystrobin-based formulation Quadris, the skin thickening came along with an increase of the epidermal index 1 (EI1, reflecting increased cellular density), which suggest the occurrence of

hyperplasia. Frogs exposed to the formulation Fasthrin 10 EC showed a clear, dose-response increase in their skin thickness that was much stronger than what was noted in animals treated with the active ingredient only at the same concentration. Although pyrethroids possess properties facilitating their diffusion through skin, a significant part of the absorbed active substance can remain in the skin; in this context, co-formulants in Fasthrin 10 EC would have helped the active ingredient the penetrate the dermis and reach the circulatory system, which would be consistent with the observation of a higher toxicity caused by Fasthrin 10 EC as compared to the active ingredient alpha-cypermethrin. We also observed some non-monotonic responses to EI1. This index can be affected by para-physiological or pathological changes that alter the overall area occupied by cells. In addition, it may also be influenced by the area occupied by the epidermis; thus, changes in EI1 can be originated not only by modification in cell numbers but also by how the entire structure responds to these modifications.

Finally, the fourth specific objective was to determine for which types of substances their toxicity could be extrapolated from surrogate taxa. Despite the few published studies reporting toxicity data from amphibian terrestrial stages, some chemical-related trends in differential toxicity between terrestrial amphibians and their surrogate taxa (i.e. birds or mammals) have been suggested, the clearest one being that related to pyrethroid insecticides. These insecticides are more toxic to poikilothermic (including amphibians) than to homoeothermic vertebrates, which has been attributed to the low rate of metabolism and elimination of these substances shown by the former. The same reasons to explain why substances undergoing metabolic deactivation (like pyrethroids) are more toxic to poikilothermic than to homoeothermic organisms could be used to explain why the opposite trend has been detected for chemicals subjected to metabolic activation. This is the case of some organophosphate or carbamate insecticides. As mentioned above, we found some patterns linking sensitivity of fish and daphnids to that of amphibians. In addition, acute oral and dermal toxicity indicators from mammals or bees were linked to increased accumulation of substances by amphibians.

The investigation on how surrogate taxa can serve to predict pesticide toxicity to amphibians was further supported by the determination of toxicity on earthworms of the same substances that were tested on amphibians, also using a scenario of dermal exposure for earthworms. The comparative toxicity resulted in a weak but significant correlation pointing that there is some parallelism in the mechanisms driving the occurrence of apical effects in amphibians and earthworms. The predictive power of earthworm sensitivity to explain amphibian dermal toxicity was explored, and it was found to be more accurate for larger molecules (those with bigger molecular mass) and for substances with a higher acute oral toxicity to birds. This does not mean that molecular mass or avian LD50 play a significant role in determining the toxicity of pesticides on amphibians or earthworms but provide some indications about the type of molecules for which predictability using earthworm as surrogates is more or less plausible.

In summary, the obtained results do not allow for the establishment of broad patterns to determine which substance properties are clearly contributing to amphibian toxicity or, more important, which properties determine possible associations between amphibians and surrogate taxa. However, some conclusions in this context can be extracted that will contribute to advancing pesticide risk assessment for amphibians:

- Lethal effects on juvenile amphibians following dermal exposure can be observed for active ingredients that are acutely toxic to *Daphnia*.

- To a lesser extent, associations between sensitivity of amphibians and other taxa (fish, bees by contact) or sensitivity by inhalation, are suggested.
- More lipophilic substances would have higher potential to compromise juvenile frog survival and growth.
- Formulations increase both uptake of the active ingredient, apical effects and histological alterations when compared to the exposure to active ingredients alone.
- Absorption of active ingredients following dermal exposure of amphibians either by overspray or soil contact shows a higher chance when those ingredients present an increased acute toxicity to mammals or bees, regardless of the exposure route in these organisms.
- Earthworms might act as good surrogates for amphibian dermal toxicity, especially for those substances with higher molecular mass and/or higher acute oral toxicity to birds.

Although a direct implementation of the results of the present project to a risk assessment protocol focused on amphibians is complicated, the knowledge gained throughout the development of the experiments conducted herein and the analysis of their results can support the regulatory decision making in that context. Relying on in vivo testing should be avoided, hence risks of PPP on amphibians should be analysed using surrogate information. The conclusions listed above may derive in a decision cascade for newly assessed active ingredients or formulations, in a way that substances of potential high risk to amphibians are identified, for instance if more than one of the above conditions concurs in a given product. Unfortunately, the amount and type of data generated in the present project did not allow for a better definition of substance characteristics accounting for increased risks to amphibians, even when all characteristics were combined to evaluate how they could interact to influence the toxicity of substances. Undoubtely, the analysis of a higher number of substances could have facilitated prediction, but our recommendation is not in that line. Rather, a protocol for flagging substances of concern, based on the conclusions above and on non-vertebrate testing alternatives that are being developed, should be established. The implementation and execution of that protocol should not only serve to consider amphibians in risk assessment but also to feedback for a possible re-evalution of the protocols and of the possibilities for surrogacy that have been addressed in the present project.

Zusammenfassung

Amphibien sind die am stärksten gefährdete Wirbeltiergruppe, und die anthropogene Verschmutzung gilt als einer der Hauptfaktoren, die ihre Populationen bedrohen. Unter allen Arten und Quellen der Umweltverschmutzung, denen Amphibien ausgesetzt sind, sind Pflanzenschutzmittel (PSM) die am meisten Besorgnis erregenden. Amphibien sind als besonders empfindlich gegenüber PSM bekannt, da diese Substanzen direkt in große Teile ihrer Lebensräume ausgebracht werden. Angesichts der begrenzten Verbreitung von Amphibien schränkt dies die Möglichkeiten der Populationen ein, den Wirkungen von Pestiziden zu entgehen. Darüber hinaus durchleben Amphibien in ihrem Lebenszyklus aquatische und terrestrische Phasen und kommen sowohl in Gewässern als auch in ihren terrestrischen Lebensräumen mit Pestiziden in Berührung. Ein weiteres Merkmal, das die besondere Anfälligkeit von Amphibien gegenüber Pestiziden und anderen Umweltchemikalien ausmacht, ist ihre nackte Haut. Während andere Wirbeltiere über Hautstrukturen verfügen, die die Aufnahme von Pestiziden aus der Umwelt einschränken können, begünstigen die Eigenschaften der Haut von Amphibien deren Aufnahme, da sie dazu dient, den Wasserhaushalt anzuregen und für die Diffusion von Substanzen aus den umgebenden Medien sehr durchlässig ist.

Die hohe Empfindlichkeit von Amphibien gegenüber Pestiziden ist nicht nur wegen des allgemein schlechten Erhaltungsstatus‘ dieser Tierarten besorgniserregend, sondern auch, weil die aktuellen Risikobewertungsverfahren im Rahmen des Zulassungsprozesses für PSM das Risiko für Amphibien im Allgemeinen nicht berücksichtigen. Traditionell wurde davon ausgegangen, dass das Risiko für sie durch die Bewertungen für andere Tiergruppen, darunter auch Wirbeltiere wie Fische, Vögel oder Säugetiere, bereits adressiert wird. Es gibt jedoch immer mehr Hinweise darauf, dass die Bewertungen für diese Gruppen die potenziellen Risiken von PSM gegenüber Amphibien nicht ausreichend abbilden können. Zu den verschiedenen Faktoren, aufgrund derer die aktuelle Risikobewertung als nicht ausreichend für Amphibien gelten sollte, gehört insbesondere die Wirkung, die eine dermale Exposition auf terrestrische Lebensstadien der Amphibien haben kann. Neben ihrer hohen Durchlässigkeit für die Diffusion von Chemikalien erfüllt die Haut von Amphibien verschiedene physiologische Funktionen, darunter Atmung, Immunologie oder innerartliche Kommunikation. Der direkte Kontakt der Haut mit Pestiziden kann aufgrund ihrer hohen Durchlässigkeit zu einer schnellen Absorption führen, kann aber zudem auch eine Reihe unmittelbarerer Auswirkungen mit sich bringen, wenn die Haut durch den direkten Kontakt geschädigt wird und ihre physiologischen Funktionen beeinträchtigt werden.

Um die Schutzwirkung der Umweltrisikobewertung von Pflanzenschutzmitteln zu verbessern, ist es notwendig, die Auswirkungen dermaler Exposition auf terrestrische Amphibien einzuordnen zu können. Da es sich jedoch um Wirbeltiere handelt, ist es nicht erwünscht, neue Datenanforderungen in die Risikobewertung einzuführen, die die Durchführung von Tierversuchen mit Amphibien erfordern. Daher muss eine Strategie zur Abschätzung der dermalen Toxizität für terrestrische Amphibien ohne neue *in-vivo*-Tests ausgearbeitet werden. Diese muss aktuelle Erkenntnisse über Stoffeigenschaften nutzen, um Hinweise auf Art und Ausmaß der Toxizität zu geben.

Das vorliegende Projekt zielte darauf ab, die Wissensbasis zur Toxizität von Pestiziden für terrestrische Amphibien zu erweitern, um die Ausarbeitung einer Strategie zur Charakterisierung der dermalen Toxizität für Amphibien voranzubringen. Um dieses allgemeine Ziel zu erreichen, wurden für das Projekt die folgenden spezifischen Ziele definiert:

- Zusammenstellung der physikochemischen Eigenschaften von Pflanzenschutzmitteln, die möglicherweise ihre Toxizität für Amphibien bei Aufnahme über die Haut beeinflussen.

- Untersuchung der Toxizität verschiedener Pestizide, einschließlich Wirkstoffe und Formulierungen, für terrestrische Amphibien und Feststellung von Toxizitätsmustern auf Grundlage der Eigenschaften der Substanzen.
- Feststellung von Auswirkungen auf die Haut bei direkter Exposition.
- Festlegung von Kriterien zur Identifizierung jener Wirkstoffe bzw. Stoffgruppen, deren Toxizität für Amphibien aus Surrogattaxa extrapoliert werden kann, und jener Stoffe, für die möglicherweise spezifische Toxizitätstests an Amphibien erforderlich sind.

Für das erste Ziel haben wir eine Durchsicht der verfügbaren Literatur zu den die dermale Toxizität bestimmenden Eigenschaften von Pestiziden vorgenommen. Angesichts des geringen Umfangs der Informationen zur Pestizidexposition bei terrestrischen Amphibien sind die Möglichkeiten begrenzt, hierdurch Schlussfolgerungen ziehen zu können. Der Octanol-Wasser-Verteilungskoeffizient wird üblicherweise als ein entscheidender Faktor für die Durchlässigkeit der Haut gegenüber Pestiziden angesehen, aber die wenigen verfügbaren Daten deuten entweder auf eine wichtige Rolle dieses Faktors hin (höhere Absorption von Substanzen mit höherem Koeffizienten) oder darauf, dass er dagegen überhaupt keinen Einfluss hat. Mehrere Studien sagen aus, dass der organische Kohlenstoff-Wasser-Verteilungskoeffizient und die Wasserlöslichkeit eines Stoffes bessere Vorhersagen der Körperbelastung und der Hautabsorption bei Amphibien zulassen als der Octanol-Wasser-Verteilungskoeffizient. Außer den physikochemischen Eigenschaften der Substanzen könnte auch die Beschaffenheit der Haut eine große Rolle spielen. Diese unterscheidet sich nicht nur zwischen verschiedenen Amphibienarten, sondern auch zwischen ihren einzelnen Körperregionen deutlich, wobei die ventrale Beckenregion vergleichsweise durchlässiger ist als andere Regionen der Körperoberfläche. Aus der Literaturrecherche folgerten wir, dass die Erhebung von Daten über die Absorption von Wirkstoffen mit einem breiten Spektrum chemischer Eigenschaften erforderlich wäre, um den Beitrag dieser Eigenschaften zur Absorption von Pestiziden durch die Haut terrestrischer Amphibien aufzuklären.

Danach entwickelten wir eine Strategie zur Testung verschiedener Wirkstoffe, Formulierungen und Beistoffe an terrestrischen Amphibien. Die zu testenden Substanzen wurden so ausgewählt, dass sie ein breites Spektrum verschiedener physikochemischer und toxikologischer Eigenschaften abdeckten, mit dem Ziel, die von Amphibien gezeigten Reaktionen mit den jeweiligen Eigenschaften zu korrelieren. Die ausgewählten Substanzen umfassten 16 Wirkstoffe, von denen fünf Fungizide (Metrafenon, Oxathiapiprolin, Benzovindiflupyr, Azoxystrobin und Tebuconazol), sechs Herbizide (MCPA, Fluazifop-p-butyl, Isoxaben, Pendimethalin, Metsulfuron-methyl und Mesotrion) und fünf Insektizide (Pirimicarb, Acetamiprid, Flupyradifuron, Alpha-Cypermethrin und Lambda-Cyhalothrin) waren, sechs Formulierungen (Vivando, Quadris, Folicur 25 EW, Fusilade Max, Sivanto Prime und Fasthrin 10 EC) und zwei Beistoffe (Naphtha und N,N'-Dimethyldecanamid). Mittels dieser Strategie wurde das zweite spezifische Ziel des Projektes umgesetzt. Die experimentelle Exposition einer Modellspezies wurde an juvenilen iberischen Wasserfröschen (*Pelophylax perezi*) durchgeführt. Die Individuen wurden den verschiedenen Substanzen in einem simulierten Sprühszenario ausgesetzt. Dabei wurde die übliche zugelassene Aufwandmenge der Substanzen verwendet, bei einigen Stoffen auch eine erhöhte und eine reduzierte Anwendungsmenge, um mögliche dosisabhängige Effekte zu untersuchen. Darüber hinaus wurde die Hälfte der Substanzen auch auf Bodensubstrat (LUFA Standardböden) aufgetragen, um ihre Auswirkungen auf Frösche zu testen, die durch Kontakt mit den so behandelten Substraten den Substanzen ausgesetzt sind. Hierbei wurde ein zusätzliches Szenario untersucht, bei dem die Frösche einer kombinierten Exposition sowohl durch einmaliges Übersprühen als auch ständigen Kontakt mit behandeltem Boden ausgesetzt

wurden. Die Tiere wurden nach der Exposition 21 Tage lang beobachtet, wobei am 7. Tag nach der Exposition eine Zwischenbewertung erfolgte.

Die Mehrzahl der getesteten Substanzen verursachte unter den in dieser Studie betrachteten Versuchsbedingungen keine tödlichen Auswirkungen auf juvenile *P. perezi*. Unter den betrachteten Wirkstoffen wurde nur bei drei der 16 Substanzen eine signifikante Mortalität festgestellt: Isoxaben, Pirimicarb und Lambda-Cyhalothrin. Es ist bemerkenswert, dass das Herbizid Isoxaben tödlichere Auswirkungen auf einem höheren Niveau verursachte als einige der erwartungsgemäß giftigeren Insektizide, wie Flupyradifuron, Acetamiprid oder insbesondere das Pyrethroid Alpha-Cypermethrin. Formulierungen wirkten deutlich giftiger als Wirkstoffe allein; dies wurde insbesondere bei den Formulierungen festgestellt, die Alpha-Cypermethrin und Tebuconazol enthielten. Wir konnten jedoch keine signifikanten Auswirkungen von zwei der in diesen Formulierungen enthaltenen Beistoffe feststellen. Es muss davon ausgegangen werden, dass die Kombination der Wirkstoffe und Beistoffe die erhöhte Toxizität verursacht hatte.

Um Muster in der toxischen Wirkung auf Grundlage der Stoffeigenschaften zu ermitteln, haben wir jedem Wirkstoff einen Toxizitätsindex nach den beobachteten apikalen Effekten zugewiesen und diese mit den physikochemischen und toxikologischen Eigenschaften korreliert. Unsere Ergebnisse zeigten, dass lipophile Substanzen ein höheres Potenzial haben, das Überleben und Wachstum juveniler Frösche zu beeinträchtigen. Dies könnte mit dem höheren Potenzial dieser Substanzen zusammenhängen, durch Körpermembranen zu diffundieren. Ebenso schienen Substanzen mit höheren Sorptionskoeffizienten für Böden für Amphibien giftiger zu sein als solche mit niedrigeren. Unter den toxikologischen Eigenschaften war die Empfindlichkeit der juvenilen Frösche größtenteils mit der von aquatischen Organismen vergleichbar, einschließlich akuter oder chronischer Toxizität für Fische und akuter Toxizität für Daphnien. Letzterer war der Parameter, der die Empfindlichkeit juveniler Frösche gegenüber Pestizidübersprühung am besten beschrieb. Substanzen mit einem 48h-EC_{50}-Wert für Daphnien unter 0.02 mg/l waren tendenziell mit dem Auftreten von Mortalität bei den juvenilen Fröschen verbunden.

Anhand der Rückstandsanalytik von bis zu elf der oben genannten Wirkstoffen in Lebern und Häuten von *P. perezi,* die durch Übersprühen oder Kontakt mit behandelten Böden exponiert wurden, konnten wir bestätigen, dass die Anreicherung von Pestiziden in den Fröschen stärker mit der Empfindlichkeit von terrestrischen Organismen gegenüber diesen Pestiziden als mit allen untersuchten physikochemischen Eigenschaften korreliert. Dies hängt möglicherweise mit bestimmten Substanzeigenschaften zusammen, die gleichzeitig zu einer leichteren Absorption und/oder zu einer Anreicherung in den Tieren und zu stärkeren Auswirkungen auf terrestrische Organismen führen, insbesondere akuten Auswirkungen. Daher nehmen wir an, dass Substanzen, die hinsichtlich der dermalen Aufnahme ein besonderes Risiko für Amphibien darstellen, solche sind, die eine hohe akute Toxizität bei Bienen oder Säugetieren aufweisen. Interessanterweise wurden diese Parallelen bei der Empfindlichkeit von Wasserorganismen nicht beobachtet, trotz des oben erwähnten Zusammenhangs zwischen der Empfindlichkeit dieser Organismen und den bei Amphibien dokumentierten apikalen Effekten. Darüber hinaus können wir aus unseren Ergebnissen keinen Einfluss des Octanol-Wasser-Verteilungskoeffizienten, des organischen Kohlenstoff-Wasser-Verteilungskoeffizienten oder der Wasserlöslichkeit auf die Wahrscheinlichkeit der Absorption der getesteten Substanzen über die Haut von Amphibien ableiten. Generell scheint es keinen Zusammenhang zwischen den Faktoren zu geben, die zur Anreicherung von Pestiziden in Amphibien beitragen, und denen, die nach Hautkontakt toxische Wirkungen hervorrufen.

Als drittes spezifisches Ziel des Projekts wurden die apikalen Effekten auf Überleben und Wachstum der Frösche sowie die Auswirkungen auf die Haut durch histologische

Untersuchungen ausgewertet. Betrachtet wurden die Auswirkungen von Übersprühen oder Kontrollbehandlungen auf die Tiere nach einer 7-tägigen Exposition. Die deutlichste Reaktion auf histologischer Ebene war ein Anstieg des Epidermisindex 2 (EI2), der auf morphologische Veränderungen hinweist, die zu einer Veränderung der Epidermisstruktur führen. Der Anstieg des EI2 spiegelte eine Verdickung des Epidermisgewebes wider und wurde durch sieben der 16 analysierten Substanzen verursacht. Eine erhöhte Hautdicke als Reaktion auf dermalen Kontakt mit Pestiziden könnte als Schutzreaktion der Haut zur Verringerung der dermalen Aufnahme interpretiert werden. Bei Fröschen, die der Azoxystrobin-haltigen Formulierung Quadris ausgesetzt waren, ging die Hautverdickung mit einem Anstieg des Epidermisindex 1 (EI1) einher, der eine erhöhte Zelldichte widerspiegelt, was auf das Auftreten einer Hyperplasie hinweist. Bei Fröschen, die der Formulierung Fasthrin 10 EC ausgesetzt waren, zeigte sich eine deutliche dosisabhängige Zunahme der Dicke ihrer Haut, die viel stärker war als bei den Tieren, die nur mit dem Wirkstoff in derselben Konzentration behandelt wurden. Obwohl Pyrethroide Eigenschaften besitzen, die ihre Diffusion durch die Haut erleichtern, kann ein erheblicher Teil des absorbierten Wirkstoffs in der Haut verbleiben. Eine Erklärung dafür wäre, dass die Beistoffe in Fasthrin 10 EC es ermöglicht haben könnten, dass der Wirkstoff die Lederhaut durchdringt und das Blutkreislaufsystem erreicht, was mit der Beobachtung einer höheren Toxizität von Fasthrin 10 EC im Vergleich zum Wirkstoff Alpha-Cypermethrin übereinstimmen würde. Wir haben auch einige nicht-monotone Reaktionen auf EI1 beobachtet. Dieser Index kann durch paraphysiologische oder pathologische Veränderungen beeinflusst werden, die die Gesamtfläche verändern, die von Zellen eingenommen wird. Darüber hinaus kann er auch durch die Fläche beeinflusst werden, die von der Epidermis eingenommen wird. Änderungen des Index EI1 können also nicht nur durch Veränderungen der Zellzahlen, sondern auch dadurch verursacht werden, wie die gesamte Struktur auf diese Veränderungen reagiert.

Das vierte spezifische Projektziel schließlich bestand darin herauszuarbeiten, für welche Arten von Substanzen die Toxizität für Amphibien aus Surrogatendpunkten verschiedener Taxa extrapoliert werden könnte. Obwohl nur wenige Studien veröffentlicht worden sind, die Daten zur Toxizität von terrestrischen Amphibienstadien enthalten, wurden einige substanzbedingte Trends zwischen der Toxizität für terrestrische Amphibien und Surrogattaxa (d. h. Vögeln oder Säugetieren) vorgeschlagen. Am deutlichsten ist dies bei den Pyrethroiden. Diese Insektizide sind für wechselwarme Wirbeltiere (einschließlich Amphibien) giftiger als für gleichwarme Wirbeltiere, was auf die niedrige Stoffwechsel- und Eliminationsrate dieser Substanzen bei Wechselwarmen zurückgeführt wird. Dass Substanzen, die durch den Stoffwechsel toxikologisch deaktiviert werden (wie z.B. Pyrethroide), für wechselwarme Organismen giftiger sind als für gleichwarme Organismen, könnte umgekehrt auch erklären, warum bei Chemikalien, deren toxische Wirkung durch den Stoffwechsel erst aktiviert wird, der entgegengesetzte Trend festgestellt wurde. Dies trifft z.B. für einige Organophosphat- oder Carbamat-Insektizide zu.

Wie anfangs erwähnt, fanden wir einige Muster, die die Empfindlichkeit von Fischen und Daphnien mit der von Amphibien in Verbindung bringen. Darüber hinaus wurden akute orale und dermale Toxizitätsindikatoren von Säugetieren oder Bienen mit einer erhöhten Akkumulation der Substanzen in Amphibien in Verbindung gebracht.

Die Untersuchung, wie Surrogattaxa zur Vorhersage der Toxizität von Pestiziden für Amphibien dienen können, wurde durch die Bestimmung der Toxizität der Substanzen für Regenwürmer weiter unterstützt. Hierfür wurde ein Versuchsaufbau mit dermaler Exposition von Regenwürmern verwendet. Der Vergleich der toxischen Wirkung ergab eine schwache, aber signifikante Korrelation, was darauf hindeutet, dass es eine gewisse Parallelität in den Mechanismen gibt, die das Auftreten von apikalen Effekten bei Amphibien und Regenwürmern bewirken. Die Vorhersagekraft der Empfindlichkeit von Regenwürmern für die dermale

Toxizität bei Amphibien wurde untersucht. Unsere Ergebnisse zeigten, dass sie für solche Substanzen besser war, die große Moleküle, also größere Molekülmassen aufweisen, und für Substanzen mit einer höheren akuten Toxizität für Vögel. Dies bedeutet zwar nicht, dass die Molekülmasse oder die LD_{50} für Vögel eine bedeutende Rolle bei der Bestimmung der Toxizität von Pestiziden für Amphibien oder Regenwürmer spielten, liefert jedoch Hinweise auf die Art von Molekülen, für die eine Vorhersagbarkeit unter Verwendung von Regenwürmern als Surrogat mehr oder weniger plausibel ist.

Zusammenfassend ist festzustellen, dass die erhaltenen Ergebnisse es nicht ermöglichen, breite Muster zu erkennen, um zu bestimmen, welche Stoffeigenschaften eindeutig zur Toxizität einer Substanz für Amphibien beitragen oder, wichtiger noch, welche Eigenschaften mögliche Zusammenhänge zwischen Amphibien und Surrogattaxa bestimmen. Es können jedoch einige Schlussfolgerungen gezogen werden, die zur Verbesserung der Risikobewertung von Pestiziden für Amphibien beitragen:

- Bei Wirkstoffen, die eine akute Toxizität für Daphnien aufweisen, können nach Hautkontakt tödliche Auswirkungen auf juvenile Amphibien beobachtet werden.
- In geringerem Maße werden Zusammenhänge zwischen der Empfindlichkeit von Amphibien und anderen Taxa (Fische, Bienen bei Kontakt) oder der Inhalationstoxizität vermutet.
- Lipophilere Substanzen könnten das Überleben und Wachstum juveniler Frösche stärker beeinträchtigen.
- Formulierungen erhöhen im Vergleich mit dem Wirkstoff allein sowohl die Aufnahme des Wirkstoffs als auch die apikalen Effekte und histologischen Veränderungen den Fröschen
- Die Absorption von Wirkstoffen nach Hautkontakt der Amphibien durch Sprühnebel oder Bodenkontakt ist wahrscheinlicher, wenn diese Inhaltsstoffe eine erhöhte akute Toxizität für Säugetiere oder Bienen aufweisen, unabhängig vom Expositionsweg dieser Organismen.
- Regenwürmer könnten als gute Surrogate für die dermale Toxizität bei Amphibien dienen, insbesondere für Stoffe mit höherer Molekülmasse und/oder höherer akuter oraler Toxizität für Vögel.

Obwohl eine direkte Umsetzung der Ergebnisse des vorliegenden Projekts in die Risikobewertung für Amphibien kompliziert wäre, können die bei der Entwicklung der hier durchgeführten Experimente und der Analyse ihrer Ergebnisse gewonnenen Erkenntnisse die regulatorische Entscheidungsfindung unterstützen. Es sollte vermieden werden, sich hierbei auf *in-vivo*-Tests zu stützen, daher sollten die Risiken von PSM für Amphibien durch Surrogatinformationen analysiert werden. Die oben aufgeführten Schlussfolgerungen können in eine Entscheidungskaskade für neu zu bewertende Wirkstoffe oder Formulierungen einfließen, sodass Stoffe mit einem potenziell hohen Risiko für Amphibien identifiziert werden, beispielsweise wenn mehr als eine der oben genannten Bedingungen in einem bestimmten Produkt vorliegen. Leider erlaubten die Menge und Art der im vorliegenden Projekt generierten Daten keine genauere Definition der Substanzeigenschaften, die erhöhte Risiken für Amphibien erklären, selbst bei Kombination aller Eigenschaften, um zu bewerten, wie sie interagieren und die Toxizität der Substanz beeinflussen könnten. Zweifellos hätte die Analyse einer größeren Anzahl von Substanzen eine Vorhersage ermöglichen können, aber unsere Empfehlung geht nicht in die Richtung, mehr Tests durchzuführen. Vielmehr sollte ein Protokoll zur Erkennung bedenklicher Substanzen erstellt werden, das auf den genannten Schlussfolgerungen und auf in Entwicklung befindlichen Alternativen ohne Wirbeltierversuche basiert. Die Implementierung und Durchführung eines solchen Protokolls sollte nicht nur dazu dienen, Amphibien bei der

Risikobewertung zu berücksichtigen, sondern auch, um eine Neubewertung der Leitlinien und der Potenziale von Surrogatendpunkten zu ermöglichen, die im vorliegenden Projekt behandelt wurden.

1 Terms of reference

In response to the call tender 3719 65 412 2 / 93 401/65 launched by UBA on 06 March 2020, the proposal entitled "Designing a strategy based on toxicity evaluation to improve pesticide risk assessment for terrestrial amphibians **(TerAmphiTox)**" was submitted, which addressed both the mandatory and optional work included in the tender specifications.

According to the tender specifications (p. 5 of the English translation), "*this research project is intended to contribute to improving the data base on the dermal effects of PPP on amphibians in order to allow an ERA in the future. The focus will be on investigations of acute and possibly prolonged toxicity by dermal uptake in terrestrial life stages of amphibians. Both lethal and sublethal effects should be recorded".* The proposed research included testing the toxicity of a series of substances, which were selected to represent a variety of physico-chemical and toxicological properties, on a terrestrial amphibian model. As specified in the tender text quoted above, the experiments were designed to test both acute and prolonged toxicity including the monitoring of both lethal and sublethal (growth and skin histology) effects.

The tender specifications also mentioned that "*as a result of this project, it should be demonstrated whether the knowledge gained from the produced data makes it possible to predict the effect on amphibians of certain toxicological or physicochemical properties of an active substance or preparation"* (p. 5 of the English translation). TerAmphiTox has conducted an analysis of data in the requested direction, trying to identify which substance properties determine most the toxicity to terrestrial amphibians, or at least whether some associations between properties may exist that serve to extrapolate toxicity of the substances to amphibians.

The work package 1 (WP1) consisted of the development of a test strategy. In this WP1, mandatory work consisted of the development of the test strategy for amphibians, which should be supported on existing knowledge. In addition, optional work of the WP1 included an extension of the test design to consider exposure via contaminated substrates and also a design involving dermal exposure of earthworms as potential surrogates. This report addresses the development of the test strategy (both the mandatory and optional works) in section 3, while the supporting information to do so is reviewed in section 2.

The WP2 consisted of laboratory testing, which derives from the test strategy designed as part of WP1. The tender specifications establish that mandatory work should include overspray tests with amphibians to test mortality and sublethal effects to skin and/or inner organs, while optional works will include (a) prolonging the surveillance of amphibians after overspray test, (b) exposing amphibians via contaminated substrates, and (c) performing overspray test with earthworms. TerAmphiTox has addressed the mandatory WP2 work as well as items (a) and (b) of the optional WP2 in an integrated way, in order to save time and resources, and to minimize animal testing. Consequently, amphibian experiments were designed to include a short-term (7 days) and longer-term (21 days) monitoring after overspray and, for half of the substances, overspray was integrated with exposures via contaminated substrate within the same experiments in a semi-factorial design, i.e., apart from the overspray-only and contaminated substrate-only exposures, some of the treatment levels from both exposure ways were combined. The methodology and results of these experiments with amphibians, corresponding to mandatory work of WP2 and to optional works 2a and 2b, as specified in the tender, are presented in section 4 of the present report.

Regarding overspray tests with earthworms, these were performed separately from those conducted with terrestrial amphibians, although using the same substances. The methodology

and results of these tests with earthworms, corresponding to optional work 2c of the tender, are presented in section 5 of the present report.

Finally, WP 3 had the aim "to highlight the gain in knowledge and progress in ERA for amphibians provided by the studies that have been conducted in this project. It needs to be discussed if it has been achieved to identify parameters that enable us to predict if a chemical substance is probably highly toxic or rather harmless to amphibians" (p. 10 of the English translation). This discussion arises from the results and data analysis of the different experiments conducted as part of WP2, and is presented in this report within section 6. In particular, a discussion on the results of the different experiments has been included, followed by a section to identify parameters that could serve to predict the toxicity of substances to amphibians, to conclude with a section to discuss the role of earthworms as potential surrogates for dermal toxicity of Plant Protection Products (PPP) to amphibians.

2 Background

Half of the European amphibian species are present in arable lands, frequently within areas of application of PPP, which have been pointed as one of the reasons for amphibian population declines (IUCN 2024) Amphibians have not been routinely considered in environmental risk assessment (ERA) of PPP. To date, it is assumed that the risk assessment conducted for other non-target organisms (e.g. birds and mammals) is also protective for the estimated exposure of amphibians via dietary uptake of PPP. However, there is growing evidence that using these surrogate taxa to extrapolate PPP impacts to amphibians leads to an underestimation of the actual risk (reviewed by EFSA PPR Panel et al. 2018)). This is particularly relevant for terrestrial stages (i.e. juveniles and adults) not only because of available data on PPP toxicity is particularly scarce for these stages, but also because they are pivotal in the sustainability of population dynamics of most species (e.g. Schmidt et al. 2005)). Some studies have suggested a high, acute toxicity of certain PPP on amphibian terrestrial stages after dermal exposure (Belden et al. 2010, Brühl et al. 2013), which can be related to the fact that amphibian skin is an organ with important physiological functions that lacks specialized structures of protection and is very permeable to the diffusion of chemical agents. The direct exposure to PPP after overspray or because of contact with treated surfaces may affect skin functions and result in substance absorption and subsequent effects on other systems.

The aim of project TerAmphiTox is to increase the knowledgebase of PPP toxicity on amphibian terrestrial stages via dermal exposures in order to define a strategy for effect characterization within the pesticide ERA for amphibians, ensuring a protective scheme while minimizing the need for additional tests. That strategy should build upon existing knowledge, which is reviewed in the following sub-sections and that must serve to design the test strategy to be follow as part of this project in order to fill the main gaps.

2.1 Physico-chemical properties of active substances determining dermal absorption in amphibians

The scarcity of pesticide exposure data for terrestrial amphibians makes difficult to infer which of the physico-chemical properties of an active substance are more suitable to predict absorption through the amphibian skin. The octanol/water partition coefficient (K_{ow}) of the substances is typically contemplated as a determinant factor in the literature about pesticide permeability and bioaccumulation, but the conclusions that are drawn about its value as a predictor of amphibian skin permeability are highly inconsistent among studies. Quaranta et al. (2009) showed that substances with a high K_{ow} value (i.e. those showing high lipophilicity) confer a higher percutaneous passage in frog skin on a flow-through cell than those with a low K_{ow} value, suggesting that the hydrophobicity of the substances contributes to its absorption, while the molecular mass showed no predictive value. Working with reptiles, Weir et al. (2014) suggested that lipophilic substances would be more suitable than hydrophilic ones for dermal uptake, because the former can easily diffuse through membranes. On the other hand, Van Meter et al. (2014) found that K_{ow} was not a strong predictor of skin permeability in amphibians placed directly on soil treated with different pesticides. These authors suggested that physiological skin reactions occurring only in living amphibians (not in in vitro models like that used by Quaranta et al. (2009), such as hydration, explains the differences between studies. Van Meter et al. (2015), corroborating with their previous study, showed that both in overspray and soil exposure treatments, the body burden and bioconcentration factors resulting from the exposure to pesticides such as atrazine, imidacloprid and pendimethalin were not related to their hydrophobicity, indicating that the role of K_{ow} in skin permeability is not relevant in living

amphibians. Differences in the relative importance of K_{ow} to determine percutaneous passage of chemicals in amphibians occur even when comparing in vitro studies, as some studies have reported, unlike that by Quaranta et al. (2009), that flux of chemicals through the excised frog skin decreases with an increase in K_{ow} (Kaufmann and Dohmen 2016, Llewelyn et al. 2020).

Other chemical properties related to the absorption of the substances through the soil, such as the organic carbon-water partition coefficient (K_{oc}) or the water solubility, have been shown to be better predictors of body burdens and skin absorption than K_{ow} (Van Meter et al. 2014, Van Meter et al. 2016). K_{oc} indicates the capacity of a chemical to adsorb to the soil. Pesticides generally bind less to soils with a lower organic matter content, so they become more available to be absorbed by terrestrial organisms (Wauchope et al. 2002). Van Meter et al. (2016) compared two soils with different organic content matter treated with five active substances to determine how bioconcentration in amphibians would be affected. Amphibians dermally exposed to pesticides on low organic matter soils presented higher body burdens and bioconcentration for all tested active substances, in contrast to those exposed to high organic matter soils, which presented lower body burdens and bioconcentration. Cusaac et al. (2016) found a low accumulation of pyraclostrobin in frogs exposed by contact with previously treated soils (only 5% of the predicted initial exposure), which was attributed to the quick binding of this fungicide to soils. However, preliminary findings from that study, as well as from other studies examining exposure to pesticides via soil (e.g. Henson-Ramsey et al. 2008, Van Meter et al. 2014), suggest that soil uptake may be important because the majority of observed toxicity occurred during the initial hours of exposure, when fungicide bioavailability would be still high. Regarding water solubility, polar pesticides could dissolve in the water fraction of the soil matrix becoming more bioavailable to be absorbed by amphibian skin (Wauchope et al. 2002).

Some alternative studies have tried to go beyond the understanding of physico-chemical parameters involved in dermal absorption of PPP by amphibians and have looked directly for patterns determining toxicity of these products to terrestrial amphibians. This is the case of the study by Weltje et al. (2017), who developed a non-testing method that established a relationship between fish and terrestrial amphibian toxicity values using available data (i.e. LC_{50} and BCF for fish). However, this model makes strong assumptions, such as presuming that the high correlation of the sensitivity to chemicals between fish and aquatic amphibian life stages translates to terrestrial amphibians.

In summary, the current data do not reveal any clear trend between the physico-chemical properties of the active substances and their dermal permeability in terrestrial amphibians. In addition, skin characteristics could be at least as important as physico-chemical properties in determining chemical diffusion, and such characteristics are highly variable across different species, and also within a single individual across different body regions. For instance, Brühl et al. (2013) found a high sensitivity of European common frog (*Rana temporaria*) juveniles to overspray with a pyraclostrobin-based formulation, which contrasts with the result of a previous assay that, with a similar methodology, had been conducted with the Great Plains toad (*Anaxyrus cognatus*) (Belden et al. 2010). They attributed this variation in sensitivity to the differences between species in skin properties, although no specific parameters were investigated in this context. Likewise, absorption of contaminants, especially of most hydrophilic ones, is particularly active through the ventral pelvic region compared with the ventral thoracic or dorsal skin ones Llewelyn et al. (2019a), as the ventral pelvic region is the part of the body normally in contact with the substrate when animals are standing by, and it is there where soil water is absorbed to keep body moisture.

The collection of data about absorption of active substances covering a wide range of chemical properties is needed in order to elucidate the contribution of these properties to the absorption of pesticides through terrestrial amphibian skin.

2.2 Mechanisms of toxicity accounting for differential toxicity to amphibians compared to other taxa

Despite the few published studies reporting toxicity data from amphibian terrestrial stages, some chemical-related trends in differential toxicity between terrestrial amphibians and surrogate vertebrates (i.e. birds or mammals) have been suggested. Perhaps the clearest trend is that relative to the pyrethroid insecticides, which show a higher toxicity to birds or mammals than to poikilothermic vertebrates. This difference in sensitivity to pyrethroids among vertebrate classes is thought to happen because, whereas birds or mammals can efficiently metabolise these substances, the comparatively low metabolic rate of fish, amphibians or reptiles reduces the efficacy of pyrethroid detoxification mechanisms in these animals (Haya 1989). Ortiz-Santaliestra et al. (2018) observed a consistently higher toxicity of pyrethroid insecticides to terrestrial amphibian stages than to birds or mammals, with oral LD_{50} values for amphibians ranging from two to four orders of magnitude lower than those for birds or mammals. The metabolism of pyrethroid insecticides produces reactive substances like aldehydes and cyanides that can generate oxidative stress in exposed organisms (Grajeda-Cota et al. 2004). On top of that, parent active substances that are not yet metabolised can act inhibiting antioxidant enzymes (e.g. David et al. 2012), contributing to increase the magnitude of damage to biomolecules (DNA strand breakage, lipid peroxidation) and cells (apoptosis) associated with oxidative stress.

Those substances like pyrethroids, whose toxicity is reduced as a result of biotransformation, are expected to cause higher toxicity to those organisms with slower metabolic activity (Nagy et al. 1999), which could explain why amphibians are more sensitive to pyrethroids than birds or mammals. This hypothesis is also supported when toxicity of pyrethroids is compared between homeothermic vertebrates and reptiles, another group of poikilothermic animals with a comparatively low metabolic rate. Reptiles have been found to be consistently more sensitive than homeothermic vertebrates to oral exposures to pyrethroids (Weir et al. 2010, Ortiz-Santaliestra et al. 2018). Likewise, increasing temperatures causes the metabolic rate to raise in poikilothermic animals, which should result in a reduction in toxicity of these substances as the biotransformation into their less toxic metabolites increases. Although data regarding amphibians in this context do not exist, Weir et al. (2015) observed how increasing temperature with the help of heat lamps during exposure of Western fence lizards (*Sceloporus occidentalis*) to lambda-cyhalothrin reduced the toxicity caused by this pyrethroid to the animals, and the same pattern was reported by Talent (2005) while investigating the effects of natural pyrethrins on green anoles (*Anolis carolinensis*).

Organochlorine insecticides are another group of substances that has been found to be more toxic to terrestrial amphibians than to birds or mammals following oral exposures (Crane et al. 2016, Ortiz-Santaliestra et al. 2018). These substances, as well as pyrethroid insecticides, are characterized by a high lipophilicity as shown by their high K_{ow} values. Actually, Ortiz-Santaliestra et al. (2018) found that K_{ow} was significantly explaining the differential toxicity of substances between amphibians and homeothermic vertebrates, although given the low number of comparable substances, this could be a side effect resulting from the specific influence of

pyrethroids and organochlorines, and it is unclear whether the trend can be extrapolated out of those chemical groups.

As explained in the previous section, K_{ow} has been suggested to play a role in the susceptibility of chemical substances to diffuse through amphibian skin following dermal exposures, although results of the diverse studies point to different effects. Apart from whether high lipophilicity contributes or not to increased percutaneous passage, lipophilic substances tend to show high bioaccumulation potential (e.g. Goutner et al. 2012), which would relate to increased toxicity to those organisms that have low metabolic rates (e.g. Mathieu-Denoncourt et al. 2016) given the longer times that accumulated substances persist in the organisms due to slow elimination. In addition, amphibians have irregular feeding regimes, with important seasonal fluctuations, as opposed to birds or mammals that should ingest food on a daily basis. Amphibians experience starving or low ingestion periods during metamorphosis and breeding season, mobilizing their fat reserves, and eventually accumulated lipophilic compounds, as energy sources (Hourdry et al. 1996).

If substances for which parent compounds are more toxic than metabolites cause stronger effects to animals with lower metabolic rates, substances in which biotransformation tends to increase toxicity will have stronger effects in organisms with higher metabolic rates, like homeothermic vertebrates are compared to poikilothermic ones. This is the case of some organophosphate or carbamate insecticides (Sams et al. 2000), which have been found to be less toxic to amphibians than to birds or mammals (Ortiz-Santaliestra et al. 2018). This lack of toxicity because of the absence of metabolic activation was used by Sparling et al. (1997) to explain why green frog (*Lithobates clamitans*) tadpoles exposed to the organophosphate temephos showed no symptoms of acetylcholinesterase inhibition, despite the well-known inhibitory effect that organophosphate insecticides have on this enzyme. They concluded that the parent compound of temephos would have relatively low toxicity and should be metabolized to sulfone or sulfoxide forms to be toxic, and that such transformation could not be happening in exposed frogs or happening at a rate too low to cause measurable effects.

All those trends of differential toxicity between amphibians and birds or mammals always refer to oral toxicity. Thus, they can be related to either specific modes of action accounting for certain mechanisms of toxicity being more or less severe to either group, or to the expected differences between groups in the intestinal absorption of chemical compounds. Inter-taxonomical comparisons following dermal exposures in the terrestrial environment cannot provide any trend given the low availability of comparable data, but a parallelism can be made relative to what happens after oral exposure. If differential sensitivity were intrinsically associated with the modes of action of the toxicant when reaching their receptors in the organism the exposure route would have little relevance (i.e. it would be of low importance if the toxicant reaches its target receptor after oral or after dermal uptake). An exception to this would occur when target receptors are present in directly exposed tissues, which could be the scenario of dermal exposures of amphibians to compounds with little specific modes of action. Non-specific toxicants will affect the skin in dermally exposed amphibians and also in other animals, but because of the relatively major physiological importance of amphibian skin as compared to other groups, severe effects could result from the action of these non-specific modes of action on amphibians following dermal exposures. This possibility has been suggested to explain the severe and quick effect that some strobilurin fungicides cause to terrestrial amphibians following overspray (Brühl et al. 2013). Strobilurins inhibit mitochondrial respiration by

blocking electron transfer at the Q_o site, prohibiting electron passage from cytochrome b to cytochrome c, thus inhibiting ATP production (Balba 2007).

Alternatively, if inter-taxonomical variations in sensitivity were not associated with the mode of action but with a differential absorption, the exposure route would then play a major role. There are no reasons to infer any parallelism between those compounds that are easily absorbed in the intestine following oral uptake and those that are easily absorbed via the skin following dermal exposure. Therefore, solving the question of which factors most influence absorption of contaminants through the amphibian skin, which as explained in the previous section is far to be clear, is pivotal to answer the question about what influences differences in sensitivity between amphibians and birds or mammals.

2.3 Dermal toxicity of PPP co-formulants of PPP on amphibians

One of the main areas of concern in the ERA of PPP is the fact that additive compounds, also called inert ingredients or co-formulants, present in commercial formulations may cause different effects from those of the active ingredient and be a distinctive attribute in the toxicity of pesticides (Bloch et al. 2020). Despite their name, inert ingredients may be biologically or chemically active and are labelled inert only because of their function in the formulated product (Cox and Surgan 2006). These co-formulants include, among others, solvents, surfactants or synergists that are mixed with the active ingredient to improve the efficacy of the product. Co-formulants are often kept confidential by the manufacturing companies and are not disclosed in product labels, which complicates the traceability of their toxicity to non-target organisms. Comparative toxicity of active ingredients and their formulations to amphibians has been studied for several pesticides, although in most of these cases the co-formulants eventually modulating formulation toxicity remain confidential. Increased toxicity of formulations compared to active ingredients has been reported in amphibians for, among others, azinphos-methyl (Güngördü and Uçkun 2015), cypermethrin (Agostini et al. 2010, Svartz and Pérez–Coll 2013, Majumder and Kaviraj 2015), diazinon (Harris et al. 1998), permethrin (Boone 2008), malathion and imidacloprid (Puglis and Boone 2011). In a few cases, however, the opposite trend has been observed, with technical formulations being more toxic than commercial ones (see examples of carbaryl, permethrin, and b-cyfluthrin in Puglis and Boone (2011)).

Some cases exist, however, of known co-formulants whose toxicity to amphibians has been investigated. The best studied case is that referred to the glyphosate-based herbicides, and in particular to the polyethoxylated tallow amine (POEA). POEA is a non-ionic surfactant added to glyphosate formulations to increase cell membrane permeability and allow increased absorption of the active ingredient (Giesy et al. 2000). A number of experimental studies conducted mostly on aquatic amphibian stages have demonstrated that POEA is the main driver of toxicity of glyphosate-based herbicides to these animals (Mann and Bidwell 1999, Edginton et al. 2004, Howe et al. 2004, Relyea 2005a,b, Carvalho et al. 2019, Turhan et al. 2020). For instance, Perkins et al. (2000) found that formulations containing POEA were 700 times more toxic to African clawed frog (*Xenopus laevis*) embryos than formulations without it. Actually, similar toxicity to amphibian embryos and tadpoles has been reported for Vision® (a glyphosate-based formulation containing POEA) and POEA alone (Perkins et al. 2000, Edginton et al. 2004), while a low toxicity of the active ingredient alone has also been reported for these stages (Mann and Bidwell 1999, Daam et al. 2019, Moutinho et al. 2020). The mechanisms by which POEA causes toxicity seems to be related to its cytotoxic potential and capacity to disrupt the membrane of

sensitive respiratory surfaces, leading to altered gill morphology and function in aquatic organisms (Partearroyo et al. 1991). POEA toxicity increases at high pH levels, which has been attributed to the fact that, in alkaline conditions, POEA appears as the non-ionized form, which readily accumulates in tadpole gills (Chen et al. 2004, Edginton et al. 2004).

The majority of studies dealing with the toxicity of glyphosate and its co-formulants to amphibians refer to aquatic stages. POEA, as well as other surfactants, are typically added to formulations approved for terrestrial applications and their incidence to aquatic stages is likely to happen after applied herbicides reach water bodies by runoff or spray drift. The glyphosate-based formulations approved for use in aquatic systems do not contain surfactants, which would reduce their toxicity to non-target aquatic fauna (Giesy et al. 2000, Levis et al. 2016, but see Brodman et al. 2010, Krynak et al. 2017). However, these aquatic herbicide formulations are typically combined with surfactants before being used (Hewitt et al. 2009).

In the terrestrial environment, both glyphosate and POEA bind rapidly to soil (Giesy et al. 2000, Malone et al. 2004), thus becoming little bioavailable for dermal uptake. However, terrestrial stages can be directly exposed to glyphosate-based formulations containing POEA and other surfactants via overspray, and under this scenario acute and chronic effects of these formulations to terrestrial life stages of amphibians have been reported. For instance, Relyea (2005b) reported between 68% and 86% mortality of juvenile wood frogs (*Lithobates sylvaticus*), Fowler's toads (*Anaxyrus fowleri*) and grey treefrogs (*Driophytes versicolor*) within one day after overspray with the original, POEA-containing Roundup® formulation. Bernal et al. (2009) found 30% of individuals of several anuran species dying within 24 h after overspray with a glyphosate mixture at application rates commonly used for coca plant eradication in Colombia. This mixture included a surfactant-free formulation for aquatic use mixed with Cosmo-Flux®, an adjuvant containing non-ionic surfactants and isoparaffins (Hewitt et al. 2009). Conversely, other studies have found little or no effects of glyphosate-based herbicides on juvenile or adult amphibians (Relyea et al. 2005, Edge et al. 2011, Edge et al. 2013, Wagner and Lötters 2013, Ujszegi et al. 2015, 2016). Although, as mentioned above, surfactants like POEA that are included in glyphosate formulations may impair the functioning of the gills and other respiratory membranes in aquatic organisms, including larval amphibians (Dinehart et al. 2009, Relyea and Jones 2009, Dinehart et al. 2010, Williams and Semlitsch 2010), they seem to have no significant effect on lung-breathing adults (Ujszegi et al. 2016).

POEA has been progressively removed from glyphosate formulations and replaced by other proprietary surfactants, some of which could also have increased toxicity to amphibians. For instance, studies conducted with new POEA-free formulations like Roundup OriginalMAX or Roundup WeatherMAX have shown these formulations are as toxic to tadpoles as, or even more toxic than POEA–containing glyphosate-based herbicides (Relyea and Jones 2009, Relyea 2011). Vincent and Davidson (2015) evaluated acute toxicity on western toad (*Anaxyrus boreas*) tadpoles of glyphosate alone (in the form of isopropylamine salt) or mixed with two surfactants: Agri-dex® (a blend of heavy range petroleum-based oil, polyol fatty acid esters, and polyethylated derivatives, designed to improve the wetting, spreading, and deposition characteristics of the pesticide) and Competitor® (a modified vegetable oil containing ethyl oleate sorbitan alkylpolyethoxylate ester and dialkyl polyoxyethylene glycol, designed to both enhance the ability of the pesticide to enter the cuticle of the plant and to increase the area that a droplet of spray mixture will cover). Glyphosate mixed with Competitor, the surfactant including polyethoxylated substances (analogous to POEA), was six times more toxic than glyphosate

mixed with Agri-dex, and both mixtures were more toxic than glyphosate isopropylamine salt alone. Among polyethoxylated substances, nonylphenol polyethoxylates are also used as surfactants in pesticide formulations. Environmental degradation of these products results in nonylphenol, which has been shown to be more toxic than many of the herbicidal active ingredients to amphibian embryos and tadpoles. For instance, Aronzon et al. (2016) reported that nonylphenol was between 11 and 18 times more toxic than diazinon to embryos and larvae of the South American toad (*Rhinella arenarum*). In addition, the toxicity of the mixture of both substances tended to be significantly higher than predicted if additive effects were considered. In the same line, glyphosate-based herbicides with nonylphenol surfactants have been found to be more toxic than glyphosate alone (Trumbo 2005).

Non-ethoxylated surfactants added to glyphosate formulations have also shown some risk for amphibian terrestrial stages. A differential toxicity between glyphosate-based formulations was reported after overspray of juvenile *A. cognatus* and New Mexico spadefoot toad (*Spea multiplicata*) (Dinehart et al. 2009). Survival of both species was drastically reduced when exposed to Roundup Weed and Grass Killer Ready-To-Use Plus® but not after exposure to Roundup Weed and Grass Killer Super Concentrate®, with both formulations being applied at the same glyphosate rate. The only known difference between the two formulations is that the toxic one contains pelargonic and related fatty acids, suggesting these compounds would be the responsible for the observed mortality. Pelargonic acid is a natural fatty acid that acts as an herbicide by quickly desiccating plant tissues (Pline et al. 2000). It has been found to be non-toxic to fish, birds or honeybees (USEPA 2000), but nothing is known about its toxicity to terrestrial amphibians. However, it is also possible that the differences in toxicity to juvenile toads between the two formulations tested by Dinehart et al. (2009) are due to some of the undisclosed inert ingredients, like the surfactants, added to either formulation.

Another common non-ionic surfactant present in PPP formulations is polyoxyethylene-alkylether sulphate (Koyama et al. 1997). Although the toxicity of this surfactant to amphibians has not been directly assessed, Lajmanovich et al. (2014) compared the effects on these animals of the herbicidal active ingredient glufosinate ammonium and a formulation of this substance containing polyoxyethylene-alkylether sulfate. They observed that exposure of *R. arenarum* tadpoles to Liberty®, a formulation containing 20% glufosinate ammonium and an unknown proportion of polyoxyethylene-alkylether sulfate and other excipients, induced a concentration-dependent increase in the frequency of micronucleated erythrocytes, suggesting a genotoxic effect, which was not observed in animals exposed to the active ingredient alone. In a recently published study, Babalola et al. (2021) reported a weak thyroid disrupting effect after conducting a standardized Amphibian Metamorphosis Assay (OECD 2009) with the formulation Basta® (18.5% glufosinate ammonium as active ingredient, 30% sodium polyoxyethylene alkylether sulphate), although no comparison with the effect of the active ingredient alone was conducted in that case.

Although sometimes used as synonym terms, emulsifiers constitute a specific class of surfactants. These substances weaken lipid membranes by fractioning lipids into small droplets, which become suspended in the water portion of the membrane. This detergent action alters membrane structure and makes cells more permeable (Mathews and van Holde 1990). Swann et al. (1996) found that chlorpyrifos formulations containing emulsifiers were more effective than the active ingredient in decreasing ciliary beat in the frog palate epithelium in vitro. This effect was supposed to be related to the acetylcholinesterase inhibition, which is the mechanism of

action of chlorpyrifos (an organophosphate insecticide), hence increased toxicity of the formulation was not initially expected. The reason for the observed results would be that either emulsifiers facilitated chlorpyrifos reaching its target receptors in the epithelial cultures (Dearden 1990), or that emulsifiers had their own mechanism of toxicity different from that of the active ingredient (Domenech et al. 1977). Chlorpyrifos formulations tested by Swann et al. (1996) caused disruption of mitochondria, which has been associated with calcium saturation resulting from changes of the inner membrane permeability (Lapidus and Sokolove 1993).

Solvents constitute another important group of co-formulants that are commonly added to pesticides and can pose some risk to amphibians and other non-target organisms. Considering what is disclosed by manufacturing companies about composition of formulations, one of the most typically added solvents seems to be naphtha, a flammable liquid mixture of aromatic hydrocarbons. Wagner et al. (2015) studied the acute toxic effects of the herbicide Focus® Ultra (containing 50% of naphtha) and its active ingredient, cycloxydim, on *X. laevis* embryos and early-stage larvae. The formulation resulted significantly more toxic than the active ingredient, with 96h-LC_{50} values for Focus Ultra between five and ten times lower that the values for cycloxydim. Although the formulation included other toxic co-formulants, like the surfactant dioctyl sodium sulfosuccinate (in a proportion of 2.4%), the high proportion of naphtha together with its high acute toxicity to fish (ECHA 2020) led the authors to suggest that the solvent would be the main responsible for the herbicide toxicity.

Regarding terrestrial stages, Belden et al. (2010) exposed *A. cognatus* juveniles via overspray with two formulations of the fungicide pyraclostrobin: Headline® and Stratego®. Mortality rates were >50% and 7%, respectively. Although both formulations contain petroleum distillates, the authors suggested that the active ingredient, based on its toxicity to fish, could have had the potential to cause the observed toxicity. Brühl et al. (2013) replicated the design of Belden et al. (2010) with *R. temporaria* juveniles, exposing them to two formulations containing the same amount of pyraclostrobin but differing in their naphtha content (67% vs <25%). Mortality rates were 100% and 20% among froglets exposed to the formulations with high and low naphtha content, respectively.

Belden et al. (2010) and Hooser et al. (2012) discussed the potential of two of the co-formulants present in Headline, naphthalene and 2-methylnaphthlane (6.2 and 13.7 % of the formulation, respectively) to influence fungicide toxicity. These chemicals can be part of the aromatic hydrocarbons that form naphtha mixtures. Both studies agreed to suggest pyraclostrobin as the main reason for the toxicity of the formulation to tadpoles and juveniles. Although naphthalene could influence the toxicodynamic of pyraclostrobin and increase its toxicity, it is volatile and unlikely to stay in the testing chambers or wetlands for very long. However, increased toxicity could occur during the initial exposure and may explain why most of the mortality caused by Headline that was observed by both Hooser et al. (2012) on *A. cognatus* tadpoles and Brühl et al. (2013) on *R. temporaria* juveniles was within 24 h of exposure. Later, Cusaac et al. (2016) compared the toxicity of Headline with that Headline AMP, the formulation recommended to replace Headline, after overspray of juvenile Blanchard's cricket frog (*Acris blanchardi*). Headline AMP contains no naphthalene, but includes propylene glycol and a second active ingredient, metconazole. They found similar LC_{50} values for the active ingredient (expressed as application rates) in both formulations, which would support that pyraclostrobin is the main contributor to toxicity of the fungicides. However, the fact that Headline is presented as an emulsifiable concentrate while Headline AMP is presented as a suspension concentrate could

account for some differences in absorption that might influence toxicity. Actually, differences in toxicity to amphibian tadpoles between soluble and emulsifiable concentrates have been recently reported for dimethoate formulations (Martinuzzi et al. 2020). The emulsifiable concentrate Perfekthion®, containing 50% of the active ingredient and 50% cyclohexanone as solvent, had an LC_{50} for *R. arenarum* tadpoles more than four times lower than that of the soluble concentrate Arpon® Plus, also containing 50% dimethoate together with undisclosed co-formulants.

Kerosene is another petroleum derivate solvent added to some pesticide formulations whose effects on amphibians have been addressed. For instance, kerosene is one of the inert ingredients of the triclopyr-based herbicide Release® (Chen et al. 2008). Wojtaszek et al. (2005) observed no unusual sensitivity to the toxic effects of this formulation after exposing northern leopard frog (*Lithobates pipiens*) and *L. clamitans* tadpoles. From the outcome of that and other studies it can be inferred that kerosene would not have a strong influence in the toxicity of the formulation, since the formulation and its active ingredient (triclopyr in the form of butoxyethyl ester) have similar effects not only on amphibians but also on fish, invertebrates, zooplankton and plants (Roshon et al. 1999, Wojtaszek et al. 2005). Conversely, Aronzon et al. (2011) observed that a 2,4-D formulation containing kerosene as solvent was, depending on the experimental conditions, up to ten times more toxic than the active ingredient alone to *R. arenarum* embryos. However, it cannot be concluded that kerosene was the reason for the increased toxicity of the formulation, since other co-formulants, particularly emulsifiers, were also present in that formulation.

Another substance known to be used as solvent in some formulations whose toxicity to amphibians has been subject of some study is xylol. Namely, da Silva et al. (2020) reported immunosuppression in amphibians exposed to the insecticide Klorpan® 480 EC, an emulsifiable concentrate containing 49% chlorpyrifos and 49.6% xylol. The affected animals showed lymphopenia, neutrophilia, and eosinophilia, which compromised tadpole efficiency to respond to pathogens. Although the authors did not compare the effects of the formulation with those of chlorpyrifos, they suggested that the observed immunosuppression could be in part related to the action of co-formulants.

Synergists constitute a last group of co-formulants that appear in pesticide formulations. The best-known synergist added to the pesticide formulations is piperonyl butoxide. This substance is usually added to a wide variety of insecticides and fungicides to inhibit the action of metabolic enzymes that break down active ingredients (Wilkinson et al. 1984). This way, the toxicity of the formulation is greatly increased. In the only study we are aware of that has addressed the toxicity to amphibians of piperonyl butoxide added to pesticides, Berrill et al. (1993) found no noticeable effects of the synergist when combined with two insecticides, the pyrethroid permethrin and the organophosphate fenvalerate, on tadpoles of several anuran species, although experimental design did not allowed for a direct comparison (mixtures and active ingredients were tested on either different species or developmental stages, which are known sources of variability). The action of piperonyl butoxide in pyrethroid formulations is especially relevant for birds and mammals, which, as explained above, are little sensitive to pyrethroids because of their active metabolization of the active ingredients. However, amphibians do not look like to have a very active metabolization of these substances, hence it is expected that the action of synergists would have a relatively low influence, as suggested by Berrill et al. (1993).

2.4 Influence of formulation types on PPP dermal uptake and toxicity

As reviewed above, the information relative to the factors influencing pesticide uptake via amphibian skin is limited to a few studies evaluating how this compares among certain substances and does not provide any concluding pattern. Perhaps the only trend that can be deduced from those studies is the obvious relationship between potential for dermal uptake and product bioavailability (e.g. substances less bound to soils would have more potential to diffuse through animals' skin). Thus, formulations including additives that increase bioavailability, like surfactants, will logically have a greater potential for absorption than others.

Cox and Surgan (2006) reviewed how inert ingredients can increase exposure of non-target organisms, including humans, to pesticide formulations. For the case of dermal exposures, some co-formulants have been shown to increase absorption or penetration of the active ingredients. For instance, solvents added to formulations of the insecticide lindane and the wood preservative pentachlorophenol increased dermal absorption of the active ingredients through the skin in humans and pigs, respectively (Dick et al. 1997a,b, Baynes et al. 2002) and the surfactant sodium lauryl sulphate increased absorption of carbaryl through porcine skin (Baynes and Riviere 1998).

The surfactants used to increase herbicide efficacy, in particular POEA, have been identified as the chemicals responsible for toxicity of glyphosate-based herbicides not only to amphibians but also to several other non-target species (Tsui and Chu 2003, Annett et al. 2014, Cattani et al. 2014, Jacques et al. 2019), which is in part related to enhanced absorption due to the action of those surfactants. For instance, POEA has been shown to increase membrane permeability of skin melanophores of *X. laevis* (Hedberg and Wallin 2010). Apart from this evidence, nothing is known about the specific influence of co-formulants or formulation types on the amphibian skin. If high lipophilicity is assumed as a contributing factor for dermal uptake of chemicals by amphibians, formulations including solvents like naphtha or other petroleum derivates that contribute to increased lipophilicity should be more susceptible of absorption. However, as reviewed above, reports about the influence of K_{ow} on percutaneous passage of chemicals through amphibian skin led to contradictory conclusions.

Finally, some inert ingredients can increase persistence and concentration of pesticides in the environment, which has been speculated that could increase uptake simply because of prolongation of the exposure period or quantity. For instance, microencapsulated formulations have been shown to be more persistent than other presentations (Wilson et al. 1995, Montemurro et al. 2002), while concentrations of some pesticides in runoff from treated fields are higher when a granular formulation is used than when a wettable powder is applied (Armbrust and Peeler 2002). However, we can only speculate about the possible influence of the environmental persistence of formulations, since no evidence exists indicating that persistent formulations result in higher dermal uptake than non-persistent ones.

In conclusion, whereas a number of studies have addressed the influence of some of the commonly disclosed co-formulants on the toxicity of PPP to amphibians, particularly of certain co-formulants like POEA or naphtha and mostly to aquatic stages, nothing is known about how these co-formulants or the formulation types including them can affect dermal uptake of PPP. Addressing this specific issue requires a focused experimental design including different co-formulants, either alone or in combination with the active ingredients they accompany in the commercial formulations.

3 Development of a test strategy

3.1 Amphibian model species

We used the Perez's frog (*Pelophylax perezi*) as study model. This species belongs to the Ranidae family, occupies the entire Iberian Peninsula and occurs also in southern France, being one of the most common amphibian species within its geographical range (Egea-Serrano 2009), and has a wide ecological range, appearing in all kinds of water bodies, either permanent or temporal (Sánchez-Montes and Martínez-Solano 2011). *Pelophylax perezi* has a common reproductive mode, with aquatic embryos and larvae, from which terrestrial juveniles emerge after metamorphosis. It is closely linked to other European water frogs like the marsh frog *Pelophylax ridibundus* (present from central France to Kazakhstan and from the Baltic Sea south of the Gulf of Finland to the Balkans, minor Asia and south-eastern Iran) and the edible frog *Pelophylax* kl. *esculentus* (present from western France to Russia and from Estonia and Denmark to northern Italy and north of the Balkans), which is in turn a hybrid of *P. rididundus* and the pool frog *Pelophylax lessonae* (with a distribution similar to that of *P. esculentus* but reaching the Gulf of Finland in northern Estonia and being absent from Denmark and northern Germany). All these species form a hybridogenetic complex known as *Pelophylax* kl. *grafi* (Crochet et al. 1995). The study model, *P. perezi*, is therefore representative of a genetic lineage widely distributed across Europe.

3.2 Selection of active substances

3.2.1 Elaboration of a pesticide database

The whole list of active substances included in the EU Pesticides Database[1] was downloaded on September 7th 2020, containing 1429 records. The list was filtered as follows:

- Removal of the 940 substances that were not approved.
- Removal of the 15 substances that are not PPP.
- Removal of the 90 substances that are not registered as pesticides (i.e. attractants, desiccants, elicitors, plant activators, plant growth regulators, repellents, safeners, soil treatments and/or virus inoculation treatments).
- Removal of the 32 substances that, even if approved at EU level, were not authorized in any Member State.

Out of the 352 remaining substances, 90 were not included in the Pesticides Properties Database (PPDB) of the University of Hertfordshire (AERU 2020), which was the major source for physico-chemical and toxicological properties that we included in our pesticide database. Despite this source is not necessarily linked to data used for regulatory, it was preferred over evaluation of each single assessment report because the access to information is quicker than in the reports. Furthermore, data in the PPDB are labelled depending on the source they come from, hence it was possible to filter only those values that were published by the European Commission or its agencies as regulatory and evaluation data. When the value source was different from verified data used for regulatory purposes, values were flagged as pending of confirmation. Those

[1] https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances

flagged values were confirmed, revised or eliminated upon consultation of the corresponding EFSA review report for the substance.

For the 262 substances that were in the PPDB, we collected information about the parameters listed in Table 1. During the collection of data, we removed another 12 substances from which almost no relevant information was available from any of the sources that we used. This left 250 substances finally considered for data analysis.

On top of these parameters, we collected information about three parameters not in the PPDB list: the avian NOEC resulting from long-term exposure tests (collected from the EFSA review reports), and the acceptable daily intake and the acceptable observed exposure level (collected from the EU pesticides database). Those parameters for which information was missing relative to more than 20% of the substances were excluded from the analysis (Table 1); which left the 23 physico-chemical or toxicological parameters considered for data analysis.

Besides data on these numerical physico-chemical and toxicological parameters, we collected information about the use classes of each substance (herbicide, fungicide, insecticide, rodenticide, bactericide, acaricide, nematicide, molluscicide), as stated in the EU pesticides database.

Table 1: Reviewed physico-chemical and toxicological parameters

The list of reviewed physico-chemical and toxicological parameters includes the main sources from which information was retrieved, and indication of whether they were included in the final data analyses.

Parameter description	Units	Comments	Primary source*	Included
Molecular mass	g/mol		PPDB	Yes
Solubility in water	mg/l	At 20°C	PPDB	Yes
Octanol-water partition coefficient (K_{ow})		At pH7, 20°C	PPDB	Yes
Dissociation constant (pKa)		At 25°C	PPDB	Yes
Soil DT_{50} in the lab	days	In aerobic conditions at 20°C	PPDB	Yes
Soil degradation DT_{90} in the field	days	In aerobic conditions	PPDB	No
Dissipation rate RL_{50} on and in plant matrix	days		PPDB	No
DT_{50} in water-sediment systems for the whole system	days		PPDB	Yes
DT_{50} in water-sediment systems for the water phase	days		PPDB	Yes
Linear organic carbon partitioning coefficient in soil (K_{oc})			PPDB	No
Freundlich organic carbon partitioning coefficient in soil (K_{foc})			PPDB	Yes

Parameter description	Units	Comments	Primary source*	Included
Bio-concentration factor	l/kg	Whole fish	PPDB	Yes
Acute oral LD_{50} in mammals	mg/kg bw	Rat (mouse or rabbit if no information from rat)	PPDB	Yes
Acute oral LD_{50} in birds	mg/kg bw	Most sensitive model	PPDB	Yes
Short term dietary LC_{50} or LD_{50} in birds	mg/kg feed	Most sensitive model	PPDB	No
96-hour LC_{50} in fish	mg/l	Most sensitive model	PPDB	Yes
Early Life Stage-consistent NOEC in fish	mg/l	Most sensitive model	PPDB	Yes
48-hour EC_{50} in Daphnia magna	mg/l		PPDB	Yes
21-day NOEC in Daphnia magna	mg/l		PPDB	Yes
96-hour LC_{50} in aquatic crustaceans	mg/l		PPDB	No
96-hour LC_{50} in sediment dwelling organisms	mg/l		PPDB	No
Contact acute LD_{50} in honeybees	µg/bee	Worst case from 24, 48 and 72 hours	PPDB	Yes
Oral acute LD_{50} in honeybees	µg/bee	Worst case from 24, 48 and 72 hours	PPDB	Yes
14-day LC_{50} in *Eisenia* sp.	mg/kg		PPDB	Yes
Reproductive NOEC in earthworms	mg/kg		PPDB	No
Dermal LD_{50} in mammals	mg/l		PPDB	Yes
Inhalation LC_{50} in rats	mg/l		PPDB	Yes
Dermal penetration	%		PPDB	No
Long-term NOEC in birds	mg/kg bw·d	Most sensitive model	EFSA review reports	Yes
Acceptable daily intake (ADI)	mg/kg bw·d		EU pesticides database	Yes
Acceptable operator exposure level (AOEL)	mg/kg bw·d		EU pesticides database	Yes

*PPDB data revised with EFSA review reports when needed.

3.2.2 Collection of information on the substance use

We reviewed the registers of approved PPP from Spain and Germany, and collected some indicators of the relative use of each reviewed substance by farmers:

- From the Spanish register: number of approved formulation compositions containing the active ingredient (e.g. glyphosate, isopropylamine salt 36% w/v as liquid solution), number

of approved commercial products (e.g. Herbolex 360, Glifochem, Spasor Plus N) and intended uses, as well as number of pests against which the substance is recommended (for non-herbicidal substances only).

- From the German register: number of approved commercial products, as well as number of pests against which the substance is recommended (for non-herbicidal substances only).

An index of relative use was calculated as follows:

The 250 substances were ranked according to their values for each of the six variables (four from the Spanish register, two from the German register). Three levels of importance were established for each variable: the first level including the substances ranked 1st to 16th, the second level including substances ranked 17th to 24th, and the third level including substances ranked 25th to 32nd. A value was given to each substance at each level of each variable as indicated in Table 2. The sum of values obtained from each variable resulted in a cumulative use value that was used to rank the reviewed substances in decreasing order of estimated use.

Table 2: Calculation of the index of relative use

Points given to each substance as a function of its importance level at any of the six variables estimative of use collected from the Spanish and German registers of Plant Protection Products.

	Spain				Germany	
Level	**N formulation compositions**	**N commercial products**	**N uses**	**N pests**	**N commercial products**	**N pests**
First	3	5	3	0.3	11	0.3
Second	2	3	2	0.2	7	0.2
Third	1	2	1	0.1	4	0.1

3.2.3 Data analysis and selection of active ingredients for testing

Within each of the 23 variables, the substances were classified in four quartiles from low (Q1) to high (Q4) values. In addition, substances with values below the 5th percentile of above the 95th percentile were classified as LOW-END and HIGH-END, respectively.

The objective was to select 16 active substances. We only considered as eligible those substances that were included in the Spanish and German register and those for which no previously published data about their dermal toxicity to terrestrial amphibians existed. Consequently, only 159 substances were eligible for selection.

Selection was performed in such a way that:

- The four quartiles, and at least one of the extreme groups (LOW-END and HIGH-END) of each variable were represented (with some exceptions, see below).
- The three main classes of use were as evenly represented as possible: herbicides, fungicides (including bactericides), and insecticides (including acaricides, nematicides and molluscicides).
- Within each class, the most important chemical families currently used were represented, for which we considered the information relative to the ECOSAR and US EPA New Chemical

categories included in the EnviroTox Database of the Health and Environmental Sciences Institute (HESI 2020).

- The selection met the above listed criteria while considering substances as high-ranked as possible according to their estimated use.

Exceptions to the first criterion applied because of the distribution of values of some of the variables, especially after removing the ineligible substances. In particular:

- More than half of the reviewed substances had a null pKa value, which made impossible to distinguish Q1 from Q2, and to establish a LOW-END category for this parameter. A HIGH-END was not included either for this parameter.
- It was not possible to select substances representing either LOW-END or HIGH-END for the long-term NOEC in birds.
- It was not possible to select substances representing Q2 for the contact acute LD_{50} in honeybees.

The list of selected substances is shown in Table 3. Details on how the selected substances represent the variability of each physico-chemical and toxicological parameter are presented in Appendix A.

Table 3: Selected active substances

Use	Family*	Substance	Test scenario
Fungicides	Benzophenone	Metrafenone	Overspray only
	Oxazole	Oxathiapiprolin	Overspray only
	Pyrolecarboxamide	Benzovindiflupyr	Overspray + soil contact
	Strobilurin	Azoxystrobin	Overspray + soil contact
	Triazole	Tebuconazole	Overspray + soil contact
Herbicides	Aryloxyalkanoic acid	MCPA	Overspray only
	Aryloxyphenoxypropionate	Fluazifop-p-butyl	Overspray + soil contact
	Benzamide	Isoxaben	Overspray + soil contact
	Dinitroaniline	Pendimethalin	Overspray + soil contact
	Sulfonylurea	Metsulfuron-methyl	Overspray only
	Triketone	Mesotrione	Overspray only
Insecticides	Carbamate	Pirimicarb	Overspray only
	Neonicotinoid	Acetamiprid	Overspray only
	Organofluoride	Flupyradifurone	Overspray + soil contact
	Pyrethroid	Alpha-cypermethrin	Overspray only
	Pyrethroid	Lambda-cyhalothrin	Overspray + soil contact

*According to PPDB (AERU 2020)

3.3 Selection of formulations and co-formulants

Six formulations, based on active ingredients out of the 16 selected active substances (see Table 3) were tested. To select the formulations to be tested, we considered that their six active ingredients should represent as much as possible the four quartiles of each parameter as explained in section 3.2.3. We also considered selecting formulations with a low percentage of active substance (i.e. a high percentage of co-formulants completing the formula) and a low number of different co-formulants that would be present in a proportion as high as possible in the formulation. Since this information is normally undisclosed to the public, the composition included in the products' Material Safety Data Sheets (MSDS) was used.

Once the active ingredients had been selected, we chose formulations of each substance recommended for foliar application, and among these, those recommended for a highest number of uses. The commercial products were those available in the Spanish market, with the exception of Fasthrin 10 EC, which was the product available in the Portuguese market. The list of tested formulations their characteristics is shown in Table 4.

Table 4: Selected formulations

Formulation	Active substance (and percentage in the formulation)	Format	Manufacturer
Vivando	Metrafenone (50%)	Suspension concentrate	BASF
Quadris	Azoxystrobin (25%)	Suspension concentrate	Syngenta
Folicur 25 EW	Tebuconazole (25%)	Oil-in-water emulsion	Bayer
Fusilade Max	Fluazifop-p-butyl (12.5%)	Emulsifiable concentrate	Nufarm
Sivanto Prime	Flupyradifurone (20%)	Concentrated soluble liquid	Bayer
Fasthrin 10 EC	Alpha-cypermethrin (10%)	Emulsifiable concentrate	Sharda Cropchem

Regarding the co-formulants, after the first set of experiments, which included three of the formulations (i.e. Quadris, Folicur 25 EW and Fasthrin 10 EC) and their corresponding active substances, an increased toxicity of the formulations relative to their active ingredients was observed (see results below). In order to determine the reasons why formulations resulted so more toxic than active ingredients, we obtained the list of co-formulants detailed in the MSDS of those formulations (Table 5). It is important to mention that not all co-formulants are necessarily included in the MSDS. In the case of Fasthrin 10 EC, the composition is reasonably well known (only a maximum of 5.9% of the composition is undisclosed) and the co-formulants potentially responsible for increased toxicity could be identified. For the other two formulations, however, up to a 49 and a 72% of the product composition remains undisclosed from the MSDS, and it is uncertain whether the more toxic ingredients co-formulants could be within this unknown part of the product. From the information available in the MSDS, we selected for testing two of the co-formulants accounting for higher proportion in the formulations, hydrocarbons C9 aromatics and N,N-dimethyldecanamid. The former is, according to the

information provided by ECHA relative to its CAS number[2], "*a complex combination of hydrocarbons obtained from distillation of aromatic streams*", and is referred generically as solvent naphtha.

Table 5: Composition of the three formulations included in the first experimental batch, as displayed in their Material Safety Data Sheet

Formulation	Ingredient	CAS number	Percentage in the formulation
Quadris	Active ingredient (azoxystrobin)	131860-33-8	20-25
	Naphthalenesulfonic acid, dimethyl-, polymer with formaldehyde and methylnaphthalenesulfonic acid, sodium salt	9084-06-4	1-3
	1,2-bencisotiazol-3(2H)-ona	2634-33-5	<0.05
	(undisclosed)		*<79*
Folicur 25 EW	Active ingredient (tebuconazole)	107534-96-3	25.8
	N,N-dimethyldecanamid	14433-76-2	>25
	(undisclosed)		<49.2
Fasthrin 10 EC	Active ingredient (alpha-cypermethrin)	67375-30-8	11.07
	Hydrocarbons, C9, aromatics	64742-95-6	80
	Poly (oxy-1,2-ethandiyl), α- [tris (1-phenylethyl) phenyl] - ω-hydroxy-	99734-09-5	1-5
	Benzol sulfone acid, C10-13- (linear). Alkyl derivate, Calcium salt		1-5
	2-Methyl-1-propanol; Isobutanol; Isobutylalkohol; 2-Methylpropanol-1	78-83-1	1-5
	2-Ethylhexan-1-ol	104-76-7	0-1
	1,2,4-Trimethylbenzol	95-63-6	<0.02
	Naphthalin	91-20-3	<0.01
	(undisclosed)		<5.93

3.4 Determination of the active substances for testing by exposure through contaminated soil

Tests on the toxicity of substance when exposure happens because of contact with contaminated soil were conducted with half of the active substances and formulations considered for overspray tests. To select the active substances (and their formulations) relevant for these tests, we looked at the properties potentially accounting for increased risk associated with exposure via contact with contaminated soils. These properties were increased DT_{50} in soil, K_{foc} and K_{ow}, which led to select the following active substances (Table 3): benzovindiflupyr, azoxystrobin

[2] https://echa.europa.eu/registration-dossier/-/registered-dossier/15237

(both as active ingredient and as Quadris), tebuconazole (both as active ingredient and as Folicur 25 EW), fluazifop-p-butyl, isoxaben, pendimethalin, flupyradifurone (both as active ingredient and as Sivanto Prime) and lambda-cyhalothrin.

3.5 Determination of experimental concentrations

3.5.1 Choice of solvent controls

The initial design contemplated three experimental concentrations to be tested via overspray, consisting of the labelled application rate (1xAR, referred to the active substance) plus two treatments corresponding to one tenth (0.1xAR) and ten times (10xAR) the application rate. However, assuming that solvents would be necessary to attain the desired concentrations of active substances, we conducted experiments to determine the toxicity of potential solvents on both aquatic and terrestrial stages of our amphibian model species, *P. perezi*, as well as on earthworms of the species that was later used in the surrogate testing experiment, *Eisenia andrei*.

In order to determine solvent toxicity, we considered two options: acetone (>99% extrapure, AgrosOrganics, Cape Town, South Africa) and acetonitrile (>99.9% pure, Fisher Chemical, Pittsburgh ,PA, USA).

3.5.1.1 Experimental exposure of larval and juvenile amphibians to solvents

Pelophylax perezi were collected as eggs at development stages G8-G10 according to (Gosner 1960) from a lake at Quinta da Boavista located in Gafanha de Áquem, Aveiro, Portugal (40°35′48.8″N 8°41′43.4″W). Animals were maintained in the laboratory as explained below (section 4.1.1).

Tadpoles were tested according to the ASTM protocol E729-96 (ASTM 2002), to which some minor modifications were incorporated. Tadpoles at the free-swimming stage G25 were exposed to one out of the eight acetone (0.125, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 5%) or ten acetonitrile (0.0625, 0.125, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 5%) concentrations prepared in ASTM medium. An additional group was exposed to ASTM medium only and used as control. Each treatment was replicated five times, with each replicate including four animals in a plastic container with 200 ml of the test solution. At the beginning of the experiments, we weighed 12-13 randomly chosen tadpoles; initial body masses (mean ± standard deviation) were 13.3 ± 1.81 mg in the acetone experiment and 13.3 ± 1.17 mg in the acetonitrile one. The experiment was conducted in an environment-controlled chamber with stable temperature and fixed photoperiod, and lasted for 96 hours. Test solutions were renewed once, after 48 hours. At the beginning of the exposure and after the water change, 0.0125 g of TetraMin commercial flakes were added to each container to feed tadpoles. We checked tadpole survival every day, with dead animals being removed from the experimental containers. At the end of the assay, we also recorded total length, inter-orbital distance and body mass of the surviving tadpoles. The two former variables were recorded after taking pictures of the animals that included a reference ruler and analysing the digital images with the software ImageJ.

For juvenile exposure to solvents, we used two designs, one to determine acute effects and another one to determine longer-term effects, more consistent with the exposure scenario that was planned for pesticide toxicity experiments. The acute test consisted of exposing newly metamorphosed juveniles that were sprayed with the corresponding test solutions and

euthanized 24 h later. The long-term test consisted of exposing 10-day-old juveniles, who were euthanised 7 days later.

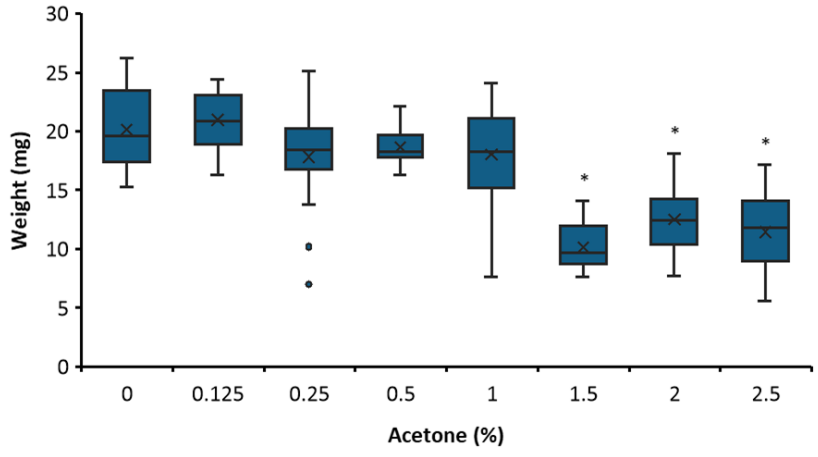
Both designs were identical in the treatments, replicate numbers and overspray protocol. Animals were exposed to one of the following acetone or acetonitrile concentrations: 0 (controls), 0.5, 1, 1.5, 2.5 or 5 %. Each treatment was replicated three times. Spraying was performed using the system described above. Juveniles were measured (snout-vent length, SVL, measured only for the long-term test) and weighed right before overspray and again at the end of the test, before euthanasia. Euthanasia was conducted by immersion in 6 g/l solution of tricaine methanesulfonate. A piece of the dorsal skin of all collected animals was extracted and preserved in 10% formalin for fixation. After elimination of the water content by ethanol flux, the skin pieces were embedded in paraffin, sliced using a microtome and stained using haematoxylin and eosin. Preparations were observed under a 100x optical microscope with objective immersion oil.

The LC_{50} values and their respective confidence limits, at each 24 h exposure period, were computed by adjusting a logistic model with three parameters to mortality data. Comparison of larval biometric measurements (weight, total body length and inter-orbital distance) among treatments was done using univariate analyses of the variance (ANOVAs) followed by the multicomparison Dunnett's test. In juvenile experiments, weight (for acute test) and body condition (calculated as the residuals of the regression between SVL and body weight, for long-term test) were compared among solvent concentrations using Generalized linear models (GzLM) with the response variable adjusted to a gamma link function due to its lack of normality.

3.5.1.2 Solvent toxicity to larval amphibians

Figure 1: Weight of *Pelophylax perezi* tadpoles after 96-hour exposure to seven concentrations of acetone

Box plots show the mean (x), median (central line), 25th and 75th percentiles (bottom and top of boxes) and 1.5 interquartile range (bottom and top of line), white circles outside the boxes represent outliers. Asterisks (*) represent treatments significantly different from controls (p < 0.05). Source: own illustration, [University of Aveiro].

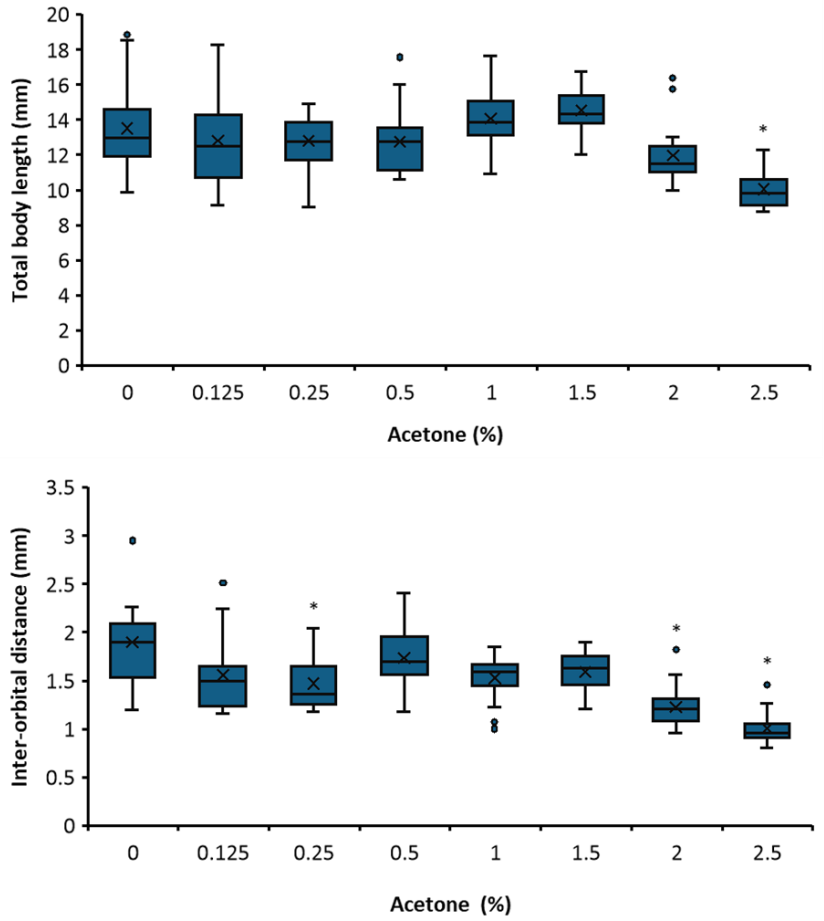


Exposure of tadpoles to 5% acetone caused 100% mortality. No mortality was observed in the control and the other tested acetone concentrations, except for 2.5%, where 5% mortality occurred. A significant reduction in body weight was observed for tadpoles exposed to 1.5, 2 and

2.5% of acetone (Figure 1; $p < 0.05$). A significant reduction in total body length and inter-orbital distance was registered at acetone concentrations equal or above 2.5% and 2%, respectively (Figure 2, $p < 0.05$).

Figure 2: Total body length (top) and inter-orbital distance (bottom) of *Pelophylax perezi* tadpoles after 96-hour exposure to seven concentrations of acetone

Box plots show the mean (x), median (central line), 25th and 75th percentiles (bottom and top of boxes) and 1.5 interquartile range (bottom and top of line), white circles outside the boxes represent outliers. Asterisks (*) represent treatments significantly different from controls ($p < 0.05$). Source: own illustration, [University of Aveiro].



Exposure to acetonitrile caused a higher lethality to *P. perezi* tadpoles than that caused by acetone. An LC_{50} of 1.91% (95% confidence interval: 1.29-2.42%) of acetonitrile was computed after the 96 h of exposure (Figure 3).

Weight revealed to be a very sensitive endpoint to acetonitrile, as all concentration of this compound caused a significant reduction in the weight of *P. perezi* tadpoles (Figure 4; $p < 0.05$).

Though acetonitrile significantly affected weight, effects were observed in total body length were slight, affecting only tadpoles exposed to 0.065% and 0.25% of the solvent as compared to the control group (Figure 5; $p < 0.05$). Finally, the inter-orbital distance was significantly reduced at concentrations equal or above 0.25% of acetonitrile (Figure 5; $p < 0.05$).

Figure 3: Cumulative mortality of *Pelophylax perezi* tadpoles after 96-hour exposure to ten concentrations of acetonitrile

Error bars correspond to the standard deviation. The median lethal concentrations (LC_{50}), with the respective confidence limits at 95%, are shown for each observation period (24, 48, 72 and 96 h). Source: own illustration, [University of Aveiro].

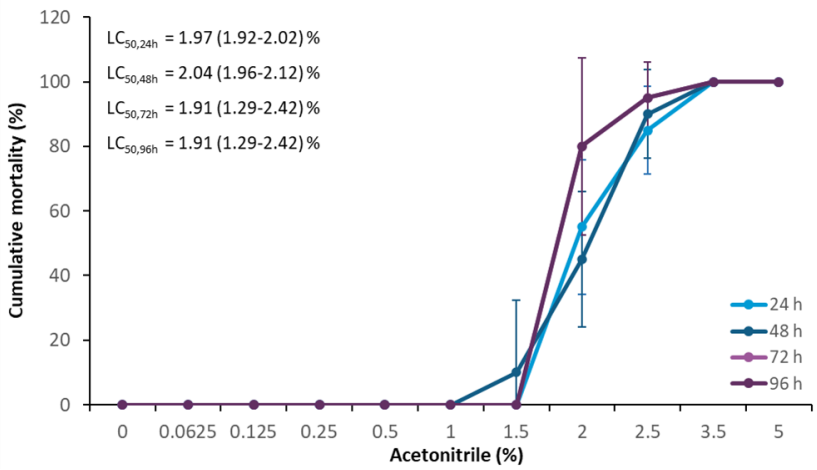


Figure 4: Weight of *Pelophylax perezi* tadpoles after 96-hour exposure to seven concentrations of acetonitrile

Box plots show the mean (x), median (central line), 25th and 75th percentiles (bottom and top of boxes) and 1.5 interquartile range (bottom and top of line), white circles outside the boxes represent outliers. Asterisks (*) represent treatments significantly different from controls ($p < 0.05$). Source: own illustration, [University of Aveiro].

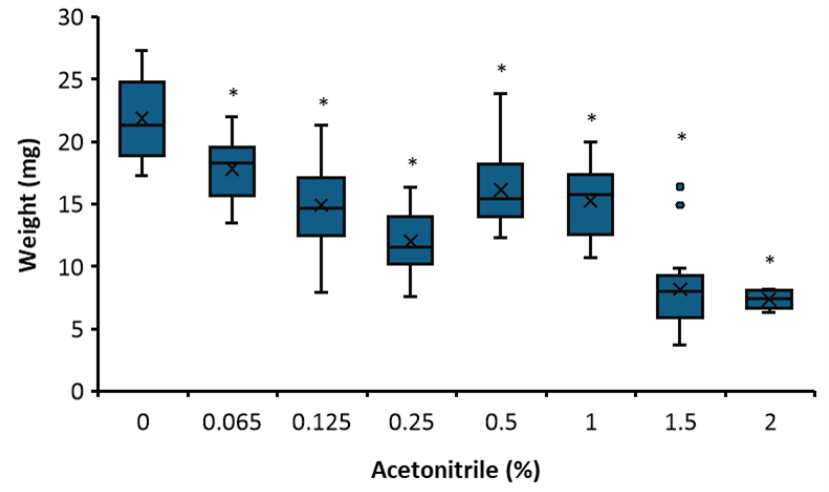
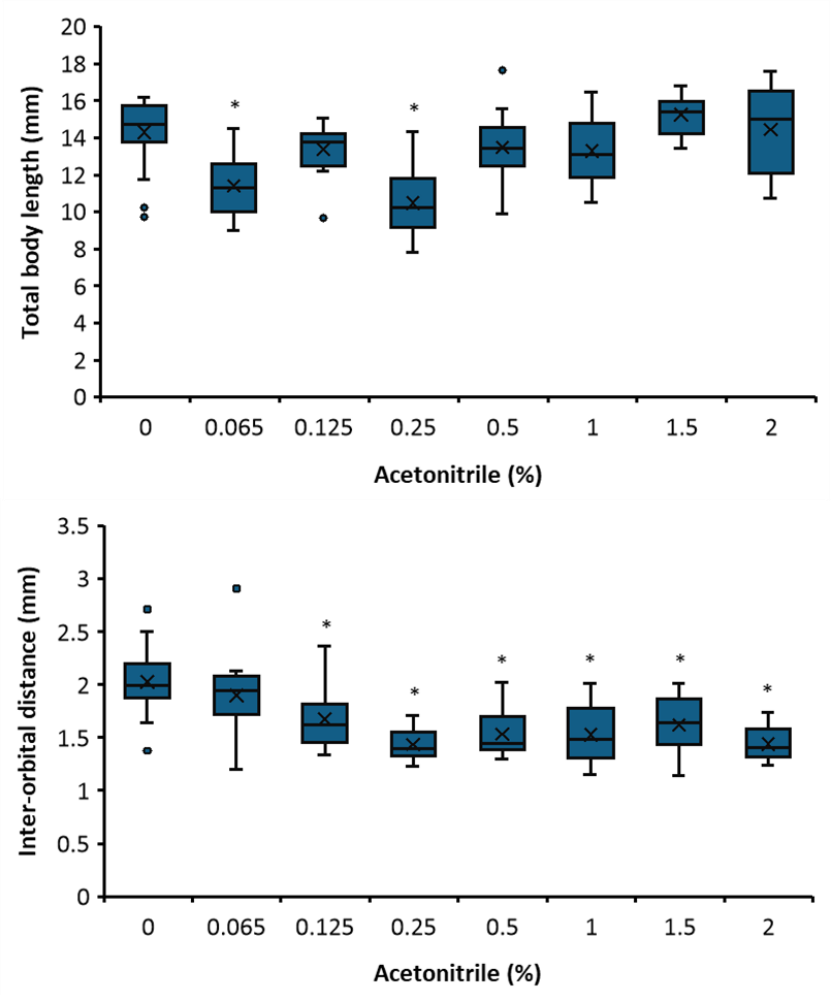


Figure 5: Total body length (top) and inter-orbital distance (bottom) of *Pelophylax perezi* tadpoles after 96-hour exposure to seven concentrations of acetonitrile

Box plots show the mean (x), median (central line), 25th and 75th percentiles (bottom and top of boxes) and 1.5 interquartile range (bottom and top of line), white circles outside the boxes represent outliers. Asterisks (*) represent treatments significantly different from controls ($p < 0.05$). Source: own illustration, [University of Aveiro].



3.5.1.3 Solvent toxicity to juvenile amphibians

No mortality was found during the 24 h of monitoring of the acute test. Change in body weight during that period did not differ significantly among any of the tested treatments, including the controls.

In the long-term test, two animals died during the 7-day monitoring period, one that had been sprayed with 1% acetone and another one sprayed with 2.5% acetonitrile. Considering the rest of the animals, no differences among treatments were detected in the change of body condition during the 7-day monitoring period (all $p \geq 0.370$).

Histopathological analyses revealed no signs of altered skin structure caused by any of the treatments.

3.5.1.4 Experimental exposure of earthworms to solvents

The origin and husbandry procedures of *E. andrei* are detailed in section 5.1.2. Adults of *E. andrei* (weighting on average ± standard deviation: 513.9 ± 86.2 mg) were exposed, through overspray, to five concentrations of acetone (0.5, 1, 1.5, 2.5 and 5%) plus a control consisting of deionised water. Approximately three to four hours before overspray, earthworms were washed and placed in Petri dishes so they could void their gut contents. Overspray was made using the system described above. After overspray, each earthworm was transferred to a Petri dish, filled with a filter paper of 85 g/m^2, 0.2 mm thick, and a diameter of 70 mm, moistened with 1 mL of deionised water. One organism was placed by Petri dish, which constituted a replicate. Five replicates were set per acetone concentrations and control. Exposure took place for 72 hours at 23 ± 1°C in total darkness. Mortality of organisms was checked each 24 h, and an earthworm was considered dead when it did not respond to a gentle mechanical stimulus for 15 seconds. Deionised water was added to the filter paper every 24 h to avoid its dryness. At the end of the assay (72 h) mortality was registered and all alive earthworms were weighted to the nearest 0.0001 g. The exposure procedures were adapted from the OECD guideline 207 for earthworm acute toxicity Tests (OECD 1984).

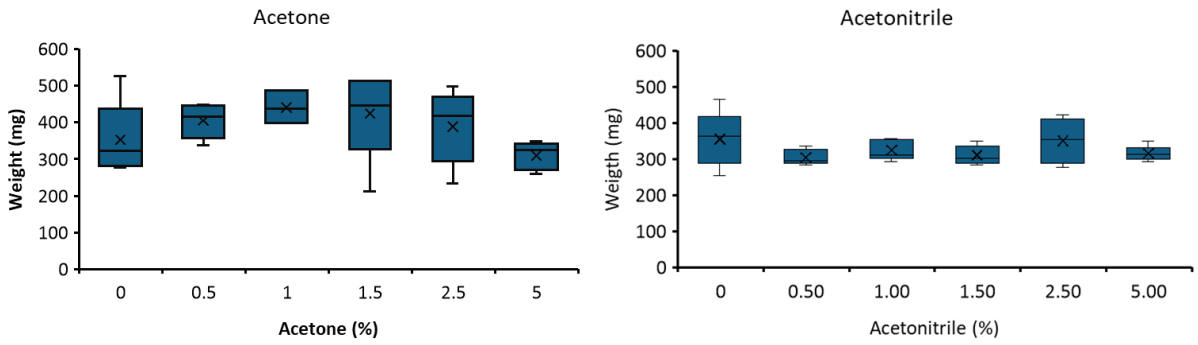
Response variables were compared among solvent concentrations using univariate ANOVA once normality of those variables had been confirmed. Cumulative survival was compared among treatments using a long-rank test.

3.5.1.5 Solvent toxicity to earthworms

The over spray of adults of *E. andrei* to deionised water (control) and to the five tested concentrations of acetone and acetonitrile caused no mortality to the organisms, for a period of 72 h. Furthermore, no significant effects were observed in the weight of the earthworms treated with the control (deionised water) and the acetone ($p = 0.251$) or acetonitrile ($p = 0.443$) concentrations, after the 72-h assay period (Figure 6).

Figure 6: Weight of *Eisenia andrei* adults after 72-hour exposure to five concentrations of acetone (left) and acetonitrile (right)

Box plots show the mean (x), median (central line), 25th and 75th percentiles (bottom and top of boxes) and 1.5 interquartile range (bottom and top of line). Source: own illustration, [University of Aveiro].



3.5.1.6 Conclusion on the use of solvents

Considering the results of the solvent toxicity tests, and despite the lack of clear effects on juvenile frogs, we did not consider adequate to use concentrations above 1% of solvents because of the high risk of interference with the toxicity caused by the products. This way, we established as highest exposure concentration the maximum that could be achieved with a concentration of

1% of acetone or acetonitrile. Although the preferred option was to use the same solvent for all products, the difference between solubility in acetone or acetonitrile of some of the active substances was so large that we were forced to adapt each test to the best possible solvent. In addition, in some cases we found that the maximum solubility in a given solvent was reachable only after a long time of mixing, which could have affected the concentration in the experimental broths. For this reason, we were forced to add surfactants (i.e. Triton® at a concentration of 1%) to improve the solubility of some of the tested substances. When relevant, a solvent or a solvent + surfactant control was included in the test design.

3.5.2 Experimental concentrations used in the toxicity assays

The application rate used as reference was determined from the approved uses of the tested substances. We prioritized application rates that were relevant to winter cereals, vineyards or maize crops. From the application instructions of formulations containing the selected active ingredients, we confirmed that the broth application volume was always 400 l / ha (i.e. 40 ml / m^2). The application rates, combined with the broth application volume, determined the concentration of active substances that was necessary to prepare the solutions that would be sprayed.

Despite the initial intention was to test always a highest level of 10xAR (for overspray treatments), the broth concentration necessary to achieve this level was not possible for all the treatments if limited to 1% solvent concentration. In these cases, the highest treatment level corresponded to that what could be achieved after diluting the active substances with 1% solvent. Details on the experimental concentrations of active substances used during the project are shown in Table 6. IN addition, the expected maximum concentration to be achieved based on the substance solubility in solvents was not possible; in those cases, and as explained above, the surfactant Triton® was added to the mixture, and to the solvent control, at a concentration of 1%.

In the case of formulations, we used the same treatment levels as for their corresponding active substances, even if in the case of formulations the level of 10xAR was always achievable; however, since our intention was to compare the toxicity between the active substance and its formulation, we maintained the same experimental concentrations. Finally, the co-formulants were tested at the concentrations that would exist if the formulations containing these co-formulants were applied at 0.1xAR, 1xAR or 10xAR.

There were, however, some exceptions to this general pattern; solubility problems prevented us from setting the highest overspray treatment levels of MCPA, isoxaben, lambda-cyhalothrin and N,N-dimethyldecanamid, while product shortage forced us to limit the pendimethalin assay to the application rate treatment only.

Table 6: Calculations to determine the experimental treatments used for overspray experiments

Type	Substance	Model crop	Application rate (g active ingredient / ha)	Spraying experimental concentration (mg/l) needed for 10x AR[a]	Water solubility of the active ingredient (mg/l)	Solvent needed	Solubility of the active ingredient in the solvent (mg/l)[b]	Maximum application rate reachable with 1% solvent[c]	Experimental application rates used[d]
Active ingredients	Metrafenone	Vineyard	150	3750	0.492	Acetone + surfactant[e]	403000	13.9x	0.1x 1x 10x
	Oxathiapiprolin	Vineyard	16	400	0.1844	Acetone	162800	40.7x	0.1x 1x 10x
	Benzovindiflupyr	Cereals	75	1875	0.98	Acetone	350000	18.7x	0.1x 1x 10x
	Azoxystrobin	Cereals	250	6250	6.7	Acetonitrile	340000	5.44x	0.1x 1x 5.44x
	Tebuconazole	Cereals	250	6250	36	Acetone	>200000	>3.2x	0.1x 1x 3.2x
	MCPA	Cereals	700	17500	29390	None[f]	-	-	0.1x 1x
	Fluazifop-p-butyl	Cereals	250	6250	0.93	Acetone	>1000000	>16x	0.1x 1x 10x
	Isoxaben	Cereals	57,95	1448,75	0.93	Acetone	270000	18.6x	0.1x 1x
	Pendimethalin	Cereals	1600	40000	0.33	Acetone	>1000000	>2.5x	1x
	Metsulfuron-methyl	Cereals	6	150	483	None	-	-	0.1x 1x 10x
	Mesotrione	Maize	150	3750	1500	Acetone + surfactant[e]	93300	2.49x	0.1x 1x 2.49x
	Pirimicarb	Cereals	100	2500	3100	None	-	-	0.1x 1x 10x
	Acetamiprid	Vineyard	75	1875	2950	None	-	-	0.1x 1x 10x
	Flupyradifurone	Vineyard	96	2400	3200	None	-	-	0.1x 1x 10x
	Alpha-cypermethrin	Cereals	15	375	0.004	Acetone	>250000	>66.7x	0.1x 1x 10x

Type	Substance	Model crop	Application rate (g active ingredient / ha)	Spraying experimental concentration (mg/l) needed for 10x AR[a]	Water solubility of the active ingredient (mg/l)	Solvent needed	Solubility of the active ingredient in the solvent (mg/l)[b]	Maximum application rate reachable with 1% solvent[c]	Experimental application rates used[d]
	Lambda-cyhalothrin	Cereals	7,5	187,5	0.005	Acetone	500000	266.7x	0.1x 1x
Formulations	Vivando	Vineyard	150	-	-	None	-	-	0.1x 1x 10x
	Quadris	Cereals	250	-	-	None	-	-	0.1x 1x 5.44x
	Folicur 25 EW	Cereals	250	-	-	None	-	-	0.1x 1x 3.2x
	Fusilade Max	Cereals	250	-	-	None	-	-	0.1x 1x 10x
	Sivanto Prime	Vineyard	96	-	-	None	-	-	0.1x 1x 10x
	Fasthrin 10 EC	Cereals	15	-	-	None	-	-	0.1x 1x 10x
Co-formulants	Naphtha	Cereals	120[g]	-	-	None	-	-	0.1x 1x 10x
	N,N-dimethyldecanamid	Cereals	250[h]	-	-	None	-	-	0.1x 1x

[a] For an application broth volume of 400 l / ha

[b] Information extracted from the pesticide assessment reports

[c] Considering the maximum solubility in that solvent

[d] For overspray treatments. Exposure via soil contact was always performed at 1xAR

[e] 1% Triton®.

[f] Acetone was finally used because of solubility problems of the active ingredient in water

[g] Calculated from the application rate of Fasthrin 10 EC. Value in ml / ha.

[h] Calculated from the application rate of Folicur 25 EW.

4 Laboratory tests with amphibians

4.1 Material and methods

4.1.1 Animal collection and husbandry

Embryos of *Pelophylax perezi* were collected from three different places with no known history of pesticide use in the vicinities, by the two consortium partners:

- An artificial pool at El Chaparrillo agri-environmental centre, Ciudad Real, Spain (39,003°N, 3,961°W).
- A pond at Santa Quiteria, Ciudad Real, Spain (39,263°N, 4,369°W).
- A lake at Quinta da Boavista located in Gafanha de Áquem, Aveiro, Portugal (40°35′48.8″N 8°41′43.4″W).

In all cases, embryos were collected at development stages G8-G10 (Gosner 1960). Embryos were transported to the laboratories of the Institute of Game and Wildlife Research (IREC) in Ciudad Real, Spain, or of the University of Aveiro (UA) in Aveiro, Portugal, in water from the water bodies they were collected. Upon arrival, the embryos were carefully separated without damaging their protective membrane and kept in FETAX medium (renewed every two days) at constant temperature (23 ± 1°C) and photoperiod (14h light: 10h dark). Animals were maintained (Figure 7) until the emergence of the forelimbs (stage G42). Larvae from GS25 until GS42 were fed six times per week with a mixture of commercial TetraMin flakes and Sera Micron powder. The feeding amounts were adjusted to the growth of the animals, conveying approximately 2% of the biomass at each feeding event.

Figure 7: Detail on the aquaria where tadpoles were grown (left) and containers with emerging individuals completing their metamorphosis (right)

Source: own illustration, [IREC-CSIC].

At stage G42, animals were moved to new containers with an immersed and an emerged section in order to facilitate metamorphosis (Figure 7), where they were maintained until the termination of metamorphosis, when the tail was completely reabsorbed (stage G46). During this period, no feed was provided to animals as they obtain their nutrients from the mobilization of reserves accumulated in the tails.

After GS46, juveniles were placed in individual containers with 100 ml of soil with 30% water content (Figure 8). During the first 10 days post metamorphosis, juvenile frogs were fed four times with 0.6-1 cm crickets that were previously spiked with vitamin and calcium powder. After day 10 post-metamorphosis, once experimental trials began, frogs were fed three times during the first week of experiment with eight crickets of 0.6-1.0 cm size; from day 7 post-exposure to the end of the experiments, frogs were fed with three times per week with six crickets of 1.0-1.5 cm size.

Figure 8: Containers used for juvenile frog housing and experiments

Source: own illustration, [IREC-CSIC].

4.1.2 Experimental exposure and sample collection

4.1.2.1 Set up of the spraying system

A spraying system was set up to control the spraying volume with the purpose of establishing a spraying scenario consistent with what happens in the field. The system (Figure 9) consisted of a 12-volt pump with an aspiration hose connected to a container and an impulsion system with three sections:

- A solenoid valve connected to a digital temporizer that controlled the impulsion time of the pump.
- A t-valve for purging and emptying the system.
- A nozzle fixed to an anti-drop holder.

The whole system was fixed to a stainless-steel structure in order to keep a stable distance between the nozzle and the floor. Considering the target volume that we wanted to spray per surface area unit (i.e. 400 l / ha, or 40 ml / m^2), three variables were monitored to set up the system:

- Nozzle type. We tested two types of full-cone nozzles, varying in the caudal they spray for a given pressure. The tested nozzles corresponded to the green (lower caudal) and yellow (higher caudal) colours according to the ISO 110 system.
- Pump impulsion time. This variable was controlled with the solenoid valve; the temporizer allowed for a range of impulsion times from 0.1 to 99 seconds.

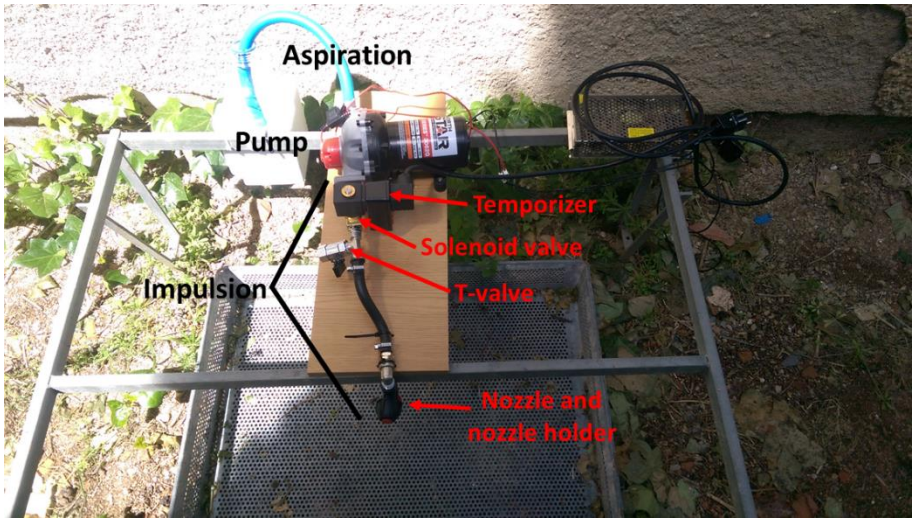
- Distance from the nozzle to the floor, where items to be subject to overspray are placed. The maximum distance that did not cause the spraying cone to lose uniformity was checked to be approximately 50 cm. Taking this into account, several options for nozzle height were tested.

After combining these three parameters, we confirmed that the combination resulting in the most accurate spraying volume relative to the target was that consisting of the yellow nozzle, an impulsion time of 0.2 seconds and a nozzle height of 35 cm.

Spraying volume at each single flush was measured by placing 9-mm petri dishes with behind the nozzle, and then measuring the volume within the dish by collecting the broth with a micro-pipette. Assuming a petri dish surface of 63.6 cm^2 the expected volume to be picked up for a spraying ratio of 40 ml / m^2 was 0.2544 ml. The mean±SD collected volume in the different spraying simulations (N=16) was 0.2591±0.0298 ml.

Figure 9: Spraying system

Source: own illustration, [IREC-CSIC].



4.1.2.2 Chemicals

Active ingredients (Dr. Ehrenstorfer, purity ≥ 97.34%) were purchased from LGC Standards Ltd (Luckenwalde, Germany). Vivando, Quadris, Folicur 25EW, Fusilade Max and Sivanto Prime were purchased at Agricoal Primer SL (Totana, Murcia, Spain) as Plant Protection Products registered for marketing in Spain. Fasthrin 10 EC was purchased at Mimagro Digital (Cova da Beira, Portugal) as a Plant Protection Product registered for marketing in Portugal. Solvent naphtha (Molekula Group, CAS: 64742-94-5) was purchased from ChemoSapiens S.L. (A Coruña, Spain) and N,N-dimethyldecanamid (Sigma-Aldrich, CDS001534) was purchased from Chemicalnor Lda (Valongo, Portugal).

4.1.2.3 Exposure of juvenile amphibians to pesticides

We used a pseudo-factorial design to consider both exposure scenarios (overspray and soil contact). This design allowed for testing not only the effects associated with each exposure type separately, but also the effects resulting from the combination of overspray and further soil contact (Table 7). As mentioned above, for the overspray assays we established three treatment levels consisting of the application rate of the substance (1xAR) plus two treatments resulting from multiplying and dividing such application rate by a factor of ten (i.e. 0.1xAR, 10xAR), although, as explained in section 3.5.2, the highest treatment was modified in some cases

because of the solubility of the pesticides (Table 6). For soil contact, we considered a unique treatment level consisting of the application rate (1xAR) of the tested substances, which was tested alone or in combination with the 1xAR via overspray. This design allowed for replicating a realistic worst-case scenario in which the application rate of the pesticide is sprayed onto the animal and onto the soil on which the animal would stay afterwards.

Table 7: Amphibian experimental set-up

		Overspray				
		No pesticide (negative control)	**Solvent 1% (solvent control)**	**0.1xAR**	**1xAR**	**10xAR***
Soil contact	**No pesticide**	10 replicates	5 replicates	5 replicates	5 replicates	5 replicates
	1xAR	5 replicates		-	5 replicates	-

*Modified for some substances depending on their solubility, see detail in Table 6.

Both the negative and solvent controls were, for some of the experiments that were run simultaneously in the same laboratory, shared, in order to minimize the number of animals to be used. Each replicate consisted of a single container with two *P. perezi* juveniles inside, given that the dimensions of the containers were appropriate to house two small-sized individuals according to the specifications of the Directive 2010/63/EU.

Overspray was be conducted on frogs ten days after the end of their metamorphosis. This time allowed us for checking that animals were feeding and growing without apparent problems, thus minimizing the risk for confounding effects due to impoverished condition or health of the analysed individuals. Frogs were placed on Petri dishes within a 2 x 2 m area outdoors, which was sprayed with the corresponding treatment using the automatic spraying system described in section 4.1.2.1. Negative control frogs were be sprayed with FETAX medium, while solvent control animals were sprayed with a 1% dilution of the relevant solvent in FETAX medium. Frogs were collected from the dishes immediately after overspray and placed in individual containers of the same characteristics as those where they had been housed since metamorphosis (Figure 8). Previously to the inclusion of the treated (or control) frogs, containers were filled with a 1-cm deep layer of pesticide-free LUFA standard soil 2.2 containing, previously moistened by spraying FETAX medium onto it to reach a 10% of water content. The organic carbon content of this soil (1.82 ± 0.48[3]), and the 90% humidity of the chambers where the experiments were carried out ensured that animals would maintain an appropriate moisture. For the soil contact scenario, containers with a 1-cm-deep layer of LUFA standard soil 2.2 with a 10% added water were placed within a 2 x 2 m area outdoors, which was then sprayed with the corresponding treatment. Frogs were then placed on the treated soils right after pesticide application. Soil samples (1 g) were collected right after pesticide application and two more times on days 1 and 7 after pesticide application to analyse residue levels in soils. Also, during each pesticide trial, a subsample of the overspray product was collected to confirm pesticide concentrations. The methodology used to perform these analyses is explained in section 4.1.4. The samples collected from the experiments conducted with MCPA, mesotrione, acetamiprid and alpha-cypermethrin (both the active ingredient and the formulation Fasthirn 10

[3] https://www.lufa-speyer.de/images/stories/V7_Analyses_Datashet_for_Standard_Soils_30.01.2024Formular.pdf

EC) could not be analysed because of sample lost.

One of the frogs from each container was be maintained there for seven days after pesticide application, while the second frog was be kept in the container for additional 14 days (i.e. total 21 days), in order to monitor effects of slow-acting substances.

4.1.2.4 Measures and sample collection

All animals were weighed and its SVL measured at the beginning of the experiments, right before exposure. The experiments were conducted in seven batches, four at UA lab and three at IREC lab. Initial measures were summarized per experimental batch (Table 8).

Table 8: Weight and snout-vent length (SVL) of juvenile forgs from each batch at the beginning of the experiments

Laboratory	Batch	Year tested	Initial weigh (mg, mean ± SD)	Initial SVL (mm, mean ± SD)	Substances tested
IREC	1st	2021	729.2 ± 199.7	18.13 ± 1.51	Metsulfuron-methyl* Alpha-cypermethrin Quadris Folicur 25 EW* Fasthrin 10 EC
	2nd	2021	1927 ± 374.1	24.30 ± 1.51	Azoxystrobin Tebuconazole Metsulfuron-methyl* Folicur 25 EW*
	3rd	2022	937.1 ± 263.8	19.32 ± 1.62	Metrafenone Oxathiapiprolin Benzovindiflupyr Flupyradifurone Vivando Sivanto Prime
UA	4th	2021	357.9 ± 180.3	14.15 ± 2.29	Pirimicarb Acetamiprid Folicur 25 EW*
	5th	2021	440.1 ± 128.2	14.08 ± 1.14	Pendimethalin Metsulfuron-methyl* Mesotrione
	6th	2022	293.1 ± 68.63	14.79 ± 1.22	MCPA Isoxaben
	7th	2023	345.4 ± 74.51	15.74 ± 1.30	Fluazifop-p-butyl Lambda-cyhalothrin Fusilade Max Naphtha N,N-dimethyldecanamid

*Included in more than one batch

On day 7 after treatment, one of the animals per replicate was removed from the container, while the other one was kept until day 21 after treatment. Individual survival was monitored daily. Animals removed from the containers, either on day 7 or 21, were measured and weighed (SVL), and their body condition was calculated from body mass and SVL measurements according to the scaled mass index (Peig and Green 2009).

After measuring, animals were euthanised by immersion in a 6 g/l solution of tricaine methanesulfonate whose pH was neutralized with 2N sodium hydroxide. For the animals removed on day 7 of experiments, a section consisting of the posterior part of the mid-trunk and hind limbs was separated and fixed by immersion in 10% neutral buffered formalin (pH 7.0) for further histopathological analysis. From the rest of the trunk, fresh pieces of skin and liver were separated and stored at -80°C for further pesticide residue analysis.

Total food intake was recorded by annotating the number of eaten crickets and a daily food intake rate (FIR) was calculated. While there were two frogs in the containers, the FIR was calculated assuming that both animals had ingested the same number of crickets.

4.1.3 Histological analysis

Histological analyses were conducted on animals removed on day 7 of experiment and coming from overspray or control treatments. Samples separated for histology were maintained in 10% neutral buffered formalin for 24 hours at room temperature. To facilitate subsequent processing steps, the body was further sectioned along the pelvic region, ensuring the legs remained attached to the trunk to prevent detachment of the skin from underlying tissues. The sectioned tissues were placed in labelled histology cassettes, which were immersed in 5% nitric acid for 2 hours to decalcify bones and soften the tissue for easier sectioning. Following decalcification, the cassettes were transferred back into 10% formalin and stored for at least 12 hours to ensure proper fixation before processing. The decalcified tissues were dehydrated and cleared in an automated tissue processor (STP 120, Myr), programmed to run overnight (~12 hours). Dehydration was achieved through immersion in a graded ethanol series (70%, 80%, 95%, and 100%), followed by clearing with xylene to facilitate paraffin infiltration. The tissues were subsequently infiltrated with molten paraffin under vacuum at 60°C to prepare them for embedding.

The paraffin-infiltrated tissues were embedded in paraffin blocks using a tissue embedding system (EC 350-1, Myr), ensuring correct orientation to obtain transverse sections that included the chosen anatomical region of reference, which was the region where ileum and caudal vertebrae or sacrum-urostyle are encountered (Figure 10). In this region the skin is tightly anchored to the underlining muscles, permitting a better quality of the sectioning. Moreover, the standardized sectioning of this region allows for minimizing biases in the downstream analysis. Histological pictures from this region should contain the dorsal plicae and the abovementioned bony structures, as well as a prominent pair of enlarged subcutaneous lymphatic spaces, dorsally on either side of the urostyle, as usual in many terrestrial anurans (Wright and Whitaker 2001).

The blocks were allowed to cool, after which they were removed from the moulds. If necessary, blocks that were improperly oriented or defective were remelted and re-embedded. Cold paraffin blocks were chilled on ice prior to sectioning. Chilling allowed for the sectioning of thinner, high-quality tissue slices, providing better support for harder tissue elements. After securing the microtome blade with a clearance angle of 2 to 4°, the paraffin blocks were trimmed to a depth of 10–30 µm using an old blade. Sections of 5 µm thickness were cut from the

trimmed blocks and floated on a 40°C water bath containing distilled water. The sections were carefully transferred to glass slides using a brush and allowed to air dry briefly. The slides were then placed in an oven at 37°C and left to dry overnight.

Histological examination was performed using a light microscope (ECLIPSE Ci, Nikon), focusing on the identification and characterization of the epidermis, dermis, glands, and associated structures. The analysis focused on dorso-lateral plicae because epidermis, dermis and glands are always identified in this structure (Figure 11). This choice permitted to standardize the region of interest (ROI) to be evaluated. The selected ROI allows for the evaluation of epidermis, dermis, glands and any existing inflammatory reactions (e.g. extravasated leukocytes and pigmented melanomacrophages).

Photographs of the ROI were captured for imaging analysis using a microscope-mounted camera (DS-Ri1, Nikon), providing detailed images for histological assessment. The microphotographs were all captured at the magnification of 10x, trying to maintain the same light conditions.

Figure 10: Anatomical region of reference identified to design a standardized histological analysis for overspray exposures

Left: Reference region highlighted on the anuran skeletal system, consisting of ileum, sacral vertebrae and urostyle (image from https://quizlet.com/). Right: Microphotographs of the histological region of reference. Ileum and caudal vertebrae or sacrum-urostyle with dorso-lateral plicae must be present in the section to analyse. Source: own illustration, [IREC-CSIC].

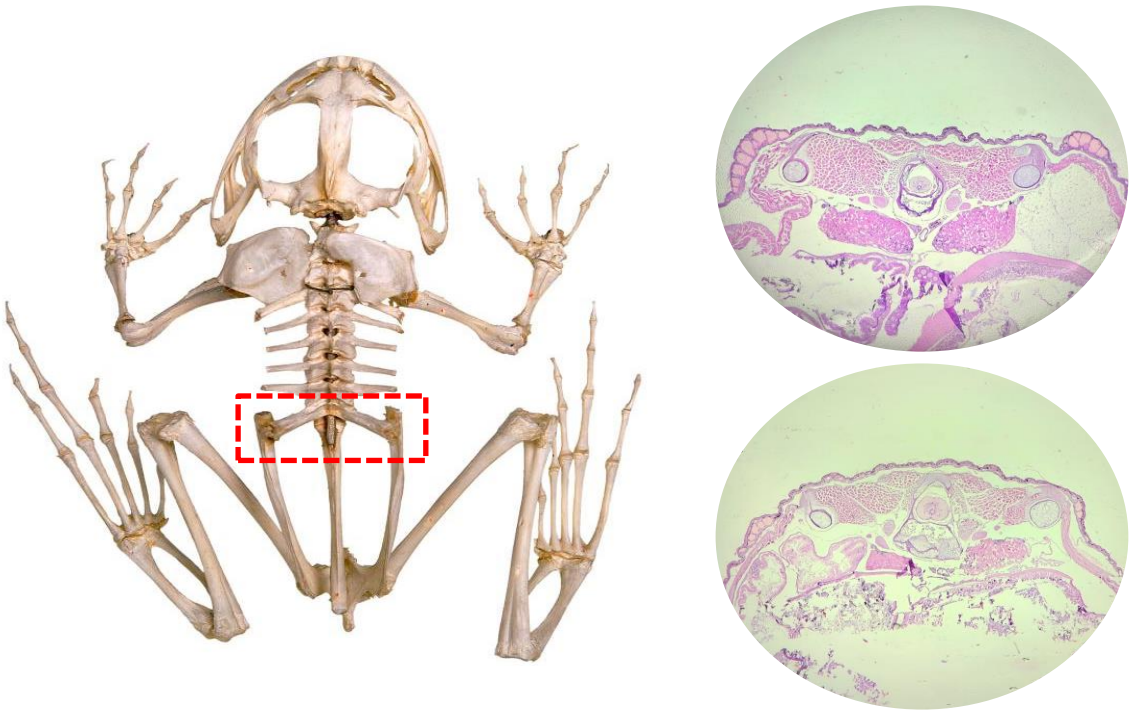
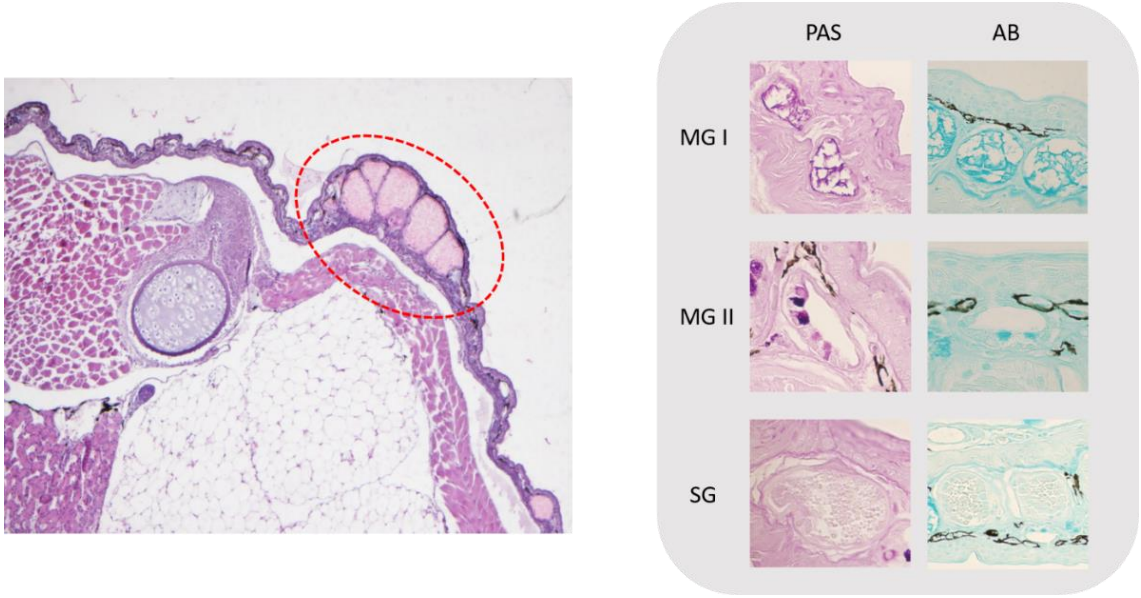


Figure 11: The dorso-lateral plicae at level of the anatomical region of reference

Left: Microphotographs of the anatomical region of reference highlighting a dorso-lateral plica. Right: Types of glands found in the dorsolateral plica and stained with Periodic acid–Schiff (PAS) and Alcian Blue (AB); MG I: mucous glands type I; MG II: mucous glands type II; SG: serous glands. Source: own illustration, [IREC-CSIC].



Epidermis was used to perform digital image analysis with QuPath v.0.5.1 software (Bankhead et al. 2017). The pixel size was identical for all images and was transformed to micrometers by setting the pixel size from a known bar of 100 µm. The annotation of epidermis was performed manually, utilizing both the wand and brush tools to define ROI on the tissue sections. Annotations of skin gland necks were specifically omitted from the epidermal ROI to ensure that only epidermal tissue was included in the subsequent analysis. Care was taken to avoid any artifacts, such as folds or out-of-focus tissue. The operator was blinded by using mask image name function in QuPath. The ROI were classified as *Region** to be later analysed in batch through script-based workflows. The variability in light exposure and staining intensity across the images presented challenges for consistent cell detection. To address this, two cell detection methods were designed to operate independently of variations in haematoxylin staining and light intensity, allowing for more robust cell identification despite the heterogeneity in image quality. The two cell analysis methods used in this study were based on automated detection processes: (i) QuPath built-in Watershed Cell Detection, and (ii) a machine-learning-based approach using the StarDist plugin. Each of these methods was implemented through script-based workflows designed for consistent and accurate identification of cells within annotated epidermal regions. The set parameters were optimized based on visual inspection of cell detection in several sample slides.

The first cell analysis method utilized QuPath's built-in Watershed Cell Detection. In this method, cells were detected by running a plugin script that applied watershed segmentation to separate individual cells based on their optical density. The script parameters were customized to fine-tune the detection: the pixel size was set to 0.5 µm to ensure adequate resolution, and a background radius of 13 µm was defined to enhance contrast between cells and their surroundings. Additionally, a median filter (1.1 µm) was applied to smooth the image and reduce noise, while Gaussian blurring (σ = 1.5 µm) further improved segmentation accuracy. The cells' minimum and maximum size limits were set at 10 and 100 μm^2, respectively, to filter out debris and large artifacts.

The second cell analysis method employed the StarDist deep-learning-based plugin for cell detection (Schmidt et al. 2018), which was specifically tailored for nuclear segmentation in histological images, such as those stained with haematoxylin and eosin. The model used for this detection was trained on images of similar tissue types and included a custom neural network model (*he_heavy_augment.pb*), which was preloaded into QuPath. The StarDist algorithm normalized the input images by adjusting the pixel intensity between the 1st and 99th percentiles to reduce the impact of outliers in staining intensity. The detection threshold was set at 0.45, meaning that only regions with a high probability of representing nuclei were segmented. The pixel size was adjusted to 0.4 µm. This method was computationally more advanced than the watershed-based detection one, relying on neural network inference to handle more complex cell shapes and overlapping structures.

Both methods were applied within the predefined annotated ROI (*Region** – see Scripts in Appendix B). To ensure accuracy, each image's cell count was validated by visualization. Typically, both QuPath's built-in Watershed Cell Detection and StarDist methods produced similar results, and in these cases, the final cell count was determined as the arithmetic mean of both automated methods. However, if one method significantly outperformed the other in terms of accuracy (based on visual inspection), the result from the better-performing method was used as the final count. For a small subset of annotations where neither automated method produced reliable results, manual cell counting was the only method used, and this count was considered the final cell count for that region.

Two indices were calculated based on the annotated epidermal regions and their associated cell counts:

- Epidermal Index 1 (EI1) reflects histological abnormalities that affect cell size, cell number or overall cellular density in a certain area. This index might be affected by atrophy, hyperplasia, hypertrophy, vacuolization, or intracellular oedema/spongiosis, which alter the overall area occupied by cells. Furthermore, it might be influenced by changes in the epidermal area, leading to variations in cellular density. Increases in EI1 indicate a larger average area per cell or reduced cellular density, suggesting either an increase in cellular size or a reduction in cell numbers. In contrast, decreases in EI1 indicate a smaller average area per cell or a higher cellular density, suggesting an increase in the number of cells or a decrease in cell size.

$$\mathrm{EI1} = \frac{Area\ of\ annotation\ (\mu m^2)}{Number\ of\ nuclei\ detected\ (\mathrm{nr})}$$

- Epidermal Index 2 (EI2) serves as an approximation of epidermal thickness and reflects morphological changes affecting epidermal structure and shape regularity. It can capture morphological changes that alter the epidermal structure, such as tissue thickening and surface irregularities (e.g., indented or folded margins). Increases in EI2 indicate greater epidermal thickness and/or a more compact or regular shape. Conversely, decreases in EI2 suggest a thinner and/or more irregular epidermal structure (surface irregularities that disproportionately increase the perimeter relative to area).

$$\mathrm{EI2} = \frac{Area\ of\ annotation\ (\mu m^2)}{Perimeter\ of\ annotation\ (\mu m)/2}$$

4.1.4 Pesticide residue analysis

Pesticide residue analyses were conducted on soils samples of those experiments in which pesticides were applied to soils, with the exception of lambda-cyhalothrin, for which no soil

sample was stored. In addition, liver and skin samples of frogs exposed as part of these experiments (including that with lambda-cyhalothrin) were also analysed for the presence of pesticide residues. Also, and as explained in section 4.1.2.3, sprayed broth levels were confirmed for all tested pesticides except MCPA, mesotrione, acetamiprid and alpha-cypermethrin, whose samples were lost during storage. Pesticide concentrations were determined using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with the exception of lambda-cyhalothrin, which was determined using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS).

4.1.4.1 Sample extraction

Frog sections that were stored for pesticide residue analysis were unfrozen and placed under a magnifying glass to be dissected and collect at least 10 mg skin and liver pieces. Skin and liver pieces were weighed inside 1.5 ml vials and spun down from the walls of these vials using a Quickspin Mini Centrifuge. The average (±SD) weight of collected samples was 97.1±57.9 mg of skin and 66.1±55.1 mg of liver.

For liquid chromatography analyses, 150 µl of acetonitrile and 30 µl of water were added to each sample vial, along with 20 µl of a mixture of deuterated Tebuconazole-d9 and Thiametoxam-d3 (both at a concentration of 0.05 µg/ml in acetonitrile), which were used as internal standards. Then, two steel balls were added to the vials and to grind and homogenize samples using a ball mixer mill (Retsch MM301, Haan, Germany) for 1 minute, followed by 5 minutes of sonication. The liquid phases were recovered after centrifugation for 10 minutes at 10000 rcf and 4°C and transferred to amber glass vials with inserts to be injected into the liquid chromatograph.

Levels of pesticide residues in soil samples from the experimental assays were extracted using similar protocols as frog tissue samples. The soil sample weights were approximately 250 mg, and the extraction solvent volumes were adjusted proportionally to the amount of sample. Final extracts were diluted 1:100 in acetonitrile before injection.

For gas chromatography analyses, the tissue samples were placed in a glass tissue homogenizer with 275 µl of ethyl acetate and 25 µl of triphenyl phosphate at a concentration of 100 mg/l in acetone, the latter being used as internal standard. Samples were ground for 2 minutes and filtered using glass wool and sodium sulphate anhydrous into amber vials with inserts to be injected into the gas chromatograph.

Sprayed broths were diluted in MilliQ-grade water to achieve a theoretical concentration of 0.5 ng/µl. The diluted samples were transferred to amber glass vials and directly analysed by liquid chromatography.

4.1.4.2 Quantification and quality controls

All pesticides but lambda-cyhalothrin were determined using LC-MS/MS with an Agilent UHPLC Serie 1290 Infinity II coupled to a triple quadrupole mass spectrometer (Agilent 6470 LC/TQ). Separation was performed using a Zorbax Eclipse-Plus-C18 column (2.1 × 50 mm, 1.8 µm). Selected chromatographic conditions included a flow of 0.250 ml/min, and a column temperature of 40°C. Elution was performed with (A) H_2O and (B) methanol 2mM, pH 7.6 + 0.1% formic acid. Initial conditions were 95% A and 5% B for 1 min, followed by gradual changes to reach 80% A and 20% B at minute 2, 60% A and 40% B at minute 4, 15% A and 85% B at minute 12, 100% B at minute 15, back to the initial conditions at minute 22 and maintenance of initial conditions until minute 25. Injection volume was 1 µL and vials were kept at 4°C in the autosampler. Calibration standards and samples were analyzed using dynamic multiple reaction monitoring in positive mode, using one transition for detection and at least two additional

transitions for confirmation. The monitored ions for each pesticide along with the retention time and the fragmentation voltages for each transition are shown in Table 9. Capillary voltage was 3500 V, drying gas flow was 8 l/min, drying gas temperature was 300°C, nebulizer pressure was 40 psi, sheath gas temperature was 350, and sheath gas flow was 11 l/min.

Lambda-cyhalothrin was analysed by GC-MS/MS using an Agilent 7890A chromatograph coupled to a 7000A MS triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara). Separation of target compounds was achieved using a HP-5MS Agilent column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness). The injection volume was 1 µl. Chromatographic conditions included a flow of 3 ml/min and a pressure of 12.432 psi. The initial temperature was set at 60 °C and kept for 1 minute, then increased to 170°C at a rate of 40°C/min and then to 310°C at a rate of 10°C/min, with maintenance of 310°C during 3 additional minutes. Lambda-cyhalothrin and the internal standard were identified by retention time and by the specific MS/MS transition (Table 9).

We performed matrix-matched calibrations. Mixed working standards prepared from stock solutions made at 1 mg/ml that were kept at 4°C at five concentration levels (0, 0.75, 1.5, 3 and 6 ng/µl). Working standards were added to control samples of each matrix type. Matrices were then extracted to create calibration curves for the different pesticides. Blank and quality control samples were prepared by fortification of 0.5 g control samples with working solutions at different levels, which were processed in the same way as the samples. Blanks and quality control samples were processed daily to calculate sensitivity and robustness of the analytical technique (% recovery ± RSD) and to obtain the limits of detection (LOD) and quantification (LOQ) for each substance, which are shown in Table 9. These were calculated as LOD = 3.3 * (s/b) and LOQ = 10 * (s/b), where “b” is the calibration curve slope and “s” is the standard deviation of the calibration curve intercept (Evard et al. 2016).

Table 9: Mass spectrometry parameters used for pesticide analysis and quality controls

Chromatographic information includes the retention time (RT) in minutes. Detection information includes the precursor and product ions with their collision energy voltage (CEV) and, for liquid chromatography analysis, the fragmentation voltage. Voltages are given in volts. Quality controls include the limits of detection (LoD) and quantification (LoQ) in ng/g wet weight, as well as the mean recovery (%) and its residual standard deviation (RSD).

Method	Substance	RT	Precursor ion	Fragmentation voltage	Product ions (CEV)				LoD	LoQ	Recovery (%, ±RSD)
LC-MS/MS	Thiametoxam-d3*	6.78	295.1	63	213.7 (12)	184 (24)	90 (56)	131.9 (24)			
	Flupyradifurone	8.15	289.1	136	126 (28)	99 (60)	73 (60)		1.5301	5.1002	119.9 ± 29.7
	Metsulfuron-methyl	8.40	382.1	78	167 (16)	77 (60)	56.2 (44)		0.2178	0.7261	170.7 ± 41.7
	Pirimicarb	11.40	239.1	97	182.1 (16)	109.1 (35)	85.1 (32)	72.1 (24)	0.1694	0.5646	130.0 ± 29.7
	Azoxystrobin	12.65	404.1	93	372 (12)	344.1 (28)	329 (35)		0.1392	0.4639	90.6 ± 25.2
	Isoxaben	13.17	333.2	100	165 (20)	107.1 (60)			0.2034	0.6780	86.6 ± 17.7
	Oxathiapiprolin	13.19	540.2	45	500.1 (28)	350.1 (36)	167 (28)	163 (56)	0.3385	1.1284	107.5 ± 30.0
	Tebuconazole-d9*	14.59	317.2	102	125 (50)	71.1 (21)	70.1 (25)				
	Bnezovindiflupyr	14.60	398.1	102	378 (16)	342 (20)	322 (28)	286.1 (36)	0.3617	1.1284	92.8 ± 16.2
	Tebuconazole	14.63	308.1	100	124.9 (47)	70 (40)	59 (36)		1.0686	3.5619	107.9 ± 10.6
	Metrafenone	15.14	409.1	100	227 (24)	209.1 (28)			0.4215	1.4049	74.6 ± 19.2
	Fluazifop-p-butyl	15.83	384.1	45	328 (16)	282 (20)	254 (32)	91 (40)	0.5945	1.9815	86.0 ± 20.2
	Pendimethalin	16.48	282.2	46	212 (8)	194 (20)	118 (24)	71.1 (16)	0.5861	1.9536	85.4 ± 27.8
GC-MS/MS	Triphenyl phosphate*	13.35	325	100	231 (20)						
			325	100	169 (20)						
	Lambda-cyhalothrin	14.68	208	100	181 (5)				0.0001	0.0004	120.8 ± 16.4
			199	100	161 (5)						
			197	100	161 (5)						

*Used as internal standard.

4.1.5 Data analysis

Cumulative survival during the 21 days following exposure was analysed using Kaplan-Meier survival analysis. Differences in cumulative survival among pairs of treatments were analysed using Log-Rank tests. In addition, individual survival differences among treatments on either day 7 or day 21 post exposure were analysed using GzLM using binomial distributions of the dependent variable. Body conditions on days 7 or 21 were analysed also with GzLM using continuous distributions of the dependent variables. Prior to analyses, normality of body condition variables was checked and those variables not fitting to a normal distribution were adjusted to a gamma distribution with logarithmic link for GzLM.

Both survival and body condition models included the pesticide treatment as fixed factor. In addition, for pesticides that were tested using individuals from different batches, the interaction of pesticide treatment with the batch of origin of the animals was included as an additional factor in the models. For body condition analysis, the body condition at the beginning of the experiment and the FIR were added as covariates to the models (although FIR was not considered in the analysis of experiments conducted during 2023 because it was similar for all frogs from those experiments). Then the interactions initial body condition*treatment and initial body condition*FIR*treatment were added to the models. All models initially incorporated all the considered terms (i.e. factors, covariates and interactions). Then, a model selection was performed by removing those factors or covariates providing non-significant effects or interactions, except for pesticide treatment that always remained in the models. The final model selection was performed based on the lowest Akaike's Information Criterion value. Specific differences among treatments were confirmed using pairwise comparison through the Least Significant Difference method.

Histological data from the same active ingredient were included in a single analysis, regardless of whether they came from exposure to the active ingredient or the formulation. As no significant differences between control batches were observed in EI1 or EI2, controls were pooled for the statistical analysis of histological data. EI1 and EI2 data were checked for normality using Kolmogorov-Smirnov tests. For each active ingredient (co-formulants were not analysed for histopathology), the effects of the overspray treatment on either EI1 or EI2 were checked using GzLM with the response variables (epidermal indices) adjusted to a linear (if normal) or a gamma (if non-normal distribution). Specific treatments among which histological differences existed were checked through the comparison of GzLM's marginal means using the Least Significant Difference procedure.

For body residue analysis, samples with concentrations below the limit of detection were assigned a value equal to half the limit of detection for the corresponding pesticide. Pesticide levels were log-transformed and used as dependent variable in a GzLM whose fixed factors included sampling day (day 7 or day 21), tissue (liver or skin) and treatment. In addition, for those substances that were tested as active ingredient or as formulation, the presentation format (active ingredient or formulation) was also included as a factor in the GzLM. The GzLM was run using the factorial approach, with all individual terms and interactions being included in the analysis.

4.2 Results

4.2.1 Apical endpoints

Detailed descriptive results for sublethal effects (i.e. body condition and FIR) as well as best-fitted GzLM to explain the effects of pesticide treatments on *P. perezi* body condition on days 7 and 21 after treatment are shown in Appendix C.

4.2.1.1 Active ingredients tested by overspray only

The active ingredients tested by overspray only (excluded those that were included in the comparisons with their formulations) were oxathiapiprolin, MCPA, metsulfuron-methyl, mesotrione, pirimicarb and acetamiprid.

4.2.1.1.1 *Oxathiapiprolin*

Oxathiapiprolin experiment was conditioned by a high mortality among both negative and solvent controls (40%) that happened from day 12 post-exposure onwards. Despite of this effect, the mortality caused by the pesticide treatments was higher than of non-exposed animals (Figure 12), although the differences among treatment levels were not significant (model for survival on day 21: Wald's X^2=2.107, 4 d.f., p=0.716).

A reduction in body condition of frogs exposed to the two highest oxathiapiprolin levels, as well as to acetone control, was noted relative to negative control frogs on day 7 (Figure 13). By contrast, on day 21 the body condition of frogs exposed to the recommended application rate of the pesticide was higher than that of frogs exposed to the rest of the treatments. This would suggest that mortality of exposed frogs occurring during the second part of the experiment would have affected mostly the smaller individuals. The differences in body condition on day 7 were explained by the body condition at the beginning of the experiment, while those on day 21 were explained by the food intake rate (Appendix C.2).

Figure 12: Cumulative survival of *Pelophylax perezi* juveniles exposed to oxathiapiprolin

Source: own illustration, [IREC-CSIC].

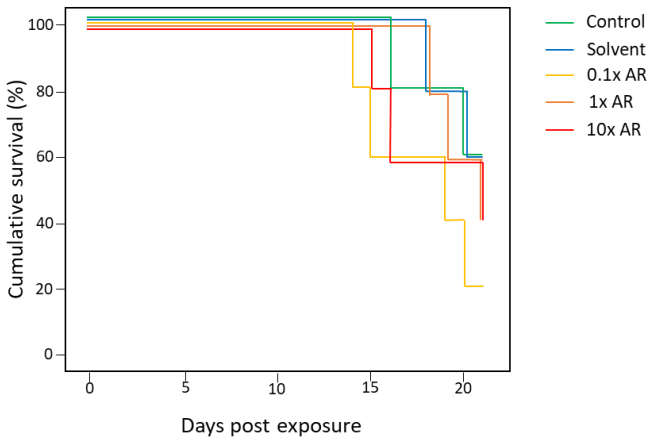
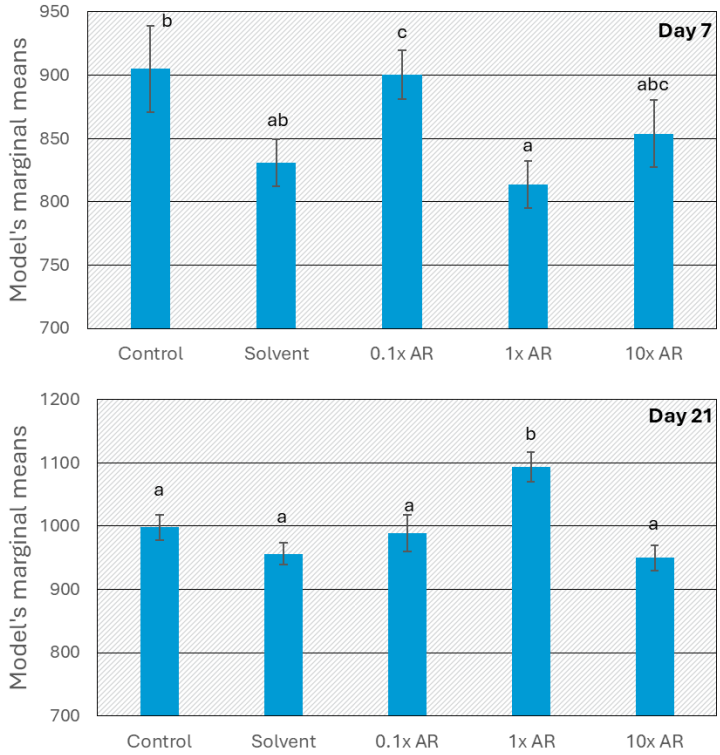


Figure 13: Body condition of *Pelophylax perezi* juveniles exposed to oxathiapiprolin on days 7 (top) and 21 (bottom) after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].

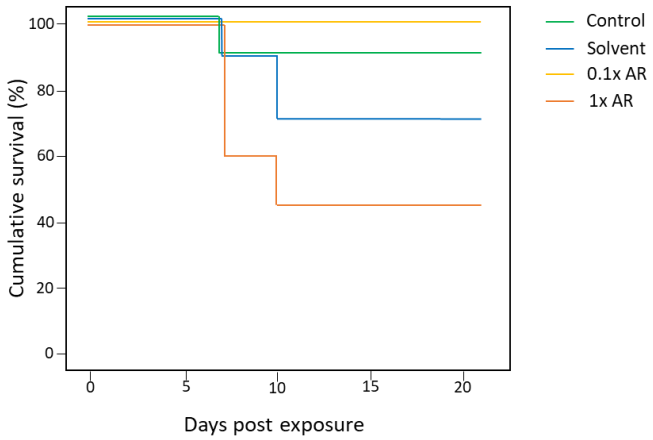


4.2.1.1.2 *MCPA*

Exposure to MCPA did not affect survivorship of animals, despite an increase of mortality associated with the solvent control (Figure 14). The herbicide caused no effects on body condition.

Figure 14: Cumulative survival of *Pelophylax perezi* juveniles exposed to MCPA

Source: own illustration, [IREC-CSIC].



4.2.1.1.3 *Metsulfuron-methyl*

Metsulfuron-methyl was tested at both laboratories. No significant lethal effects were observed in any case ($p \geq 0.414$), despite the increased cumulative mortality recorded at both laboratories in animals exposed to the 10x AR treatment (Figure 15).

Frogs exposed to metsulfuron-methyl showed increased body condition relative to the initial values when compared with controls. On day 7, this was especially evident for frogs exposed to 10x AR at UA (Figure 16) and those exposed to 1x AR at IREC, although the lowest overspray treatment also increased body condition among frogs treated at IREC (Figure 17). The selected models show an interaction of body condition at the beginning of the experiment and food intake rate in determining treatment effects on body condition on day 7 at IREC, while at UA an interaction between treatment and initial body condition was observed (Appendix C.2). On day 21, body condition could not be analysed in the IREC experiment due to low sample size of some treatments, whereas at UA no differences among treatments were observed.

Figure 15: Cumulative survival of *Pelophylax perezi* juveniles exposed to metsulfuron-methyl at both laboratories

Source: own illustration, [IREC-CSIC].

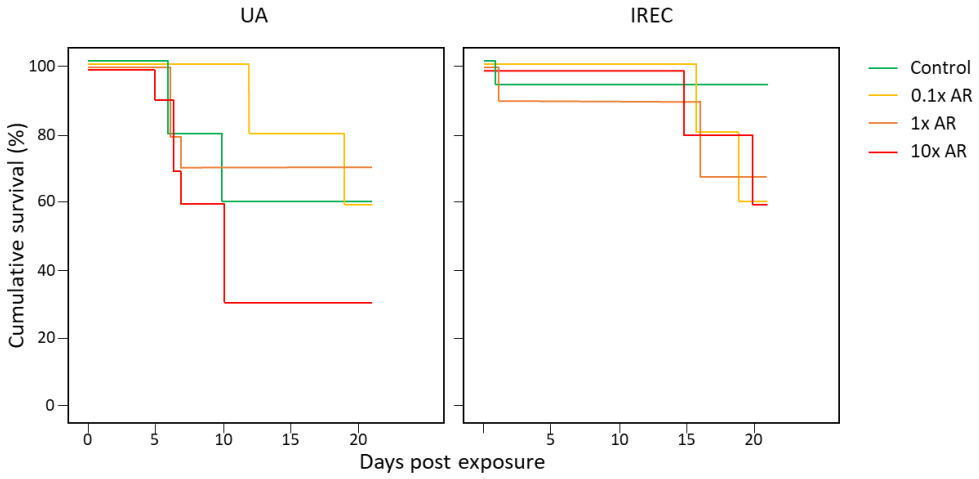


Figure 16: Body condition of *Pelophylax perezi* juveniles exposed to metsulfuron-methyl at UA lab on day 7 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].

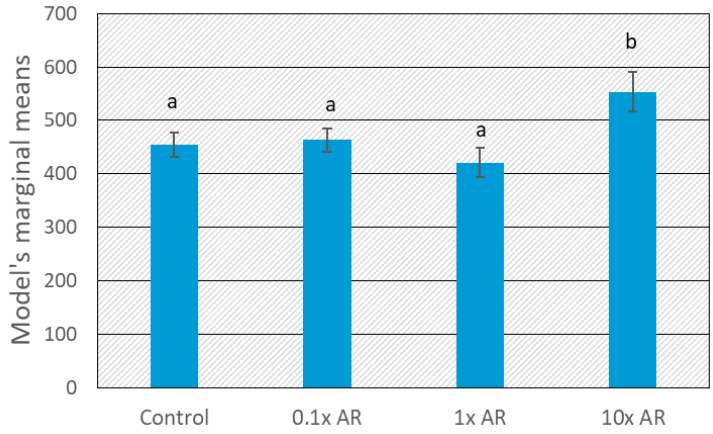
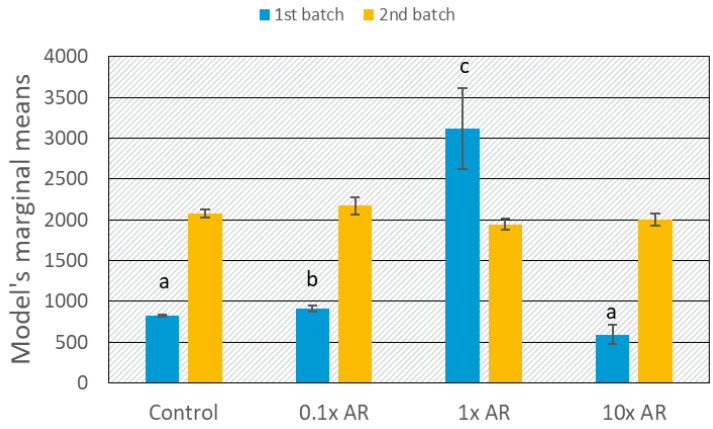


Figure 17: Body condition of *Pelophylax perezi* juveniles exposed to metsulfuron-methyl at IREC lab on day 7 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].



4.2.1.1.4 *Mesotrione*

Mesotrione caused no significant lethal effects to frogs (Figure 18) but reduced their body condition on day 7 (Figure 19). The selected model included a significant interaction with the body condition at the beginning of the trials (Appendix C.2). Exposure to any of the tested concentrations reduced body condition of frogs at day 7 compared to controls, among which the solvent one (mixture of acetone and Triton®,) resulted in an increased body condition as compared to the negative control 7 days after overspray. On day 21, no differences among treatments were noticed in body condition of frogs (Figure 19).

Figure 18: Cumulative survival of *Pelophylax perezi* juveniles exposed to mesotrione

Source: own illustration, [IREC-CSIC].

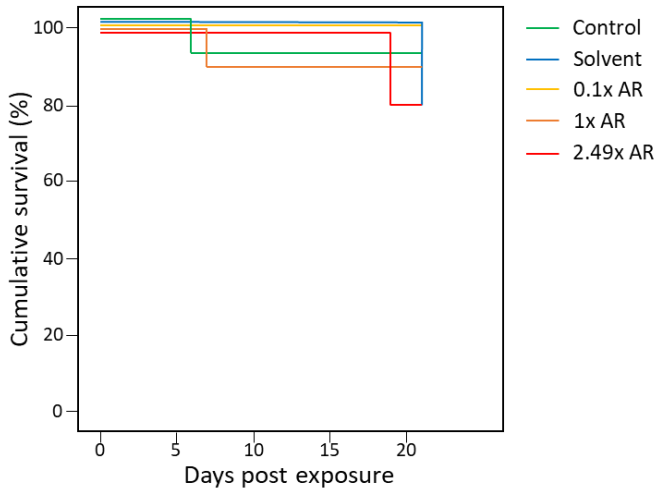
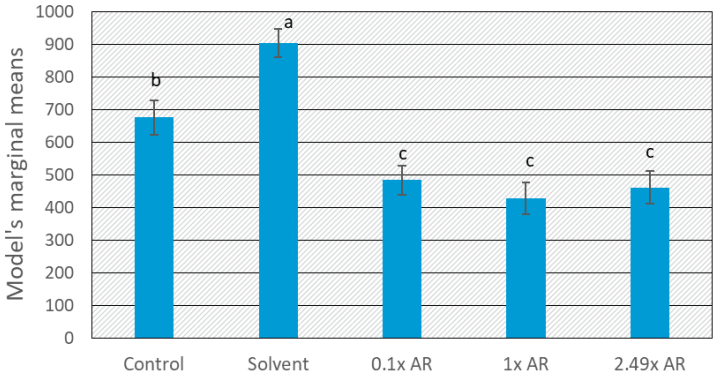


Figure 19: Body condition of *Pelophylax perezi* juveniles exposed to mesotrione on day 7 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].



4.2.1.1.5 *Pirimicarb*

A reduction of cumulative survival was detected in frogs treated with pirimicarb via overspray (Figure 20), although this effect was statistically significant only at the treatment corresponding to 1x AR (X^2=5.055; p=0.025). The highest application dose (10x AR), even if also reduced survival, did not cause such a significant effect (X^2=1.184; p=0.277). The GzLMs confirmed that lethal effect of pirimicarb happened on day 7 (Wald's X^2=9.920, 3 d.f., p=0.027), but not on day 21 (Wald's X^2=3.097, 3 d.f., p=0.377).

Body condition on day 7 was higher among frogs exposed to 0.1x AR than for any other treatment (Figure 21). On day 21, body condition compared to controls was higher in frogs exposed to 10x AR and lower in frogs exposed to 1x AR (Figure 21). The selected models revealed a significant influence of the initial body condition in the effect of treatments on body condition measured on both day 7 and day 21. In addition, on day 21, a significant interaction with the food intake rate was also observed (Appendix C.2).

Figure 20: Cumulative survival of *Pelophylax perezi* juveniles exposed to pirimicarb

Source: own illustration, [IREC-CSIC].

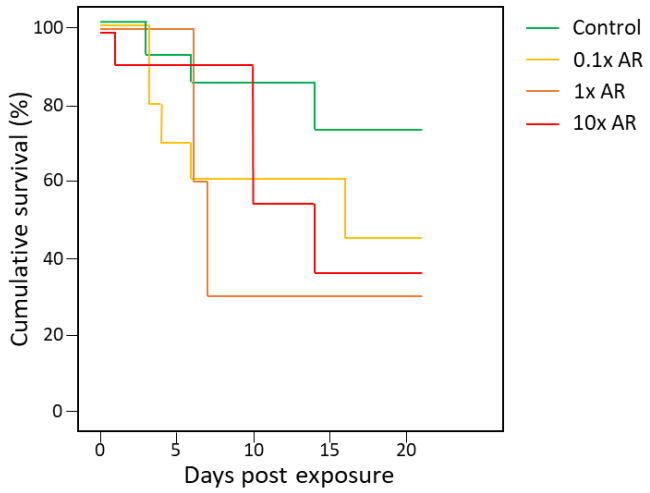
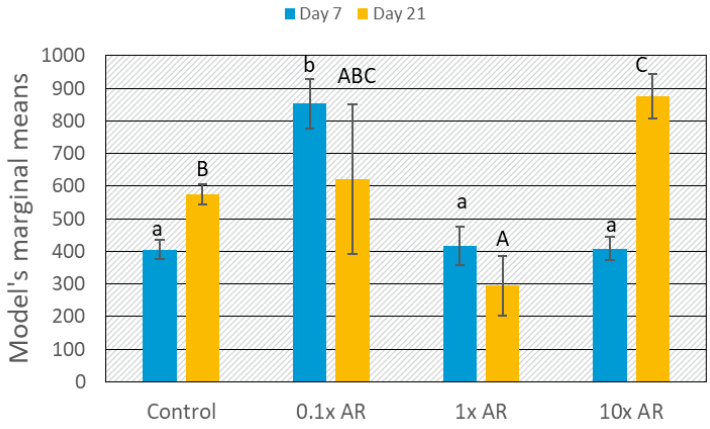


Figure 21: Body condition of *Pelophylax perezi* juveniles exposed to pirimicarb on days 7 and 21 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis (lower case for day 7 data, caps for day 21 data). Source: own illustration, [IREC-CSIC].

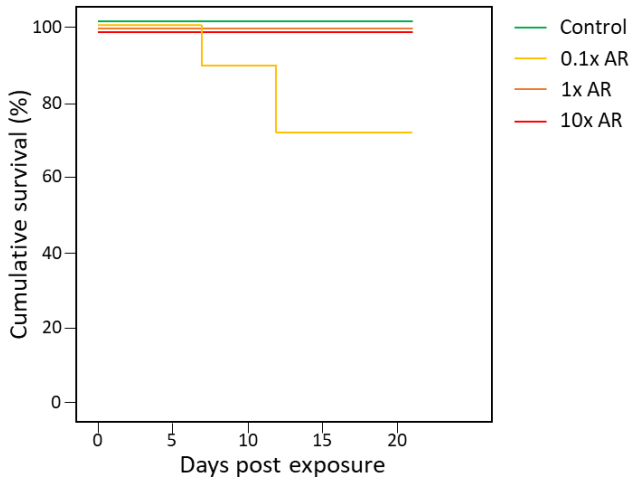


4.2.1.1.6 *Acetamiprid*

Acetamiprid caused no lethal effects (Figure 22) or affected body condition of the exposed frogs. Only a marginally significant reduction of the cumulative survival was noticed among individuals exposed to the 0.1x AR treatment (X2=2.8; p=0.094).

Figure 22: Cumulative survival of *Pelophylax perezi* juveniles exposed to acetamiprid

Source: own illustration, [IREC-CSIC].



4.2.1.2 Active ingredients tested by overspray and contact with contaminated substrate

The active ingredients tested by overspray only (excluded those that were included in the comparisons with their formulations) were benzovindiflupyr, isoxaben, pendimethalin and lambda-cyhalothrin.

4.2.1.2.1 *Benzovindiflupyr*

Benzovindiflupyr caused no significant effects on survival (Figure 23). Body condition on day 7 was affected by the interaction between the treatment and the initial body condition, but not by the treatment itself (Appendix C.2). On day 21, however, we observed an effect of the treatment alone, and in interaction with the initial body condition and with the food intake rate, on frogs' body condition (Appendix C.2). Body condition of exposed animals was increased at all treatments relative to both negative and solvent controls (Figure 24).

Figure 23: Cumulative survival of *Pelophylax perezi* juveniles exposed to benzovindiflupyr

Source: own illustration, [IREC-CSIC].

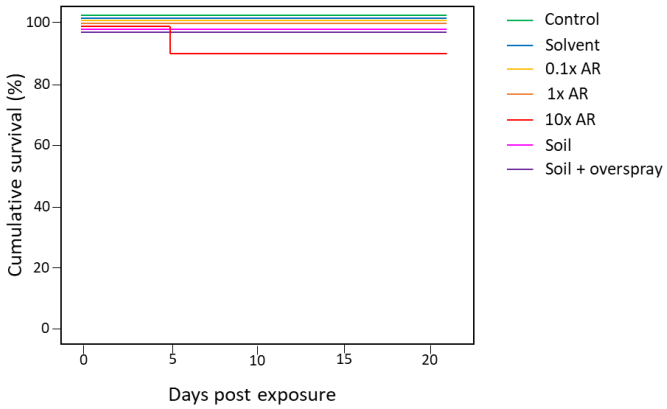
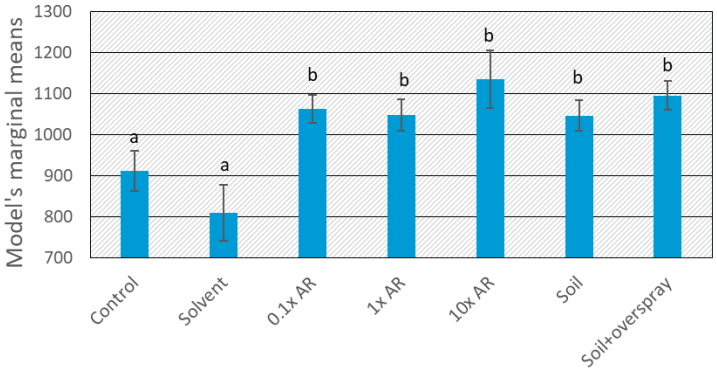


Figure 24: Body condition of *Pelophylax perezi* juveniles exposed to benzovindiflupyr on day 21 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].



4.2.1.2.2 *Isoxaben*

The combined exposure through soil and especially the combination of overspray and soil contact routes caused a significant increase of mortality compared to controls (Figure 25). Although the GzLM was not significant overall (Wald's X^2=2.495, 5 d.f., p=0.777), pairwise comparisons showed significant different of these treatments compared to controls on days 21 (for exposure through contact soil only, p=0.014) and 7 (for the combination of exposure routes, p=0.010).

Significant models for isoxaben effects on body condition were found on days 7 and 21 after exposure (Appendix C.2). Body condition of animals exposed to isoxaben through contact with treated soils or via overspray with the application rate was significantly higher than for control frogs on day 7 of the experiment. On day 21, increased body condition was observed for frogs exposed to the lowest overspray treatment (Figure 26).

Figure 25: Cumulative survival of *Pelophylax perezi* juveniles exposed to isoxaben

Source: own illustration, [IREC-CSIC].

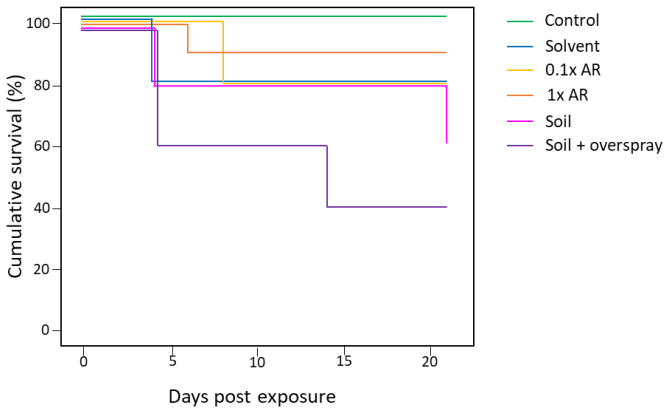
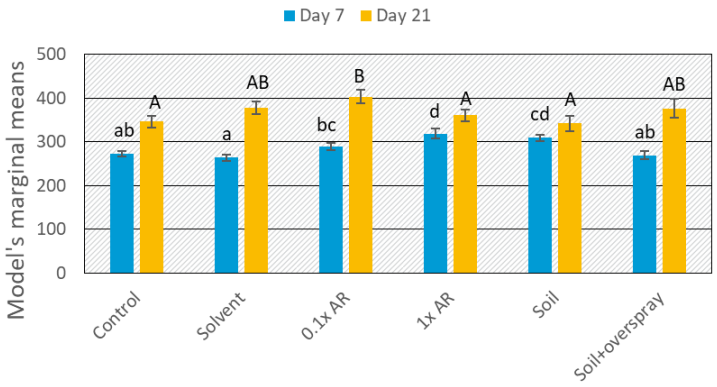


Figure 26: Body condition of *Pelophylax perezi* juveniles exposed to isoxaben on days 7 and 21 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].



4.2.1.2.3 *Pendimethalin*

Pendimethalin caused no significant effects on survival (Figure 27). Body condition on day 7 was reduced in individuals exposed through soil contact, both at the soil only and at the soil+overspray treatments (Figure 28). This effect was significant when comparing treatments with the negative control, but not when comparing them with the solvent control. The selected model revealed that the treatment effect was significant only when the interaction with initial body condition and food intake rate was considered (Appendix C.2). Body condition on day 21 was reduced at the same two treatments as above, but also lower at the solvent treatment respect the negative control (Figure 28). The selected model in that case also included a significant effect of the treatment alone (Appendix C.2).

Figure 27: Cumulative survival of *Pelophylax perezi* juveniles exposed to pendimethalin

Source: own illustration, [IREC-CSIC].

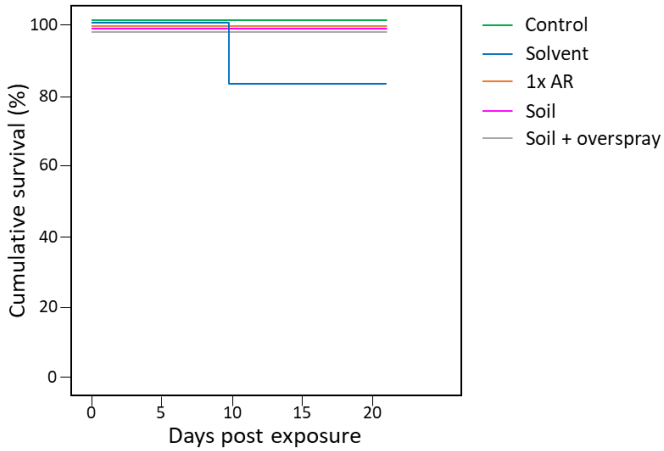
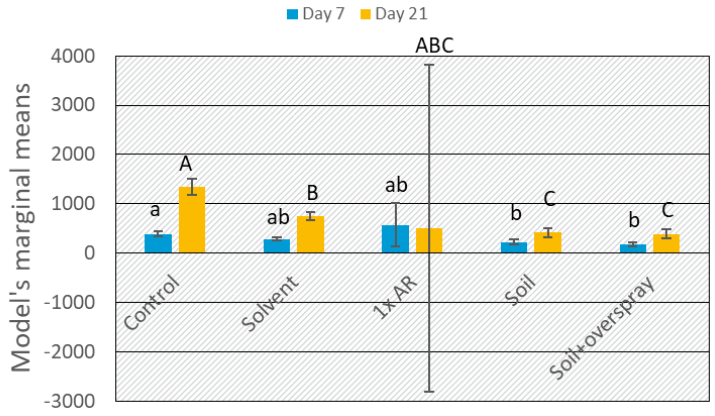


Figure 28: Body condition of *Pelophylax perezi* juveniles exposed to pendimethalin on days 7 and 21 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis (lower case for day 7 data, caps for day 21 data). Source: own illustration, [IREC-CSIC].



4.2.1.2.4 *Lambda-cyhalothrin*

The survival analysis showed that the combined application of lambda-cyhalothrin to frogs and soil caused a less-than-significant (p=0.083) reduction in survival of individuals relative to controls, being this different significant (p=0.018) when survival under that treatment was compared to that of the solvent control individuals (Figure 29). Although the overall results of the GzLM did not show significant lethal effects either on day 7 (Wald's X^2=2.833, 5 d.f., p=0.726) or on day 21 (Wald's X^2=0.027, 5 d.f., p=1.000), pairwise comparisons of the analysis conducted on day 7 confirmed the results from the survival analysis, with a less-than-significant difference in mortality occurrence between controls and animals exposed via overspray and soil contact (p=0.069) and a significant difference when exposed animals were compared with solvent controls (p=0.010).

A reduction in body condition of frogs exposed to the two lowest overspray rates of lambda-cyhalothrin was noted relative to negative control frogs on day 21 (Figure 30). This differences in body condition on day 21 were explained by the body condition at the beginning of the experiment (Appendix C.2).

Figure 29: Cumulative survival of *Pelophylax perezi* juveniles exposed to lambda-cyhalothrin

Source: own illustration, [IREC-CSIC].

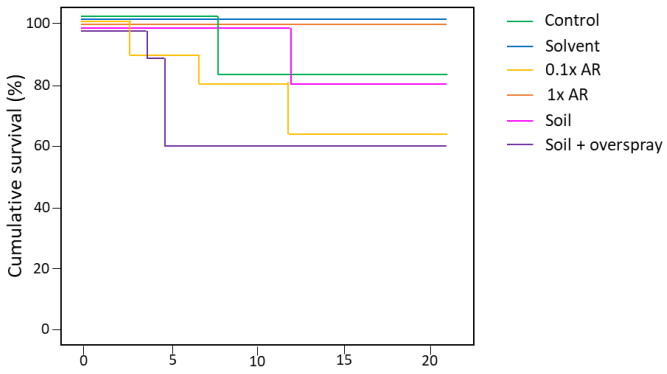
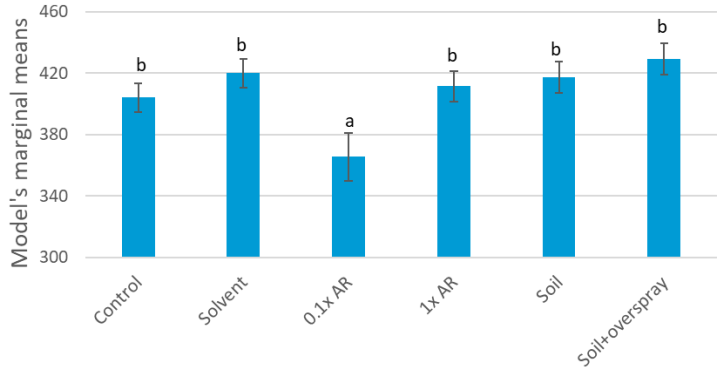


Figure 30: Body condition of *Pelophylax perezi* juveniles exposed to lambda-cyhalothrin on day 21 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].



4.2.1.3 Comparison of active ingredients and formulations

4.2.1.3.1 *Metrafenone and Vivando*

Neither metrafenone nor its commercial formulation Vivando, caused significant lethal effects to frogs (Figure 31). Exposure to metrafenone affected body condition of animals on day 21 after exposure; the selected model retained all the initially introduced terms (Appendix C.2). Control frogs showed a higher body condition than those exposed to the two lowest pesticide levels but lower than that of frogs exposed to 10 x AR (Figure 32). Unlike the active ingredient alone, body condition was unaffected by the exposure to Vivando.

Figure 31: Cumulative survival of *Pelophylax perezi* juveniles exposed to alpha-cypermethrin and to its formulation Fasthrin 10 EC

Source: own illustration, [IREC-CSIC].

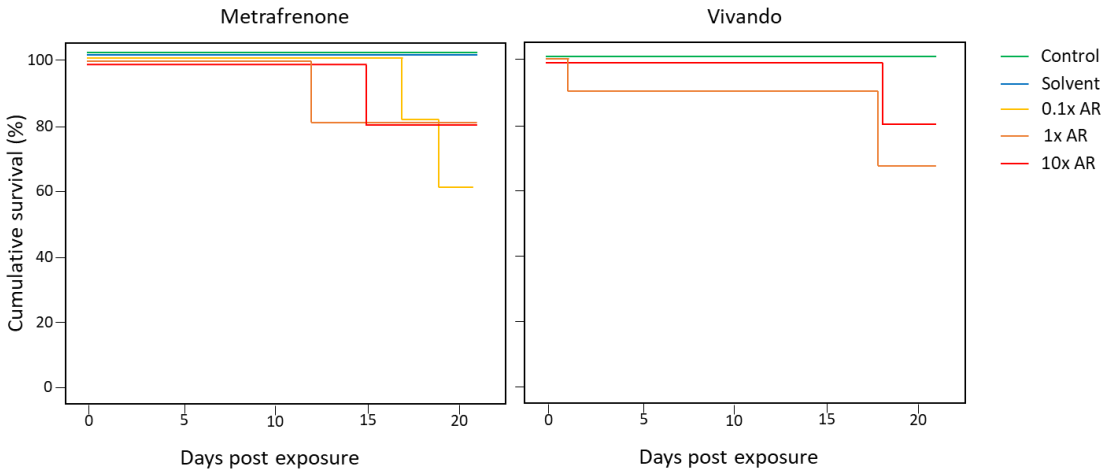
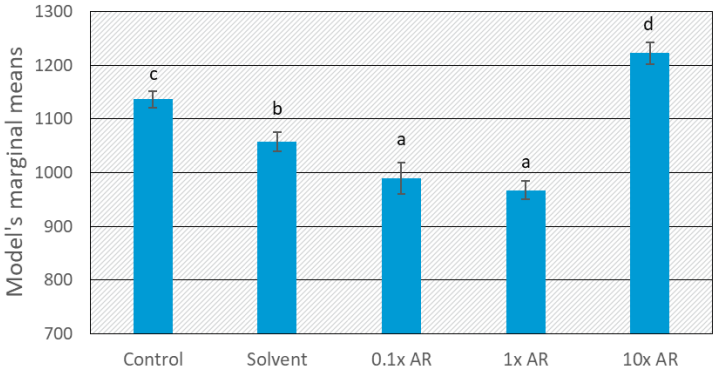


Figure 32: Body condition of *Pelophylax perezi* juveniles exposed to metrafenone active ingredient on day 21 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].



4.2.1.3.2 *Azoxystrobin and Quadris*

Neither the active ingredient azoxystrobin nor its formulation Quadris caused significant effects to frog survival (Figure 33) or body condition on day 7. However, a less-than significant increase in cumulative mortality was observed for animals exposed to the application rate of Quadris on day 21 (X^2=3.559; p=0.059).

On day 21 after exposure, body condition of animals exposed to the active ingredient was higher at the overspray treatment corresponding to 5.44x AR but lower in those animals exposed simultaneously through overspray and soil (Figure 34). The selected model showed significant effects of the treatment, as well as of their interaction with the initial body condition and with the initial body condition*food intake rate (Appendix C.2). In the case of the formulation, no effects on body condition were observed on day 21 either, although it must be noticed that the active ingredient and the formulation were tested on different batches. This could add a source of variability to the effects, despite of which the differences in effects on body condition between azoxystrobin and Quadris have been quite limited.

Figure 33: Cumulative survival of *Pelophylax perezi* juveniles exposed to alpha-cypermethrin and to its formulation Fasthrin 10 EC

Source: own illustration, [IREC-CSIC].

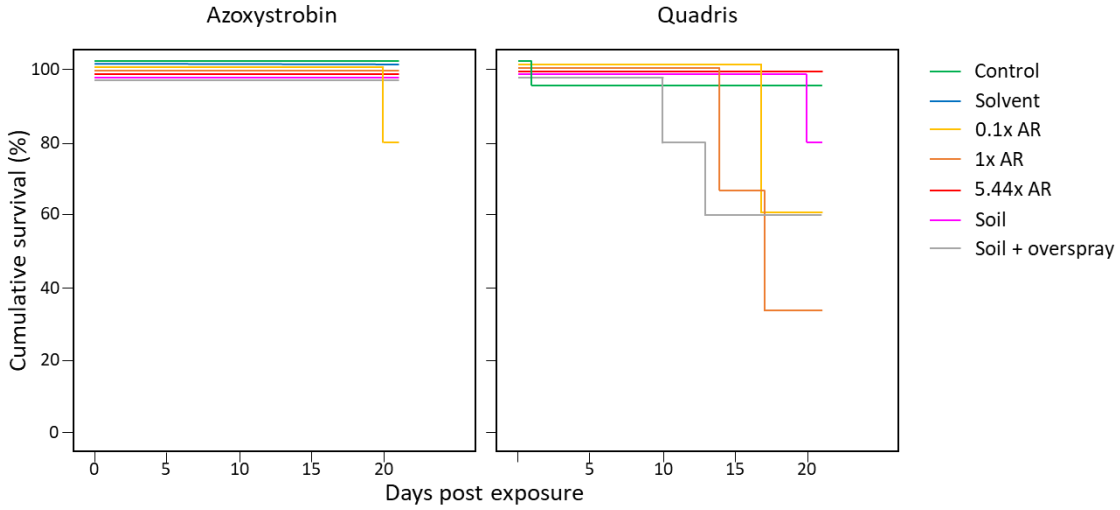
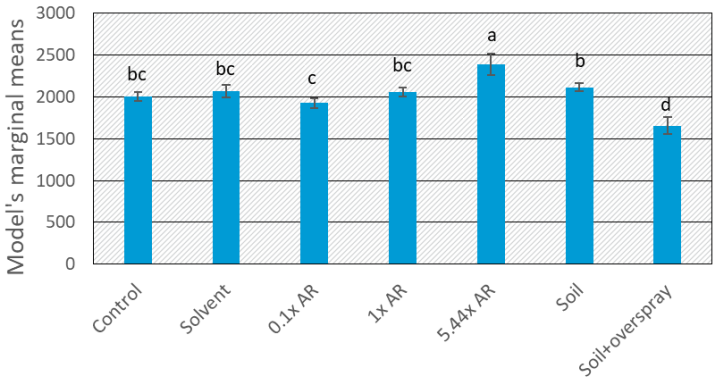


Figure 34: Body condition of *Pelophylax perezi* juveniles exposed to azoxystrobin active ingredient on day 21 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis (lower case for day 7 data, caps for day 21 data). Source: own illustration, [IREC-CSIC].



4.2.1.3.3 *Tebuconazole and Folicur 25 EW*

At the IREC laboratory, two different batches of animals were used to test both the active ingredient tebuconazole and its formulation Folicur 25 EW. This was preferred over using different batches for the formulation and the active ingredient, which would have impaired the comparisons. In addition, Folicur 25 EW was tested also at the UA laboratory.

The active ingredient caused no significant mortality to exposed frogs (Figure 35). However, the formulation Folicur 25 EW reduced cumulative survival, although with different outputs in the two laboratories (Figure 36). At IREC, significantly reduced survival was noted in frogs exposed to the application rate either as overspray (X^2=6.995; p=0.008) or through soil contact (X^2=7.709; p=0.005), but not in the combined treatment or in the highest overspray application rate (although survival in this combined treatment was clearly lower than that of controls). GzLMs showed that increased mortality was not significant on either day 7 (Wald's X^2=3.784, 5

d.f., p=0.581) or day 21 after exposure (Wald's X^2=7.548, 5 d.f., p=0.183). At UA, cumulative survival was reduced after the combined exposure to soil and overspray (X^2=19.000; p<0.001), but 100% mortality occurred before day 7, hence it was not possible to confirm through GzLM.

Figure 35: Cumulative survival of *Pelophylax perezi* juveniles exposed to tebuconazole active ingredient

Source: own illustration, [IREC-CSIC].

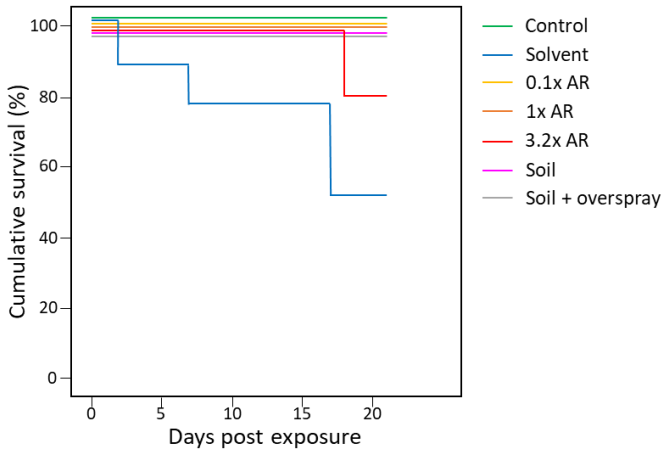
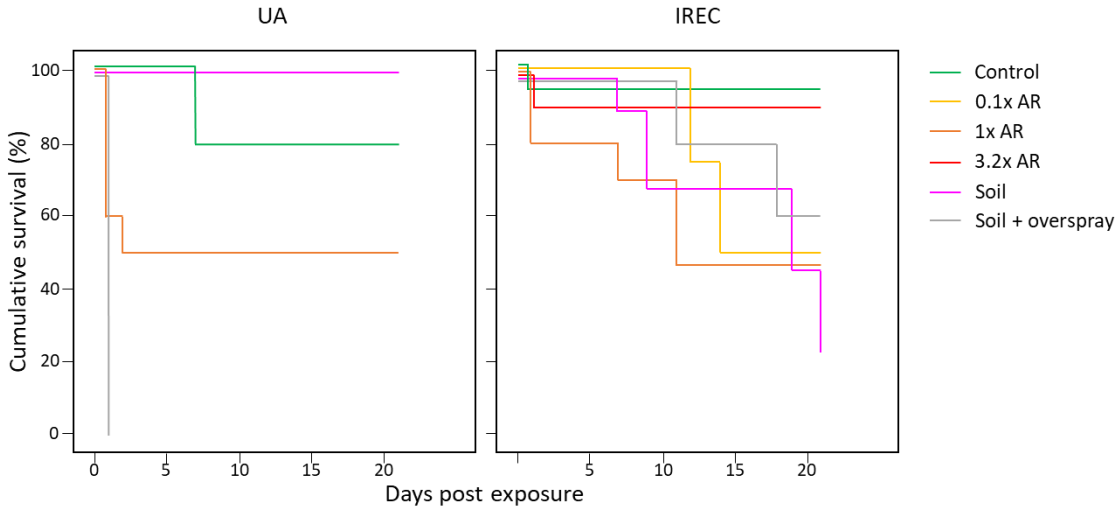


Figure 36: Cumulative survival of *Pelophylax perezi* juveniles exposed to the tebuconazole formulation Folicur 25 EW at both laboratories

Source: own illustration, [IREC-CSIC].



The solvent control was excluded from body condition analysis of tebuconazole as active ingredient because the body condition was considered for the second batch of animals while acetone was tested on first batch animals only. For the active ingredient experiment, body condition on day 7 was slightly lower at 0.1x and 1xAR, and less-than-significantly lower (pairwise comparison's p-value <0.1) at 3.2xAR and at the combination of soil+overspray exposure (Figure 37). The selected model revealed a close-to-significant effect of the treatment alone, with no effects of the interactions with the initial body condition and the food intake rate (Appendix C.2). On day 21, body condition of individuals exposed to the active ingredient did not differ among treatments.

For the formulation experiments, body condition on day 7 at IREC was reduced after exposure to overspray with 1x and 3.2x the application rate, as well as after combined exposure through soil and overspray (although only among individuals from the first batch, Figure 38). In these animals, the selected model showed that only when the initial body condition was taken into account, the effect of the treatment was significant (Appendix C.2). At this exposure time, no treatment-related differences in body condition were noted in either the second batch at IREC or at UA.

On day 21, exposure to Folicur 25 EW resulted in increased body condition with respect to controls in both laboratories, although the treatments causing such effects were not fully coincident because of differences between laboratories in experimental outputs. Thus, increased body condition was found at 1x AR, 3.2x AR and the combined soil-overspray treatment at IREC (second batch animals, Figure 38). The only treatment out of this three that was tested at UA (1x AR) also resulted in increased body condition, as did also the exposure through soil (Figure 39). The soil+overspray treatment was not analysed for body condition at UA because of 100% mortality (Figure 36) while the 3.2x AR was not tested at UA because of experimental constrictions. The selected models revealed significant interactions with both the initial body condition (at both IREC and UA) and the combination of initial body condition and food intake rate (only at UA) in the determination of body condition differences among treatments on day 21.

Figure 37: Body condition of *Pelophylax perezi* juveniles exposed to tebuconazole active ingredient on day 7 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different ($p<0.05$) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].

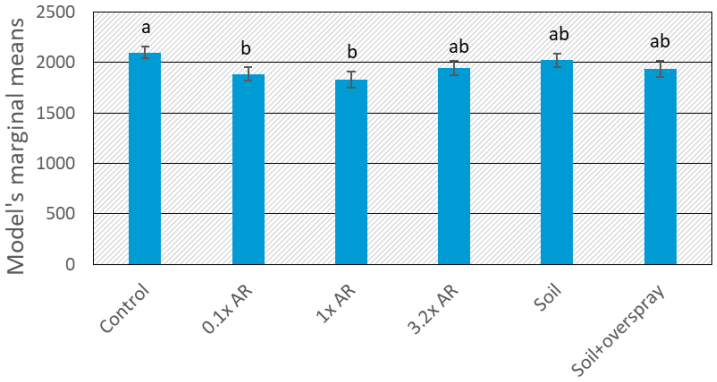


Figure 38: Body condition of *Pelophylax perezi* juveniles exposed to Folicur 25 EW at IREC lab on days 7 (top) and 21 (bottom) after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis (lower case for day 7 data, caps for day 21 data). Source: own illustration, [IREC-CSIC].

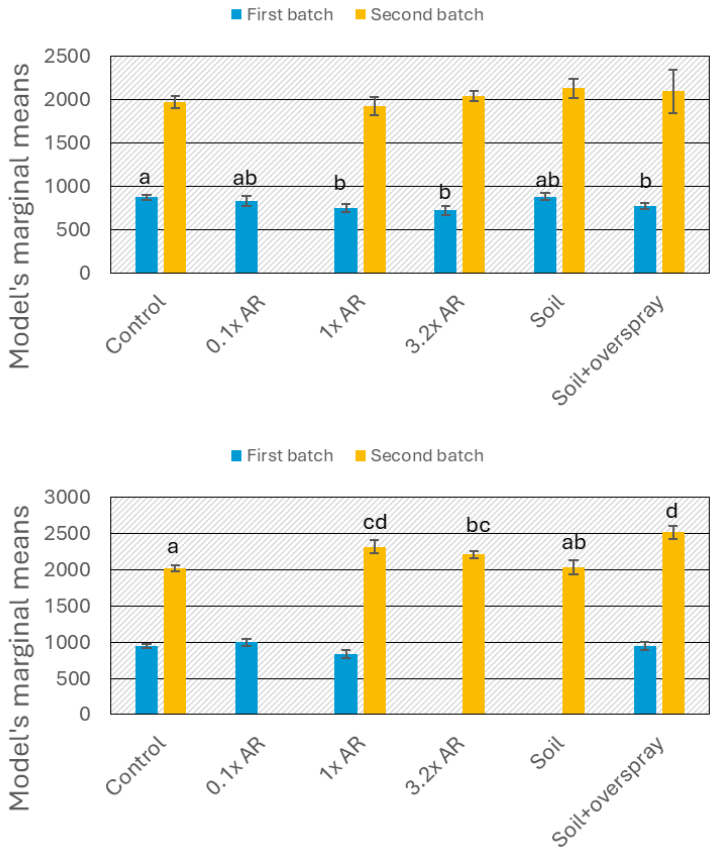
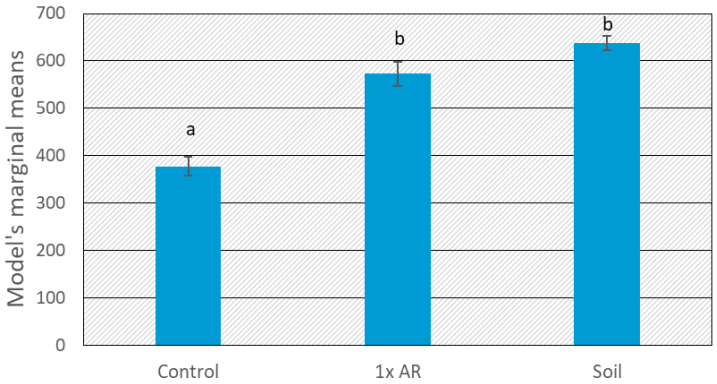


Figure 39: Body condition of *Pelophylax perezi* juveniles exposed to Folicur 25 EW at UA lab on day 21 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].



4.2.1.3.4 *Fluazifop-p-butyl and Fusilade Max*

Neither fluazifop-p-butyl nor its commercial formulation, Fusilade Max, caused significant lethal effects to frogs (Figure 40).

Exposure to Fusilade Max affected body condition of animals on day 21 after exposure (Appendix C.2). Control frogs showed a higher body condition than those exposed to the formulation application rate via overspray (Figure 41). In the case of the active ingredient alone, body condition was increased following 21 days of overspray with the lowest tested application rate of fluazifop-p-butyl (Figure 41) compared to controls, which could indicate that the few mortality cases registered under this treatment would have differentially affected the smaller individuals.

Figure 40: Cumulative survival of *Pelophylax perezi* juveniles exposed to fluazifop-p-butyl and to its formulation Fusilade Max

Source: own illustration, [IREC-CSIC].

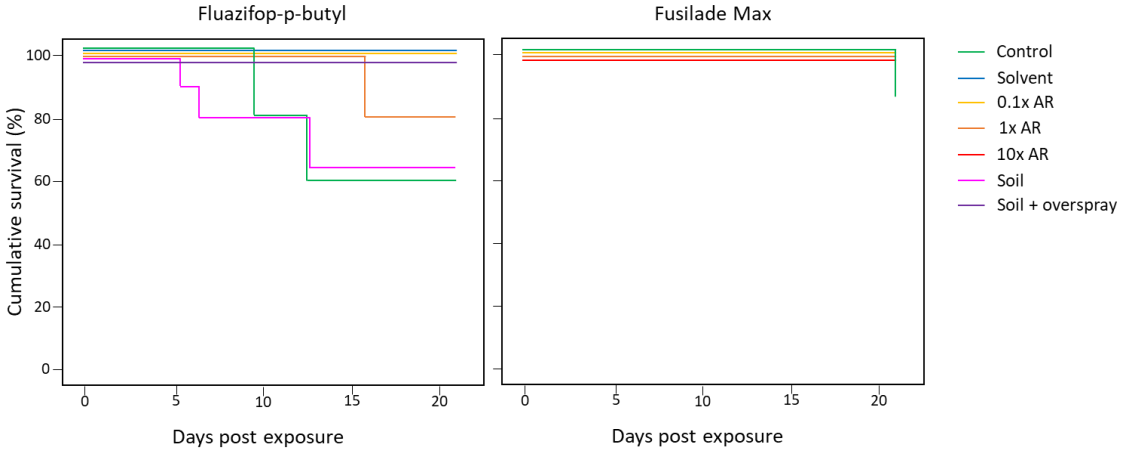
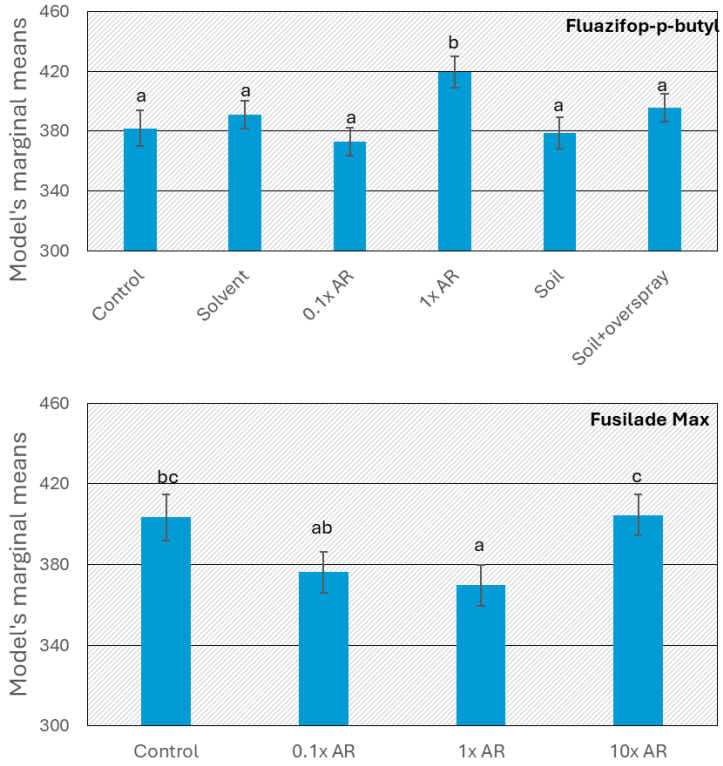


Figure 41: Body condition of *Pelophylax perezi* juveniles exposed to fluazifop-p-butyl (top) and to its formulation Fusilade Max (bottom) on day 21 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].

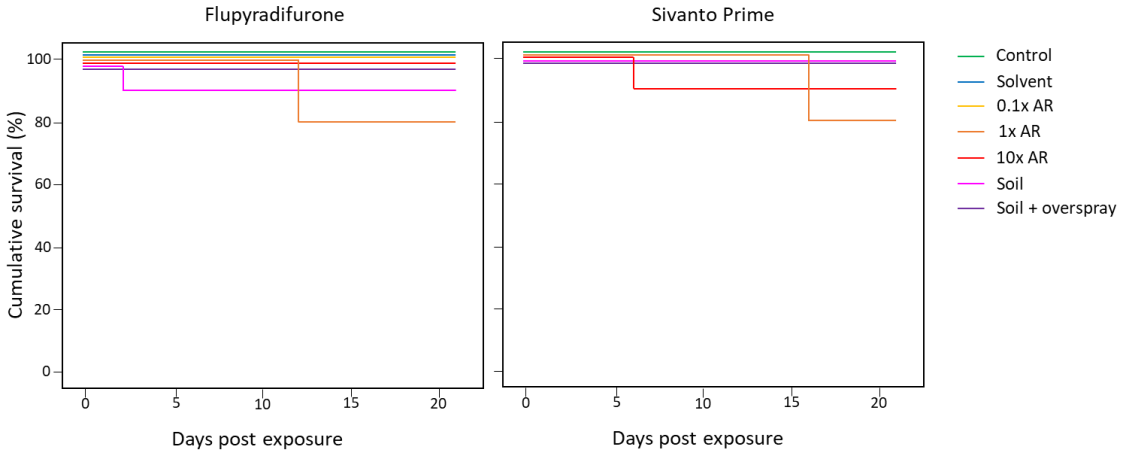


4.2.1.3.5 *Flupyradifurone and Sivanto Prime*

Neither the active ingredient flupyradifurone nor its formulation Sivanto Prime caused significant effects to frog survival (Figure 42).

Figure 42: Cumulative survival of *Pelophylax perezi* juveniles exposed to flupyradifurone and to its formulation Sivanto Prime

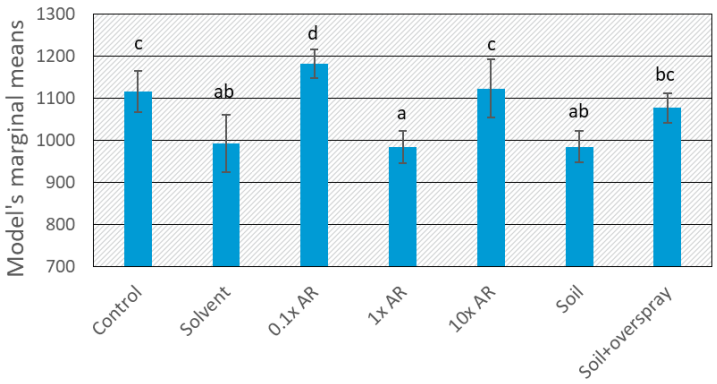
Source: own illustration, [IREC-CSIC].



Body condition of animals exposed to the active ingredient was affected by the treatment on day 21 post-exposure. The model retained also the interactions with initial body condition and with food intake rate as significant terms (Appendix C.2). However, the direction of the effect was unclear; compared to controls, frogs exposed by overspray showed better condition at 0.1x AR and poorer condition at 1x AR, while soil exposed frogs also had a lower condition than controls. By contrast, neither the highest overspray treatment nor the combination of the exposure routes caused any effect on body condition relative to controls (Figure 43). Unlike the active ingredient alone, body condition was unaffected by the exposure to Sivanto Prime.

Figure 43: Body condition of *Pelophylax perezi* juveniles exposed to flupyradifurone active ingredient on day 21 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].



4.2.1.3.6 *Alpha-cypermethrin and Fasthrin 10 EC*

The exposure to the active ingredient alpha-cypermethrin increased accumulated mortality over time, especially at the highest overspray treatment level (Figure 44). However, such increase was not statistically significant (X^2=0.943; p=0.332). On the contrary, cumulative survival of frogs exposed to the alpha-cypermethrin-based formulation Fasthrin 10 EC was reduced after overspray with 0.1x (X^2=5.822; p=0.016) and 10x (X^2=5.248; p=0.022) the application rate (Figure 44). GzLMs showed that increased mortality was not significant on day 7 after exposure (Wald's X^2=4.194, 3 d.f., p=0.241), while was close to significance on day 21 (Wald's X^2=6.613, 3 d.f., p=0.085).

Body condition on day 7 was increased in individuals exposed to the active ingredient at the application rate, but only when compared to the negative control, and no differences were found when comparing treated frogs with the solvent treatment (Figure 45). Likewise, exposure to Fasthrin increased body condition on day 7 of frogs exposed to 0.1x and 10x the application rate (Figure 45). The selected models showed a significant interaction between the treatment and the initial body condition for the body condition effects of alpha-cypermethrin, and a significant interaction between the treatment, the initial body condition and the food intake rate for both the active ingredient and the formulation (Appendix C.2).

On day 21, body condition was reduced by the treatment corresponding to the application rate in frogs exposed to the active ingredient (Fig. 10), while no differences relative to controls were observed in body condition on day 21 for any of the Fasthrin 10 EC treatments (Figure 45). The

selected models showed a significant interaction between the treatment and the initial body condition for the body condition effects of alpha-cypermethrin on day 21 (Appendix C.2).

Figure 44: Cumulative survival of *Pelophylax perezi* juveniles exposed to alpha-cypermethrin and to its formulation Fasthrin 10 EC

Source: own illustration, [IREC-CSIC].

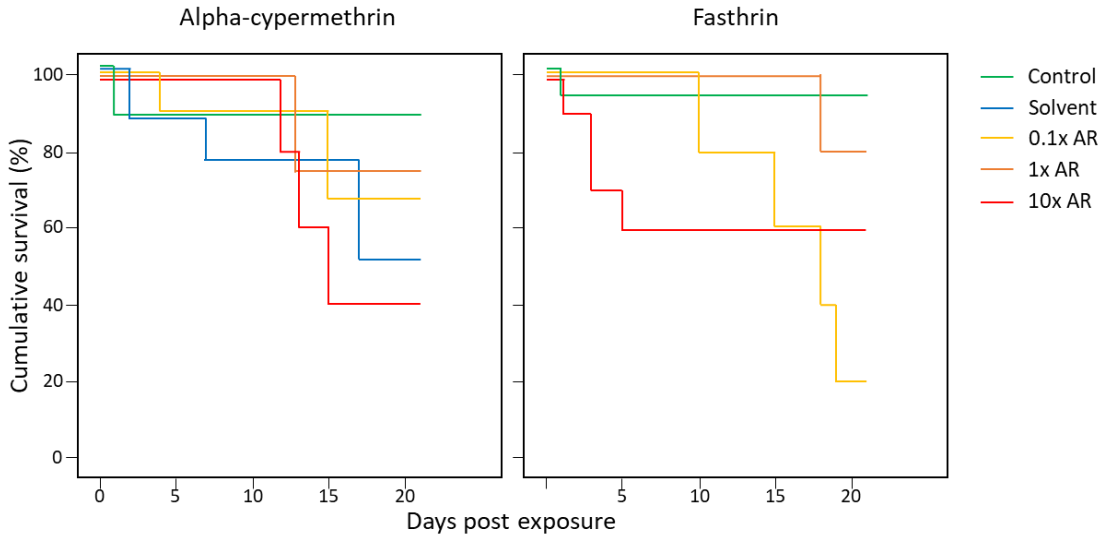
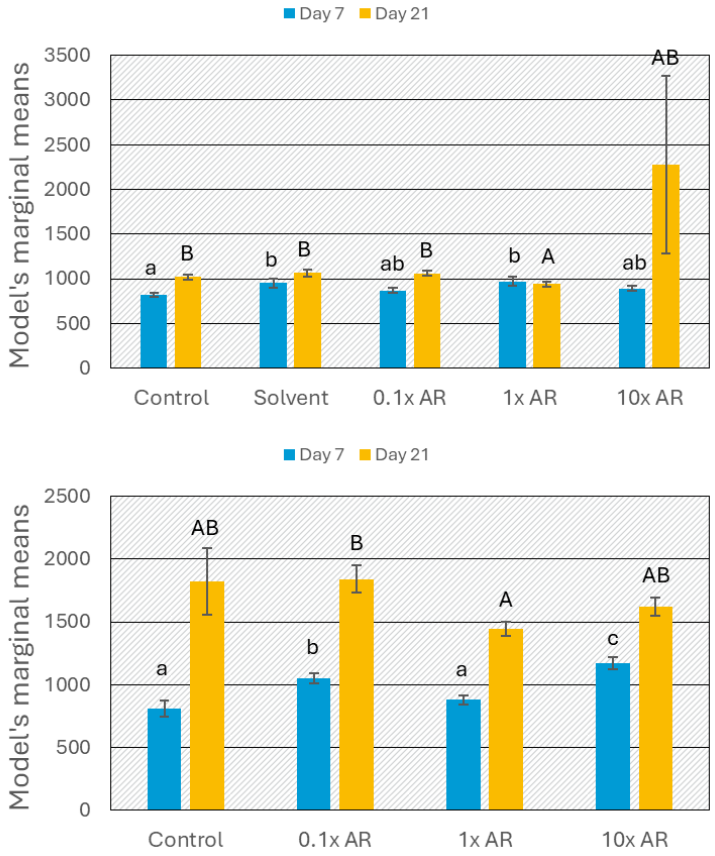


Figure 45: Body condition of *Pelophylax perezi* juveniles exposed to alpha-cypermethrin active ingredient (top) and Fasthrin 10 EC formulation (bottom) on days 7 and 21 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis (lower case for day 7 data, caps for day 21 data). Source: own illustration, [IREC-CSIC].



4.2.1.4 Co-formulants

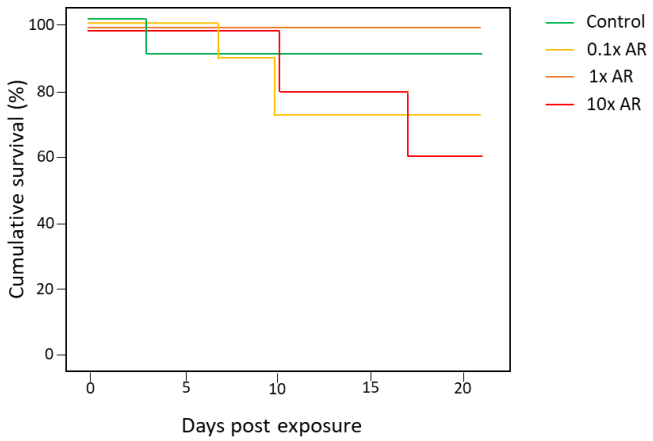
4.2.1.4.1 *Naphtha*

Although overspray of frogs with increased naphtha concentrations resulted in higher percentages of dead individuals (Figure 46), no significant lethal effects of the exposure to this solvent were detected. Nevertheless, the pairwise comparison of mortality occurrence on day 21 between controls and animals subject to overspray at ten times the application rate revealed a close to significant increase in lethality caused by naphtha (p=0.068).

Body condition of frogs exposed to Naphtha was unaffected on both day 7 and day 21 post-exposure.

Figure 46: Cumulative survival of *Pelophylax perezi* juveniles exposed to naphtha

Source: own illustration, [IREC-CSIC].



4.2.1.4.2 *N,N-Dimethyldecanamid*

The experiment with the additive N,N-dimethyldecanamid was conditioned by the high mortality among solvent controls (close to 80%) that happened from day 13 post-exposure onwards. Apart from this effect, no significant mortality caused by the substance was observed (Figure 47).

Body condition on day 7 of frogs exposed to acetone was significantly higher than that of frogs from the other treatments (Wald's X2=10.053, 2 d.f., p=0.007; Figure 48), although this effect was explained by differences in body condition at the beginning of the experiment (Appendix C.2). On day 21, differences between treatments in body condition had disappeared, probably because of the strong effect of mortality registered under the acetone treatment during the second part of that experiment.

Figure 47: Cumulative survival of *Pelophylax perezi* juveniles exposed to N,N-dimethyldecanamid

Source: own illustration, [IREC-CSIC].

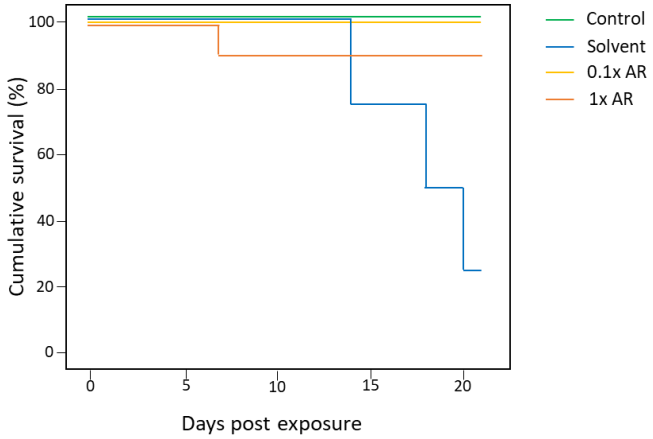
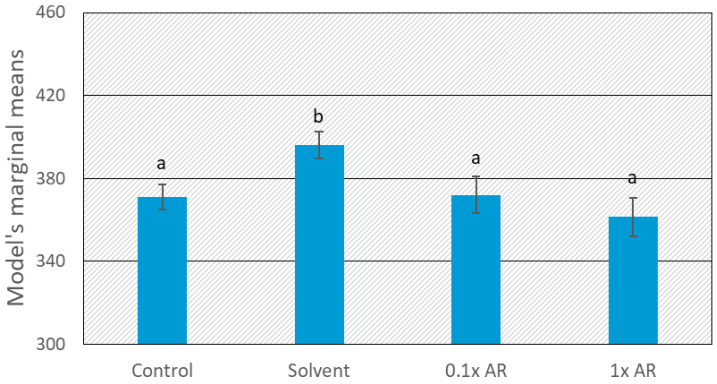


Figure 48: Body condition of *Pelophylax perezi* juveniles exposed to N,N-dimethyldecanamid on day 7 after treatment

Bars represent mean (±SD) values for the marginal means per treatment resulting from the generalized linear model. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].



4.2.2 Histological analysis

The majority (79.6%) of epidermal cell counts used for analysis was obtained from the arithmetic mean of counted cells through the QuPath's built-in Watershed Cell Detection and StarDist methods. The rest of the options were used in a much lower proportion of cases; Watershed Cell Detection was used as the only method for 7.1% of cases, StarDist was the only method for 4.4% of cases, and manual cell count was used for 8.8% of cases (Figure 49).

For some of the tests, it was not possible to include all treatments in the histological analysis, either because of the mortality of frogs prior to day 7, the loss of sample integrity while processing or the bad quality of the stained products. Nine out of the 16 tested pesticides caused some effect on any of the epidermal indices (Table 10). In general, exposure to pesticides reduced EI1 and increased EI2 when compared to control samples, although there were some exceptions to this general pattern.

Figure 49: Example images comparing the cell count methods used

a: StarDist (SD) vs. QuPath's built-in Watershed Cell Detection (CD); the visual inspection confirms the highest accuracy of CD compared to SD, due to the ability of CD to count nuclei based on optical density. b: QuPath's built-in Watershed Cell Detection (CD) vs. StarDist (SD); in this case the SD significantly outperformed CD since the latter method counts as nuclei the outer epidermis with higher optical density values. c: StarDist (SD) vs. Manual Cell Count (MCC); in this case both SD and CD (not shown) lacked acceptable detection, and MMC (yellow dots) was used.

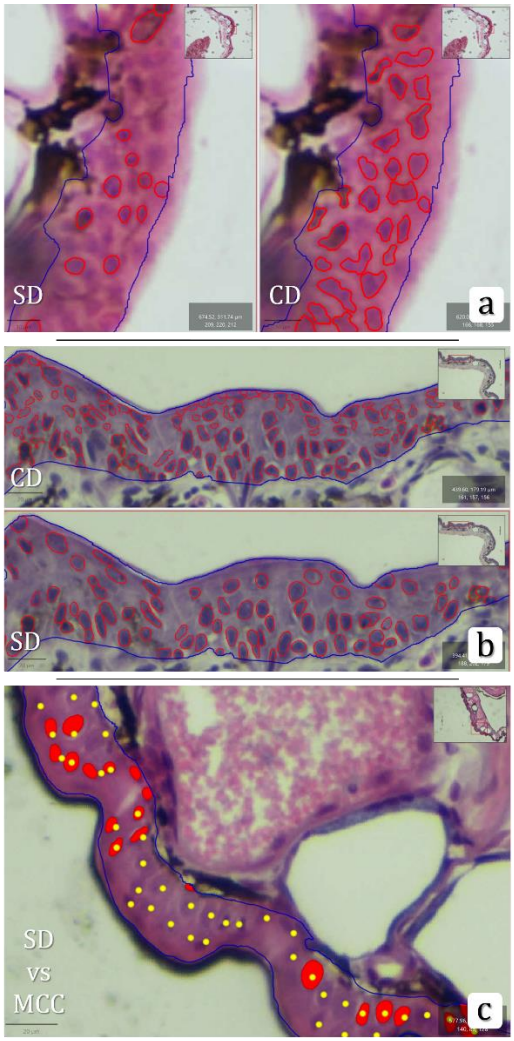


Table 10: Results of the overall generalized linear models to test the overall effects of pesticides on epidermal indices

Substance	Treatments included in the analysis (xAR)	EI1			EI2		
		Wald's X^2	d.f.	p	Wald's X^2	d.f.	p
Metrafenone (including Vivando)	1, 10, 1*, 10*	10.677	4	**0.030**	10.409	5	**0.034**
Oxathiapiprolin	0.1, 1, 10	7.677	3	0.053	4.775	3	0.189
Benzovindiflupyr	0.1, 1, 10	4.684	3	0.196	6.931	3	0.074
Azoxystrobin (including Quadris)	0.1, 1, 5.44, 1*, 5.44*	30.459	5	**<0.001**	35.490	5	**<0.001**
Tebuconazole (including Folicur 25 EW)	1, 3.2, 0.1*, 1*, 3.2*	12.658	5	**0.027**	9.054	5	0.107
MCPA	0.1, 1	3.126	2	0.209	13.600	2	**0.001**
Fluazifop-p-butyl (including Fusilade Max)	0.1, 1, 0.1*, 1*, 10*	5.065	5	0.408	18.594	5	**0.002**
Isoxaben	0.1, 1	4.509	2	0.105	4.331	2	0.115
Pendimethalin	0.1, 1	2.059	2	0.357	1.995	2	0.369
Metsulfuron-methyl	1, 10	0.097	2	0.953	6.282	2	**0.043**
Mesotrione	1, 2.49	1.706	2	0.426	8.006	2	**0.018**
Pirimicarb	1, 10	13.839	2	**<0.001**	2.936	2	0.230
Acetamiprid	0.1, 1, 10	0.708	3	0.871	2.071	3	0.558
Flupyradifurone (including Sivanto Prime)	0.1, 1, 10, 1*, 10*	7.322	5	0.198	8.046	5	0.154
Alpha-cypermethrin (including Fasthrin 10 EC)	0.1, 1, 10, 0.1*, 1*, 10*	5.077	6	0.534	63.290	6	**<0.001**
Lambda-cyhalothrin	0.1, 1	4.542	2	0.103	1.992	2	0.369

Bold characters indicate epidermal indexes that were significantly affected by each pesticide.

* referred to the formulation.

Among fungicides, metrafenone, azoxystrobin and tebuconazole caused effects on epidermal indices. Azoxystrobin exposure increased the EI2 at the highest treatment level (5.44xAR) of both the active ingredient and the formulation Quadris. In addition, overspray with the application rate of Quadris also increased EI2, but at this exposure level such effect was not detected for the active ingredient alone (Figure 50). Metrafenone also increased EI2, although only at 1xAR, and in this case the formulation Vivando had no effects on this index (Figure 51). The effects of these two fungicides on EI1 were unclear; azoxystrobin reduced EI1 at 1xAR of the active ingredient while caused an increase in this index at 5.44xAR of Quadris when compared to controls (Figure 50). On the other hand, metrafenone exposure reduced EI1 values relative to controls, but only among frogs exposed to the highest application rate of the formulation

Vivando (Figure 51). The changes in epidermal thickness and/or harmony, as reflected by the effects on EI2, probably influenced the EI1 measures as well. Similar results were found with the effects of tebuconazole on EI1, which were only evident at low concentrations of both the active ingredient and the formulation Folicur 25 EW (Figure 52).

Figure 50: Epidermal indices 1 and 2 measured in *Pelophylax perezi* juveniles exposed via overspray to azoxystrobin or its formulation Quadris on day 7 after treatment

Bars represent mean (±SE) values for the epidermal index. Different letters indicate significantly different ($p<0.05$) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].

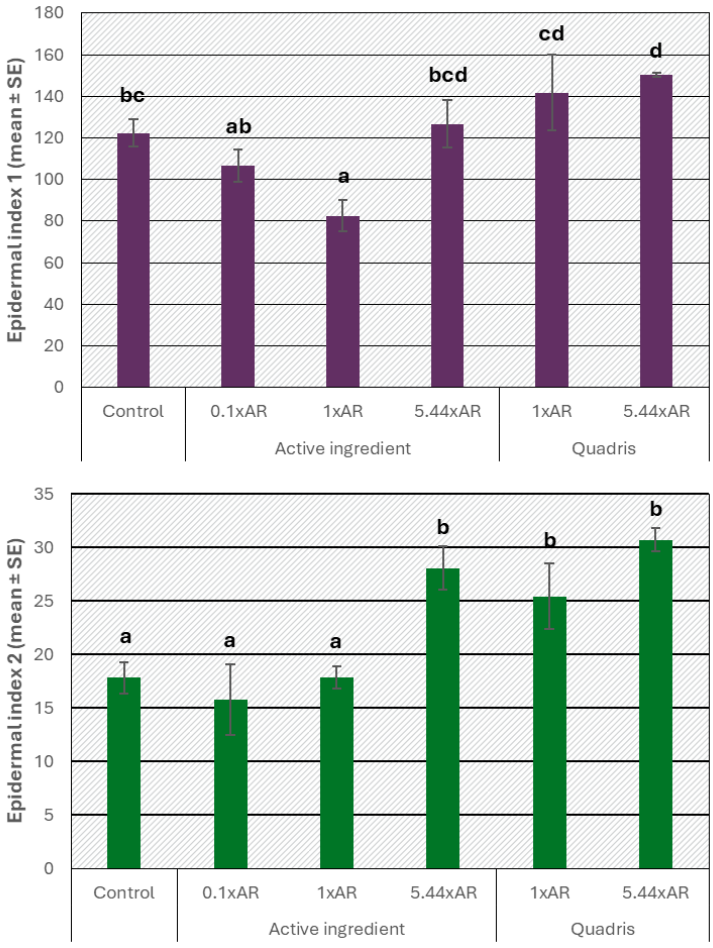


Figure 51: Epidermal indices 1 and 2 measured in *Pelophylax perezi* juveniles exposed via overspray to metrafenone or its formulation Vivando on day 7 after treatment

Bars represent mean (±SE) values for the epidermal index. Different letters indicate significantly different ($p<0.05$) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].

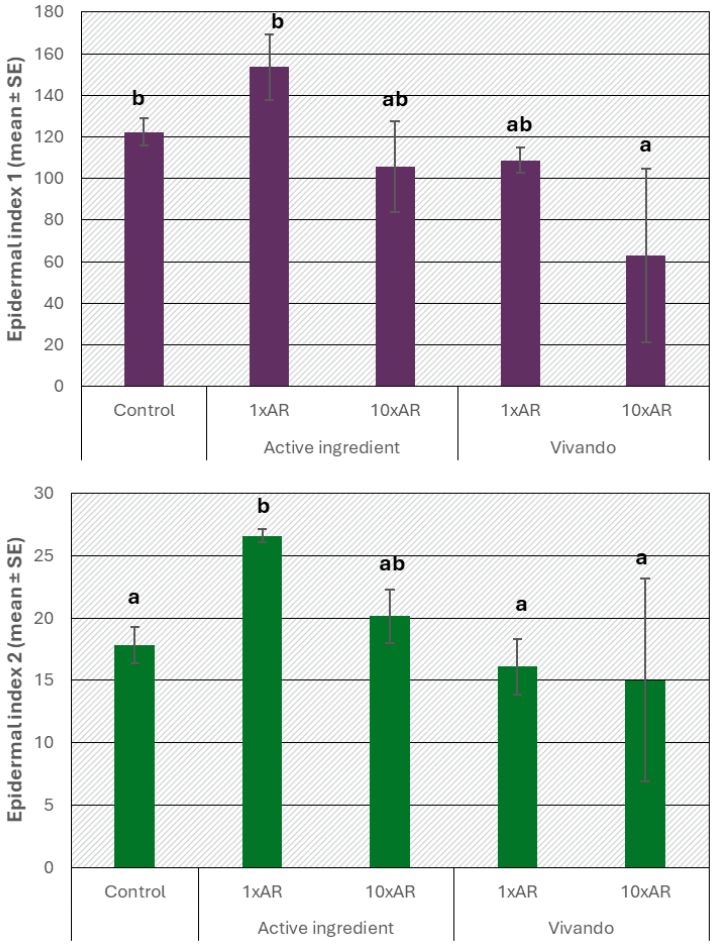
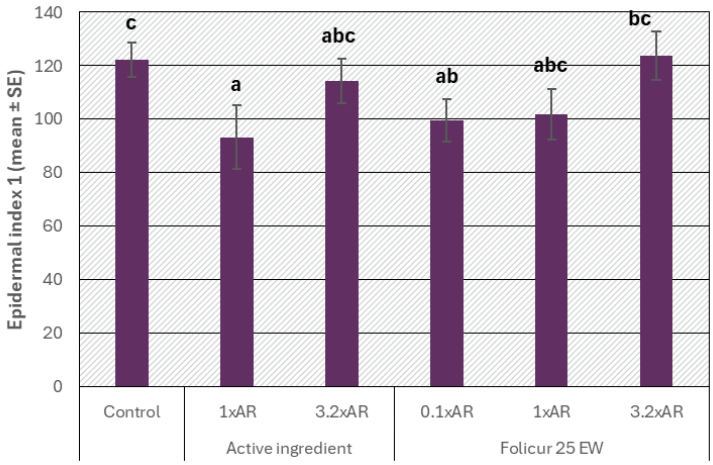


Figure 52: Epidermal index 1 measured in *Pelophylax perezi* juveniles exposed via overspray to tebuconazole or its formulation Folicur 25 EW on day 7 after treatment

Bars represent mean (±SE) values for the epidermal index. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].



Herbicides also affected epidermal indices, especially EI2. With the exception of isoxaben, the herbicides that were tested only as active ingredients increased the EI2 values compared to controls; the effects were observed for MCPA at application rates from 0.1xAR, for mesotrione at 2.49xAR and for metsulfuron-methyl from 10xAR (Figure 53). With regards to fluazifop-p-butyl, that was tested as active ingredient and formulation, we observed a different pattern depending on the format; the active ingredient increased EI2 compared to controls, while exposure to the formulation Fusilade Max caused no effect on this index (Figure 54).

Figure 53: Epidermal index 2 measured in *Pelophylax perezi* juveniles exposed via overspray to the herbicides MCPA, mesotrione and metsulfuron -methyl on day 7 after treatment

Bars represent mean (±SE) values for the epidermal index. Different letters indicate significantly different ($p<0.05$) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].

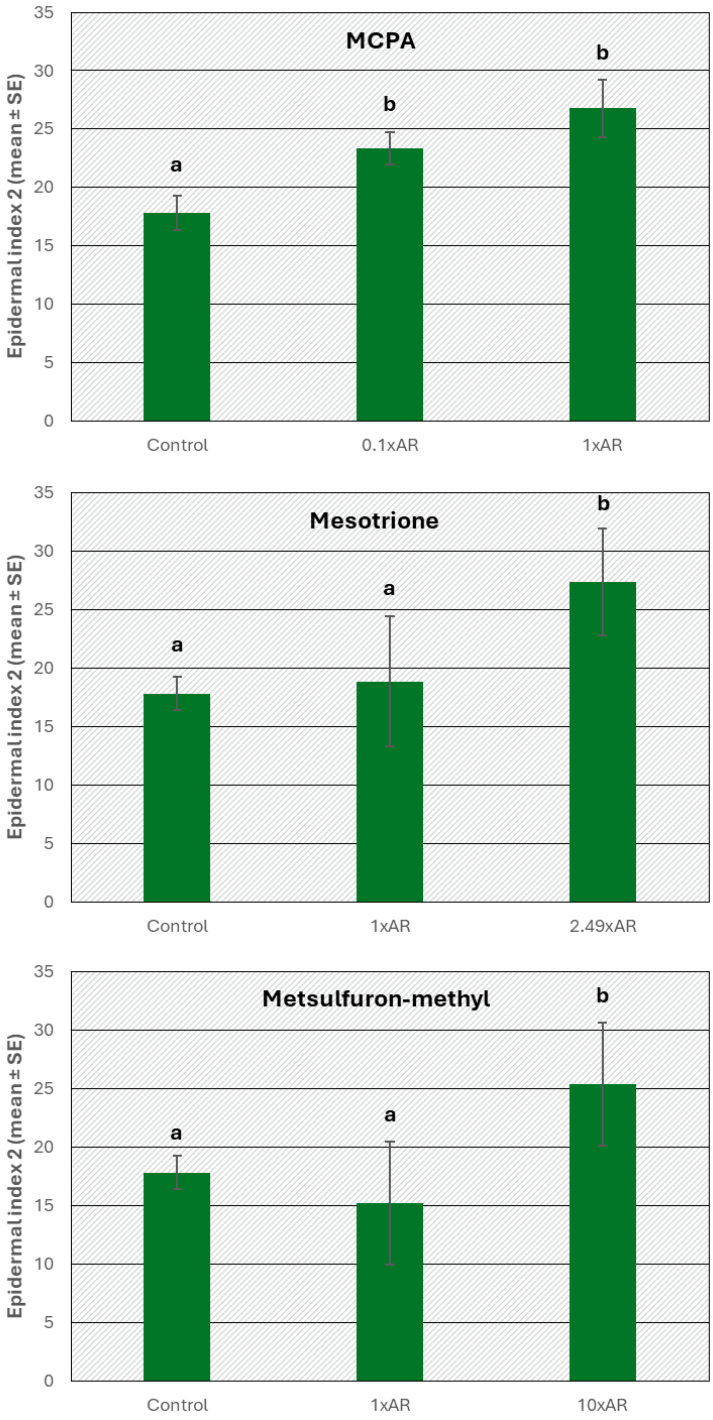
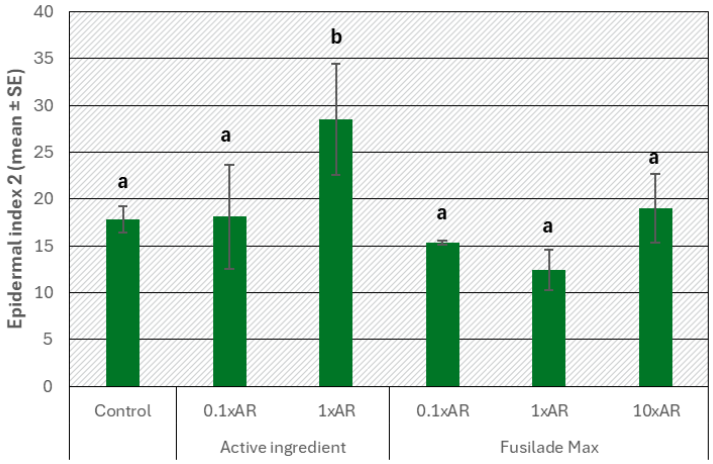


Figure 54: Epidermal index 2 measured in *Pelophylax perezi* juveniles exposed via overspray to fluazifop-p-butyl and its formulation Fusilade Max on day 7 after treatment

Bars represent mean (±SE) values for the epidermal index. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].



The effects of insecticides on epidermal indices were variable. Acetamiprid, flupyradifurone and lambda-cyhalothrin caused no effects, although the latter was not evaluated in this context at its higher overspray level (10xAR). Pirimicarb reduced EI1 values compared to controls at treatments from 1xAR (Figure 55). Finally, alpha-cypermethrin was the tested substances with clearest effects on EI2 (Figure 56); an increase in EI2 values linked to the exposure of this insecticide and its formulation Fasthrin 10 EC was observed, with a dose-dependent response in both cases. Furthermore, the effects were stronger when frogs were exposed to Fasthrin 10 EC than when they were exposed to the active ingredient alone (Figure 57).

Figure 55: Epidermal index 1 measured in *Pelophylax perezi* juveniles exposed via overspray to pirimicarb on day 7 after treatment

Bars represent mean (±SE) values for the epidermal index. Different letters indicate significantly different (p<0.05) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].

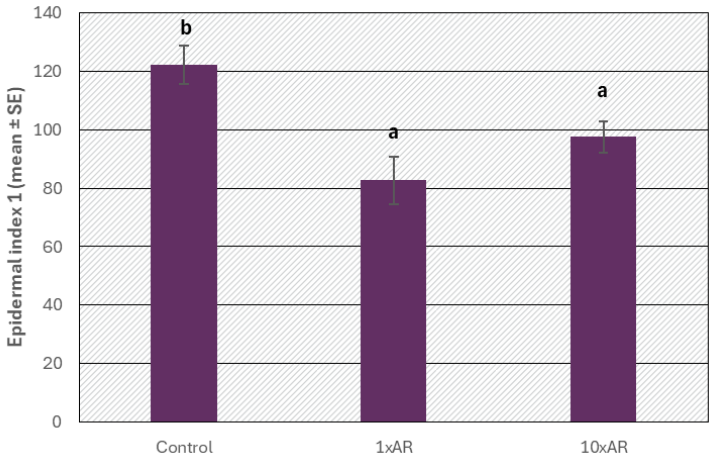


Figure 56: Epidermal index 2 measured in *Pelophylax perezi* juveniles exposed via overspray to alpha-cypermethrin and its formulation Fasthrin 10 EC on day 7 after treatment

Bars represent mean (±SE) values for the epidermal index. Different letters indicate significantly different ($p<0.05$) treatments according to Least Significant Difference post-hoc analysis. Source: own illustration, [IREC-CSIC].

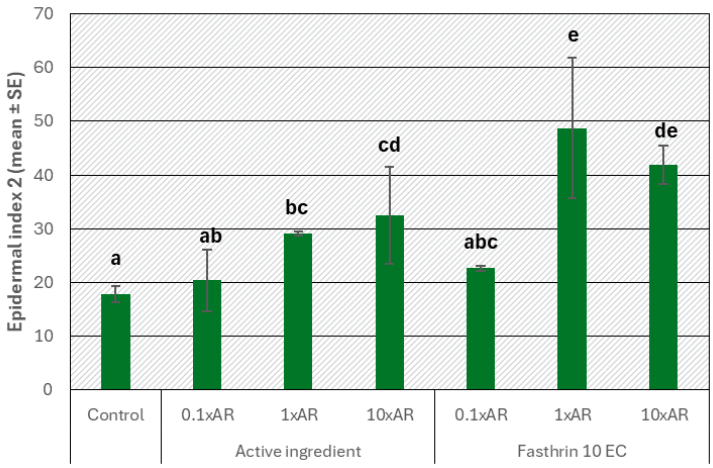
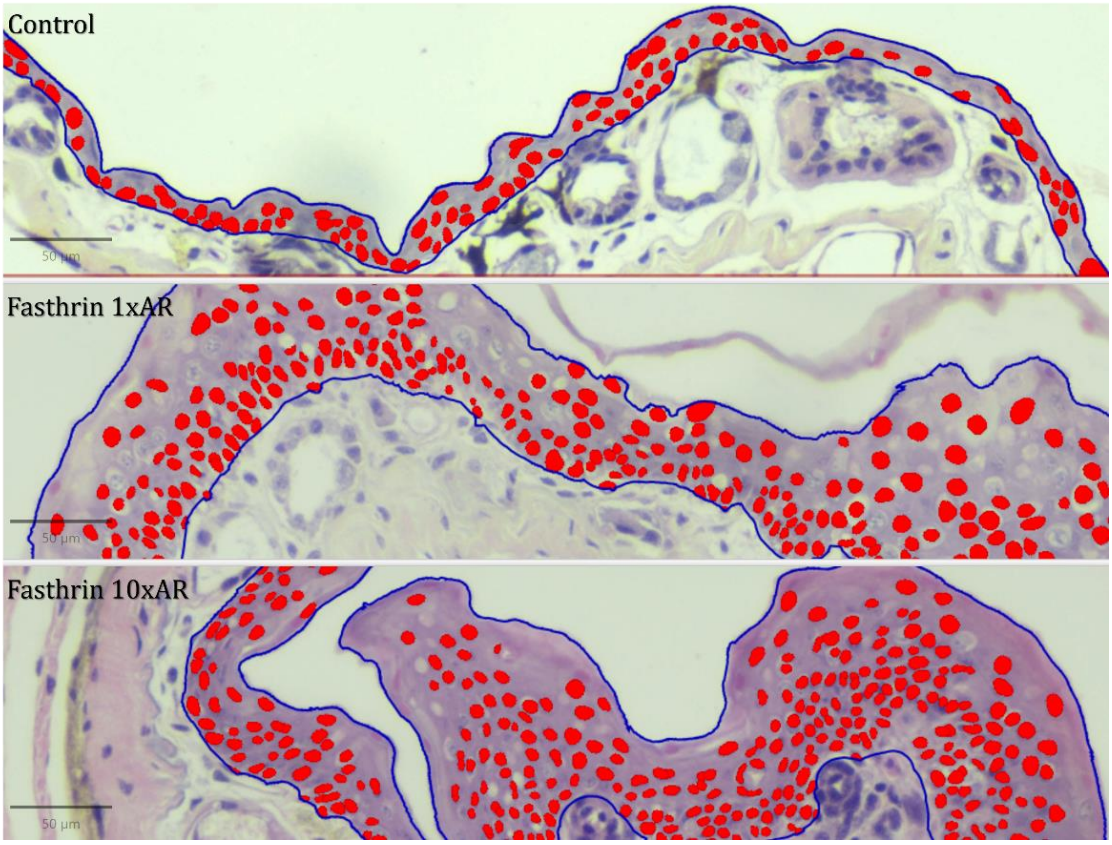


Figure 57: Comparison of epidermal area and cell count (StarDist method) between example samples from control and Fasthrin 10 EC samples

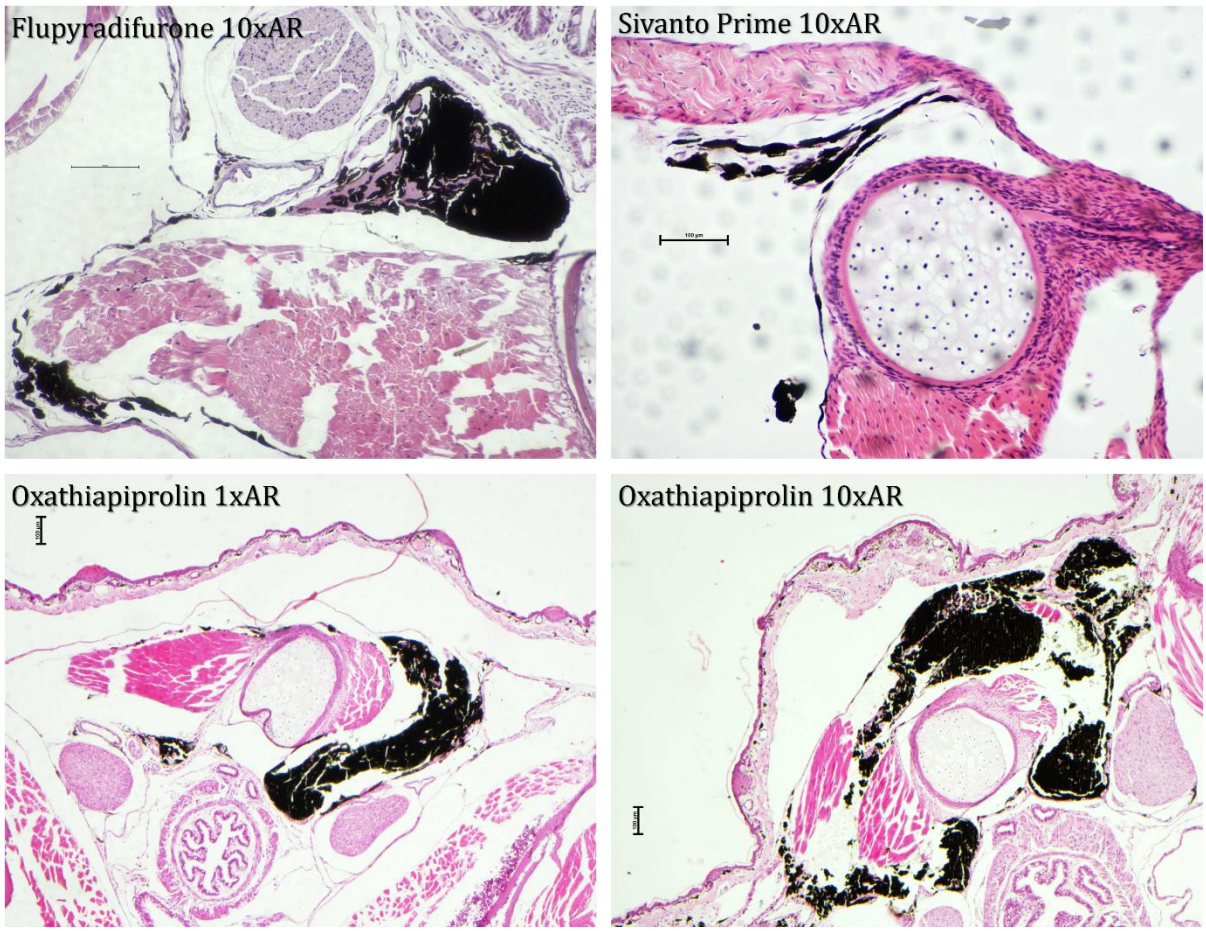
The resolution of the QuPath viewer is matching (all the images are represented at the same magnification to make them comparable). The statistically significant different between treatments in EI2 seems influenced by the annotation thickness and the irregularity of the perimeter.



The analysis of haematoxylin and eosin-stained histological sections from some frogs on day 7 after different pesticide exposure and concentration showed an intense dark stain. This increased dark brown/blackish pigment is compatible with accumulation of melanocytes and/or pigmented melanomacrophages (Figure 58).

Figure 58: Examples of sections of pesticide exposed frogs with evident clusters of pigmented melanomacrophages

Pictures correspond to frogs on day 7 after overspray with flupyradifurone active ingredient (10xAR), Sivanto Prime (10xAR) and oxathiapiprolin (1xAR and 10xAR). Clusters of pigmented melanomacrophages are located in the caudal lymphatic space surrounding the urostyle, and within the skeletal muscles, tunicae of vessels and/or subdermal space, likely subsequently to extratissutal migration and penetration of melanomacrophages or precursors. Bars: 100 µm.



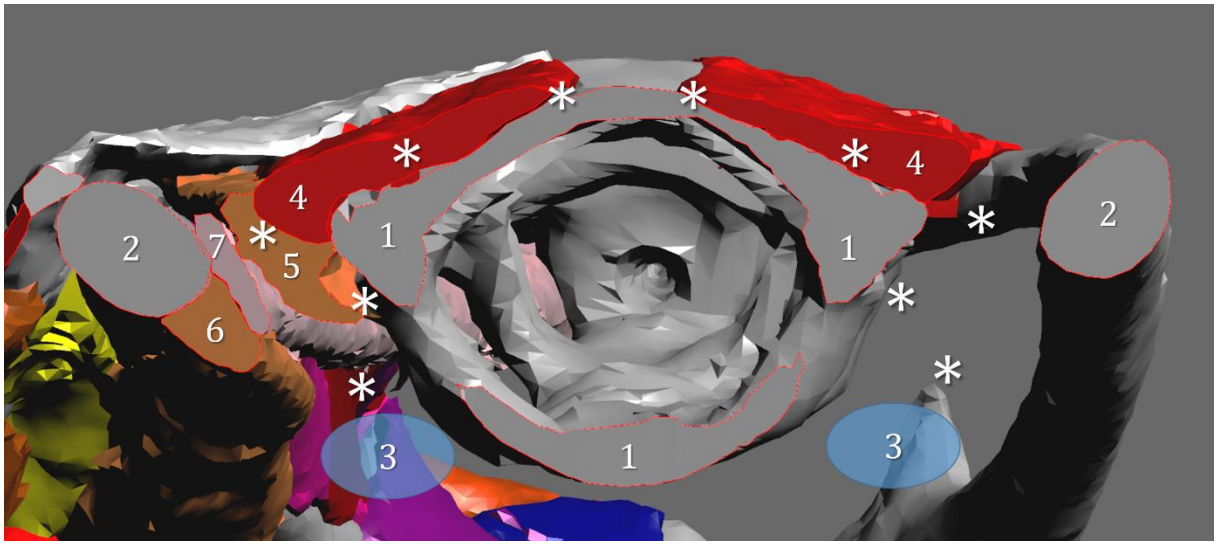
Melanomacrophage centres (MMC) in poikilotherms can be found in various organs, specifically in spleen, kidney and liver, and are specialized aggregates of pigmented macrophages involved in immunological and non-immunological functions (Steinel and Bolnick 2017). In fish, one of their non-immunological functions is to primarily act as “metabolic dumps” by storing indigestible and effete materials such as degraded erythrocytes and hemosiderin, which supports iron recycling (Agius 1979, Agius 1980, Fulop and McMillan 1984). Additionally, MMC accumulate exogenous substances, including metals and inert materials, highlighting their role in debris clearance and long-term storage of indigestible or toxic substances (Steinel and Bolnick 2017). MMC in amphibians and other poikilotherms play also a key role in both innate and adaptive immunity. They are highly phagocytic, engulfing pathogens like bacteria, fungi, and parasite eggs, and also act as antigen retention sites by storing antigens within immune complexes, potentially aiding in adaptive immune responses (Steinel and Bolnick 2017).

Furthermore, MMC increase in size or number following infection or immunization, reflecting their active involvement in immune processes (Agius 1979, Secombes 1982, Herráez and Zapata 1986, Steinel and Bolnick 2017). MMC alterations have been also related to environmental stress and chemical exposure (Agius and Roberts 2003, Franco-Belussi et al. 2016, Steinel and Bolnick 2017). Clusters of pigmented melanomacrophages can be found also in ectopic locations. In salmons, it is assumed that melanocytes, pigmented melanomacrophages or potential precursors are summoned within the skeletal muscles in case of inflammation (Larsen et al. 2012). In mammals, the deposition of melanin in visceral organs, facilitated by migrating melanophages, is a well-documented phenomenon (Plonka et al. 2005). Thus, the increase of pigmented melanomacrophage, melanocytes and melanin could be a physiological response to damage directly or indirectly produced by the pesticides in amphibians.

Hereinafter we report few qualitative data of large clusters of pigmented melanomacrophages found during the observation within the anatomical region of reference. Evident clusters of pigmented melanomacrophages (Figure 58) were found in histological sections of some individual 7 days after overspray at 10xAR with flupyradifurone, both as active ingredient and its formulation Sivanto Prime, and 7 days after overspray at 1xAR and 10xAR with oxathiapiprolin, and were not observed in the control animals. The anatomical location of these clusters is detailed in Figure 59. Clusters of pigmented melanomacrophages were found in the caudal lymphatic space around the urostyle, as well as within the skeletal muscles, in the wall of blood vessels, and subdermal areas. This likely results from the migration and infiltration of melanomacrophages or their precursors beyond their original tissue boundaries. We observed that these clusters seem not related to melanocytes are normally found in the layer of basal cells of the epidermis of the studied frog (e.g., see Figure 49). Nonetheless, dermal melanocytes might change after exposure to xenobiotics and have a significance in the inflammatory and immunity processes.

Figure 59: A transverse section of a musculoskeletal digital model of *Xenopus laevis* illustrating the anatomical reference regions used in this study, highlighting the areas where clusters of pigmented melanomacrophages were most frequently observed

Asterisks indicate areas where enlarged clusters of pigmented melanomacrophages were most frequently observed; 1: caudal vertebrae or sacrourostyle; 2: ilium; 3: kidneys; 4: longissimus dorsi muscle; 5: intertransversarii dorsi muscle; 6: deep iliacus externus muscle; 7: coccygeoiliacus muscle. Model from Porro and Richards (2017).



4.2.3 Pesticide concentrations

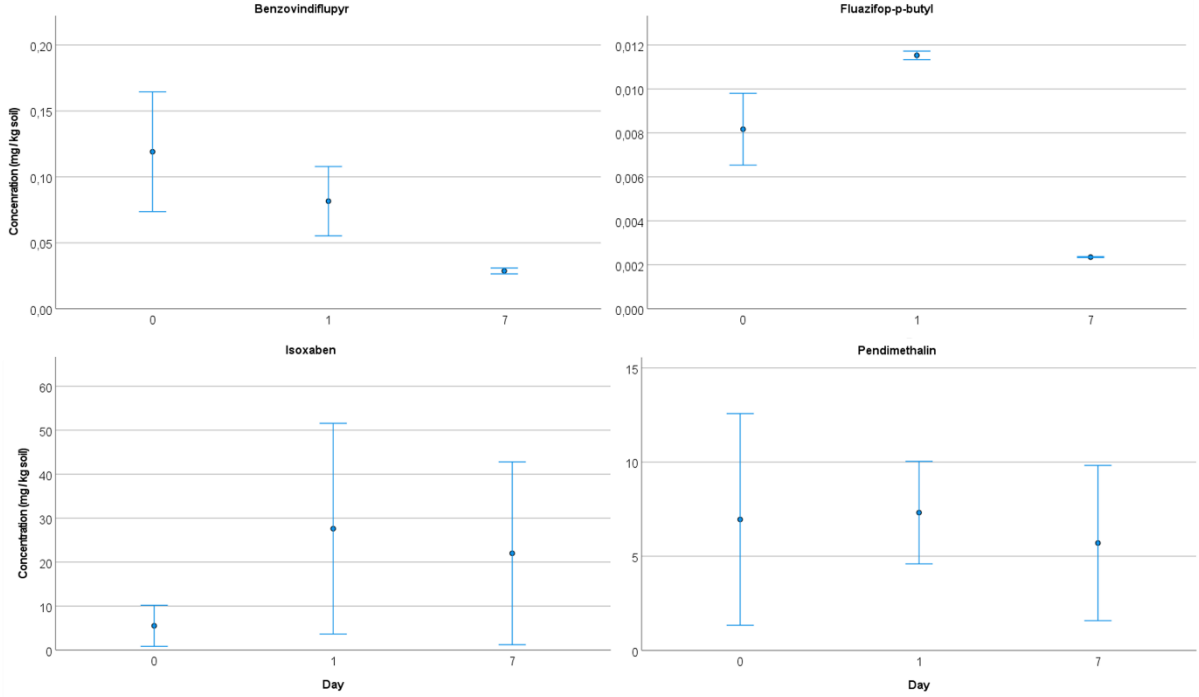
4.2.3.1 Concentrations in sprayed broths

Average measured concentrations in experimental brother relative to nominal concentrations were 56% for metrafenone, 158% for Vivando, 96% for oxathiapiprolin, 66% for benzovindiflupyr, 48% for azoxystrobin, 114% for Quadris, 64% for tebuconazole, 80% for Folicur 25 EW, 40% for fluazifop-p-butyl, 92% for Fusilade Max, 44% for isoxaben, 84% for pendimethalin, 100% for metsulfuron-methyl, 52% for pirimicarb, 40% for flupyradifurone and 114% for Sivanto Prime. Differences were due in most cases to solubility issues, as revealed also by the good adjustment when formulated products were used compared to their active ingredients alone.

4.2.3.2 Concentrations in soils

Figure 60: Concentrations of four pesticide active ingredients measured in sprayed soils at different times post-application

Bars represent mean (± standard error) values as mg active ingredient per kilogram of soil wet weight. Day 0 corresponds to the spraying moment. Source: own illustration, [IREC-CSIC].

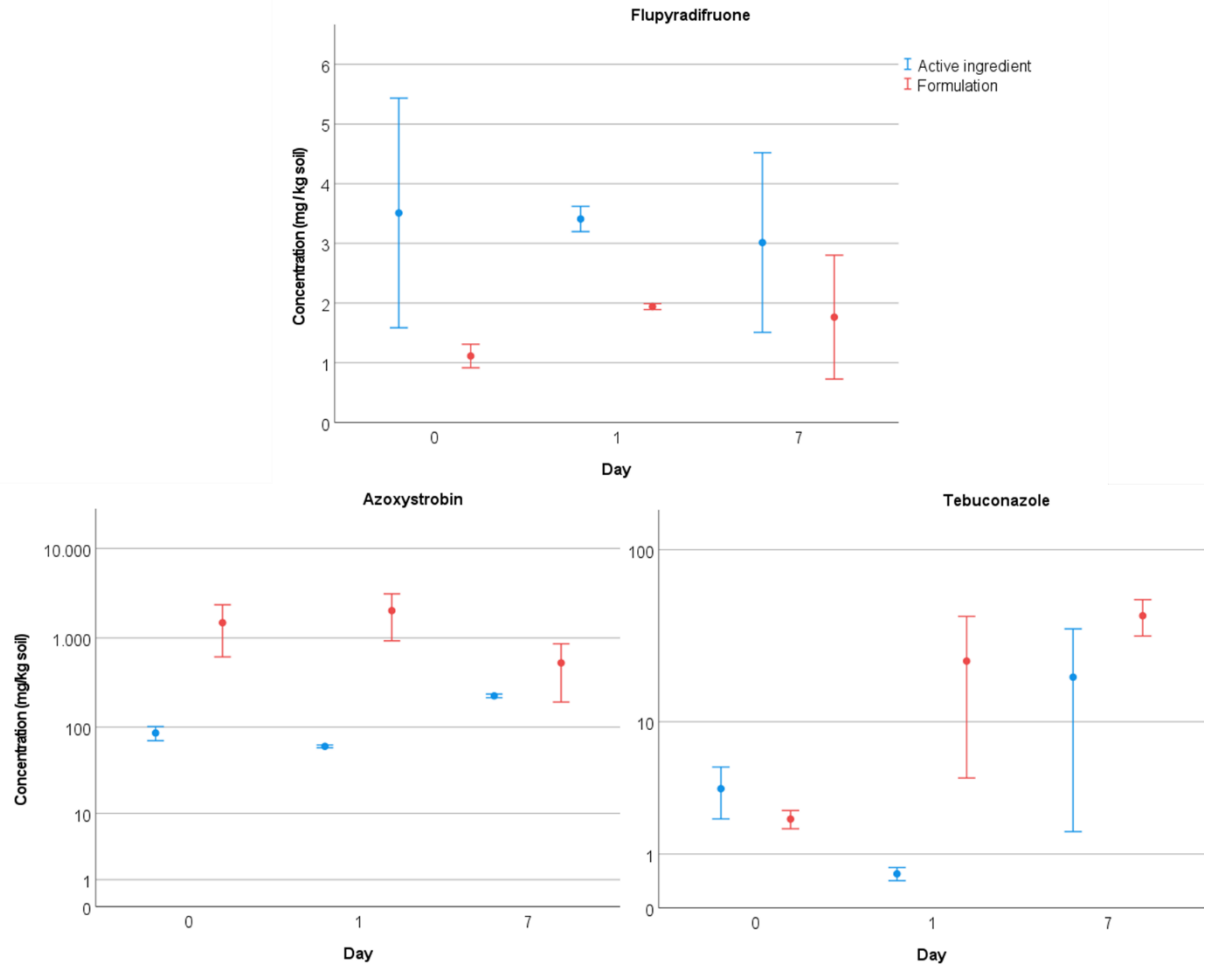


Pesticide concentrations in soils were measured right after spray of the pesticides, and on days 1 and 7 post-application. In general, the applied products showed a certain level of stability along the 7-day period. Only benzovindiflupyr and fluazifop-p-butyl presented a clear degradation over the monitoring time, which was quicker for the former as a significant drop in measured levels of benzovindiflupyr was observed already after 24 hours of application (Figure 60). Regarding the comparative behaviour of pesticides depending on whether they were applied as active ingredients or as formulations, there were no big differences in persistence, although the levels when formulations were applied were clearly higher than when the active ingredient was sprayed alone for the cases of azoxystrobin and tebuconazole (Figure 61). These differences can be explained in part because of the better dilution of the substance when applied as formulation, as reflected also by the differences observed in the concentrations measured in broths. However,

especially for the case of azoxystrobin and Quadris, the difference in soil levels cannot be explained solely by variations in the sprayed broth levels.

Figure 61: Concentrations of three pesticides measured in sprayed soils at different times after application of the substances as active ingredients or as formulations

Bars represent mean (± standard error) values as mg active ingredient per kilogram of soil wet weight. Day 0 corresponds to the spraying moment. To improve visualization, some axes are depicted in logarithmic scale. Source: own illustration, [IREC-CSIC].



4.2.3.3 Pesticide accumulation in frog tissues

Because the objective of the pesticide residue analyses in frogs was to determine the accumulation resulting from a prolonged exposure via soil, the analysis of pesticide concentrations in animals exposed to the lowest overspray application rate (0.1xAR) was not performed in some cases, namely benzovindiflupyr, azoxystrobin (both as an active ingredient and as Quadris), tebuconazole (as active ingredient), flupyradifurone (as active ingredient) and lambda-cyhalothrin on day 7, and the formulations Folicur 25EW and Sivanto Prime on days 7 and 21. In addition, samples collected on day 21 of the experiment with lambda-cyhalothrin corresponding to the treatment with 1xAR via overspray only were lost and could not be analysed. By contrast, we did analyse in all cases the frogs exposed to higher overspray application rates (≥1xAR) to compare with the treatments involving exposure via soil contact.

No detectable levels of fluazifop-p-butyl and of isoxaben were detected in any of the frogs from the experiments conducted with these two active ingredients.

Benzovindiflupyr residue levels in exposed frogs were higher on day 7 of experiment than on day 21 (X^2=43.151, 1 d.f., p<0.001). The effect of the day was also found in interaction with the sample type (X^2=3.955, 1 d.f., p=0.047). In general, concentrations of benzovindiflupyr in frog tissues on day 21 were low in all cases. On day 7, however, concentrations in liver responded to the applied doses regardless of the exposure route, whereas in the skin there was a slightly stronger effect of overspray than of exposure via soil contact in accumulated levels (Figure 62).

Figure 62: Concentrations of benzovindiflupyr measured in juvenile *Pelophylax perezi* tissues after exposure to this substance

Bars represent mean (± standard error) values as ng active ingredient per gram wet weight. Values below the limit of detection are represented with a thin line above the horizontal axis; the absence of such a line or a bar indicates that no samples were analysed for that specific group. Source: own illustration, [IREC-CSIC].

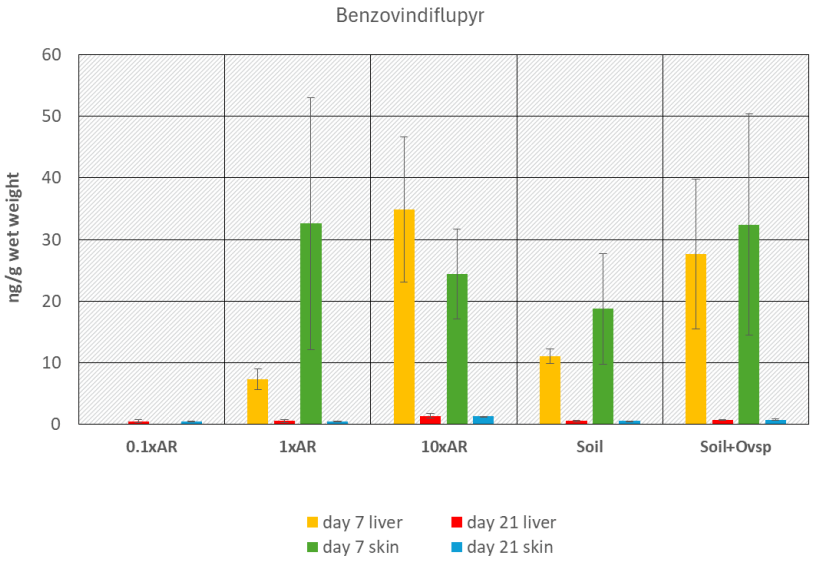
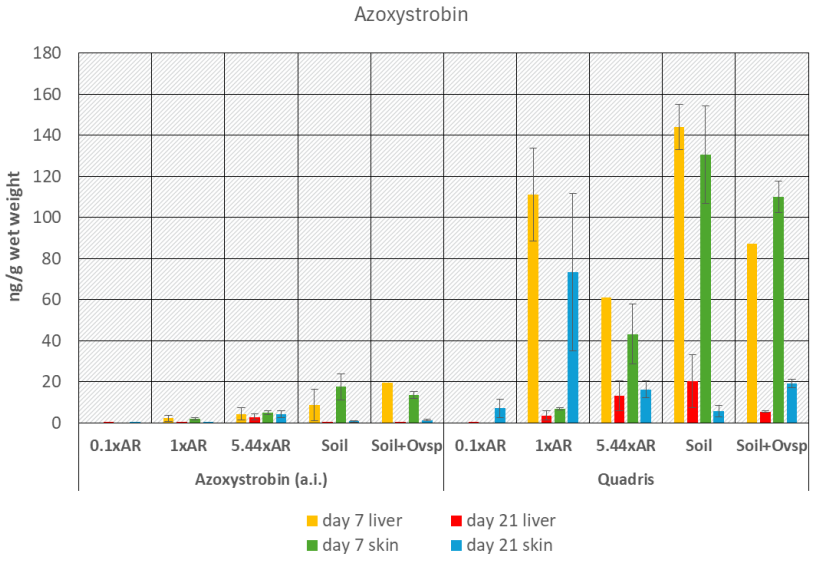


Figure 63: Concentrations of azoxystrobin measured in juvenile *Pelophylax perezi* tissues after exposure to this substance as active ingredient or as its formulation Quadris

Bars represent mean (± standard error) values as ng active ingredient per gram wet weight. Values below the limit of detection are represented with a thin line above the horizontal axis; the absence of such a line or a bar indicates that no samples were analysed for that specific group. Source: own illustration, [IREC-CSIC].

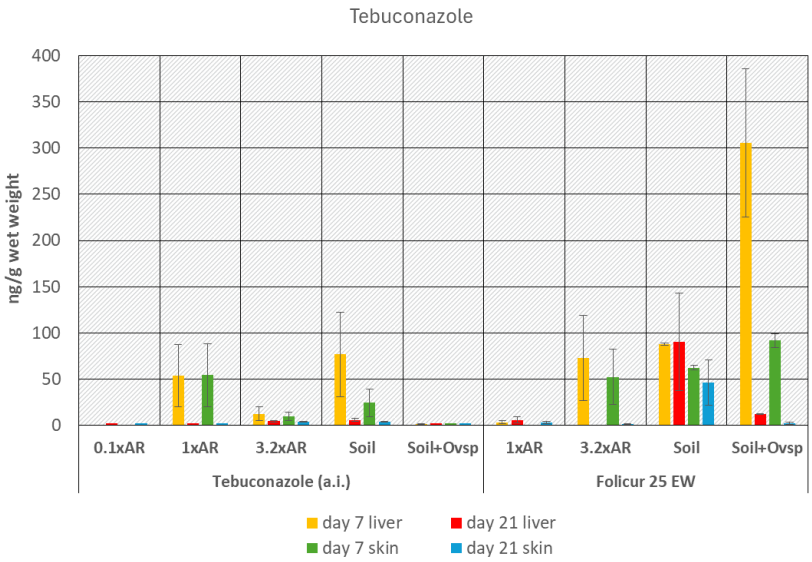


Azoxystrobin was accumulated by frogs exposed to either the active ingredient or the formulation Quadris, although the latter resulted in pesticide residues significantly higher than those measured in frogs exposed to the active ingredient alone (difference active ingredient vs. formulation: X^2=43.151, 1 d.f., p<0.001). Day of experiment also had a significant effect on azoxystrobin residues measured in frogs (X^2=21.057, 1 d.f., p<0.001); for both the active ingredient and Quadris, levels on day 7 were higher than those measured on day 21, In fact, in the active ingredient experiment, all analysed samples from day 7 showed detectable levels of the azoxystrobin in both liver and skins, whereas on day 21 the fungicide was detected only in livers and skins of frogs exposed to the highest overspray treatment and in skins of frogs exposed via soil contact. When comparing treatments subjected to the application rate, there was a clear influence of exposure route, with soil contact being more determinant than overspray for accumulation of azoxystrobin in the skin at any sampling time and in the liver on day 21 of experiment (Figure 63).

The level of tebuconazole measured in exposed frogs varied depending on the format (active ingredient vs Folicur 25 EW formulation, X^2=26.636, 1 d.f., p<0.001), sampling day (day 7 vs 21, X^2=94.958, 1 d.f., p<0.001), analysed tissue (liver vs skin, X^2=17.223, 1 d.f., p<0.001) and treatment (X^2=48.049, 3 d.f., p<0.001). As it happened with azoxystrobin, the formulation led to highest residue levels than the active ingredient alone, and the concentrations were higher in frogs measured after 7 days than after 21 days of experiment; however, this difference between sampling days was detected mostly for overspray treatments. When exposure happened via soil contact, in the case of Folicur 25 EW, no differences were found in accumulation on day 7 or 21 of experiment. Residues measured in liver were higher than those measured in skins, but this difference was detected mostly within treatments in which the exposure happened via soil contact, whereas for overspray-only treatments the difference between sample types was not so clear. Soil contact tended to be more determinant for pesticide accumulation than overspray when treatments involving the application rate were compared (Figure 64).

Figure 64: Concentrations of tebuconazole measured in juvenile *Pelophylax perezi* tissues after exposure to this substance as active ingredient or as its formulation Folicur 25 EW

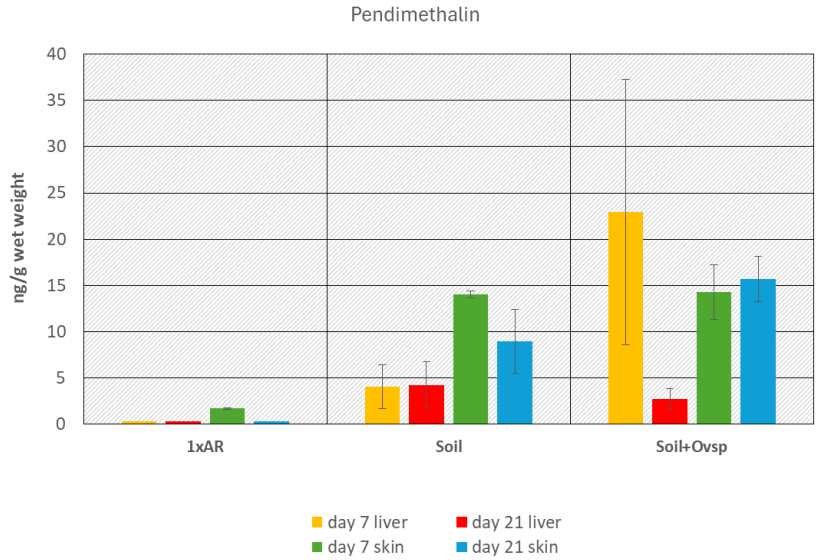
Bars represent mean (± standard error) values as ng active ingredient per gram wet weight. Values below the limit of detection are represented with a thin line above the horizontal axis; the absence of such a line or a bar indicates that no samples were analysed for that specific group. Source: own illustration, [IREC-CSIC].



The levels of pendimethalin measured in exposed frogs were clearly dependent on the exposure route; exposure via soil contact was the main determinant of accumulation of the herbicide (X^2=131.752, 21 d.f., p<0.001), and a significant effect of the sampling day was also detected for pendimethalin body residues (X^2=8.430, 1 d.f., p=0.004). In fact, for animals exposed via overspray detection of pendimethalin was restricted to the skin of frogs analysed on day 7 of experiments, although it must be reminded that the highest overspray treatment was not used in this experiment. No overall differences between tissues were detected in the accumulation of pendimethalin under the tested conditions (Figure 65).

Figure 65: Concentrations of pendimethalin measured in juvenile *Pelophylax perezi* tissues after exposure to this substance

Bars represent mean (± standard error) values as ng active ingredient per gram wet weight. Values below the limit of detection are represented with a thin line above the horizontal axis; the absence of such a line or a bar indicates that no samples were analysed for that specific group. Source: own illustration, [IREC-CSIC].



Residues of flupyradifurone determined in frog samples varied depending on the format (active ingredient vs Sivanto Prime, X^2=8.119, 1 d.f., p=0.004), sampling day (day 7 vs 21, X^2=102.773, 1 d.f., p<0.001), analysed tissue (liver vs skin, X^2=8.901, 1 d.f., p=0.003) and treatment (X^2=65.014, 3 d.f., p<0.001). The effect of formulation on accumulation of flupyradifurone was not so clear as with the previous pesticides that were tested as active ingredients and formulations. In general, for treatments involving a single exposure route, residues were higher for frogs exposed to Sivanto Prime, whereas for the treatment combining both exposure routes, higher levels were detected in frogs exposed to the active ingredient. Also, in these experiments there was a clearly higher concentration of flupyradifurone after 7 days of experiment than after 21 days, even in treatments where exposure happened via soil. Soil contact resulted a more relevant source than overspray for pesticide accumulation in the experiment with Sivanto Prime, but not in the experiment with the active ingredient flupyradifurone (Figure 66).

The only factor affecting lambda-cyhalothrin levels in frogs exposed to the application rates either by overspray, soil contact or the combination of routes was the tissue (X^2=13.567, 1 d.f., p<0.001), with livers showing higher concentrations of the insecticide than skins (Figure 67).

Figure 66: Concentrations of flupyradifurone measured in juvenile *Pelophylax perezi* tissues after exposure to this substance as active ingredient or as its formulation Sivanto Prime

Bars represent mean (± standard error) values as ng active ingredient per gram wet weight. Values below the limit of detection are represented with a thin line above the horizontal axis; the absence of such a line or a bar indicates that no samples were analysed for that specific group. Source: own illustration, [IREC-CSIC].

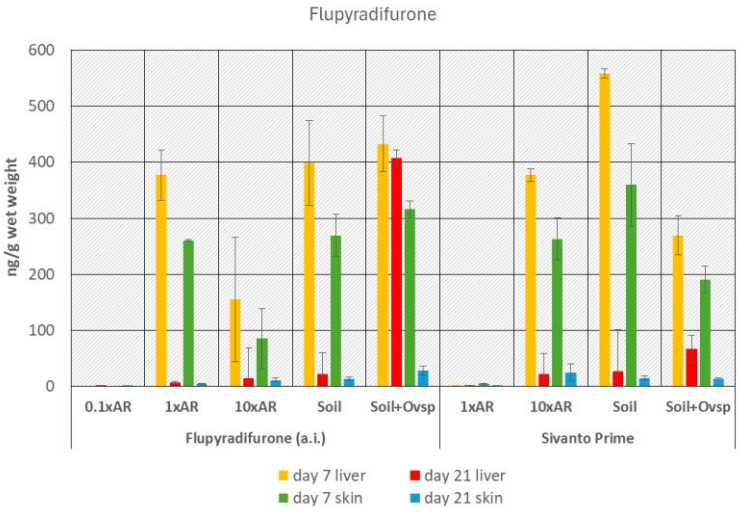
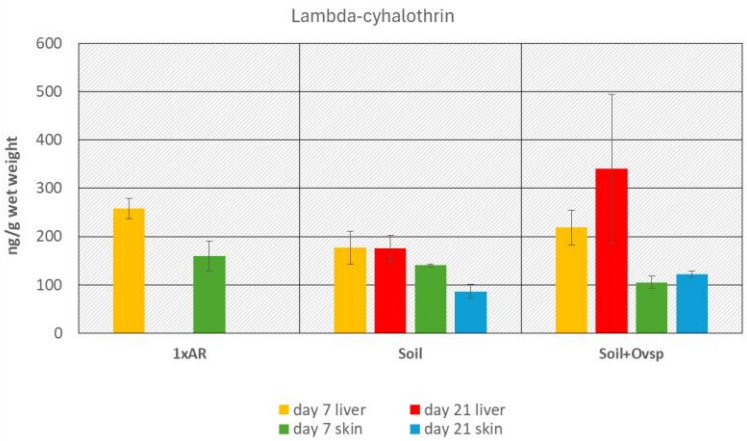


Figure 67: Concentrations of lambda-cyhalothrin measured in juvenile *Pelophylax perezi* tissues after exposure to this substance

Bars represent mean (± standard error) values as ng active ingredient per gram wet weight. Values below the limit of detection are represented with a thin line above the horizontal axis; the absence of such a line or a bar indicates that no samples were analysed for that specific group. Source: own illustration, [IREC-CSIC].



5 Laboratory tests with earthworms

5.1 Material and methods

5.1.1 Earthworm model species

The epigeic earthworm species *Eisenia andrei* was chosen as an invertebrate model to evaluate its suitability as a surrogate species for animal experimentation in assessing the risk of dermal exposure to PPP in the terrestrial life stages of amphibians. This species, which dwells in the organic layers of various soil types, is considered widespread and globally distributed. It has also been extensively used as a bioindicator of soil health and for the ecotoxicological assessment of chemicals in terrestrial ecosystems (Edwards and Bohlen 1996, Lee et al. 2008). Several factors influenced the selection of this species for the present study: (i) *E. andrei* is native from temperate regions, is abundant, easy to handle, and simple to maintain in laboratory conditions (Fründ et al. 2010); (ii) it is recommended by numerous standard guidelines for toxicity testing (OECD 1984, 2004); (iii) represent a group of organisms widely used as bioindicators for assessing the quality of the terrestrial environment (Lee et al. 2008); (iv) as an epigeic species, living on the soil surface, it is a more appropriate surrogate for terrestrial amphibians compared to other earthworms that dwell deeper in the soil; (v) its highly permeable skin allows for respiration through the skin, similar to amphibians (Laverack 1963); and (vi) like many amphibian species, *E. andrei* commonly inhabits agricultural environments and may be exposed to PPP (Miglani and Bisht 2019).

5.1.2 Origin and maintenance of earthworms in the laboratory

Adults of *E. andrei*, with clitellum, were purchased to the company CloverStrategy, Lda (Coimbra, Portugal), to initiate lab-cultures of this species. The identification of the species was confirmed by this company through barcoding. The age of the acquired earthworms was synchronized by CloverStrategy, Lda, being all less than seven months old when the culture was initiated in the laboratory.

Once in the laboratory, the cultures of *E. andrei* were maintained in dark plastic containers filled with a 1:1 mixture of *Sphagnum* peat and cow manure, the latter serving as a food source for the earthworms. The cow manure was sourced from the Escola Superior Agrária de Coimbra (ESAC), where no chemicals, such as antibiotics or PPP, are used in livestock farming, ensuring a chemical-free food source for the earthworms. Upon collection at ESAC and arrival at the laboratory, the manure was defaunated by undergoing two freeze-thaw cycles at -20°C. The substrate's moisture content was kept between 70% and 80%, and the containers were covered with dark plastic bags to reduce water evaporation. The cultures were kept in a room at a stable temperature of 23 ± 1°C, with the substrate being replaced every month.

5.1.3 Experimental exposure of earthworms to pesticides

Adults of *E. andrei*, with clitellum, that had been acclimatized to the laboratory, were exposed, through overspray, to the same concentrations of the 16 active ingredients, six commercial formulations and two co-formulants that were tested for amphibians. As with amphibians, these tests were initially designed to evaluate the effect of three concentrations of each substance, but for some of the substances, the highest concentration was not achievable even when using solvents and solvents+surfactants, hence in those cases only the treatments corresponding to 0.1xAR and 1xAR were tested (Table 11). In addition, in the assay with acetamiprid only the

lowest concentration (0.1xAR) was tested because it induced 100% of mortality in the earthworms, and, thus, the following highest concentrations were not tested.

Table 11: Treatment levels as application rates (xAR) used in the toxicity experiments with earthworms

Type	Substance	Experimental application rates used[d]
Active ingredients	Metrafenone	0.1x 1x 10x
	Oxathiapiprolin	0.1x 1x 10x
	Benzovindiflupyr	0.1x 1x
	Azoxystrobin	0.1x 1x
	Tebuconazole	0.1x 1x
	MCPA	0.1x 1x
	Fluazifop-p-butyl	0.1x 1x
	Isoxaben	0.1x 1x
	Pendimethalin	0.1x 1x
	Metsulfuron-methyl	0.1x 1x 10x
	Mesotrione	0.1x 1x 2.49x
	Pirimicarb	0.1x 1x 10x
	Acetamiprid	0.1x
	Flupyradifurone	0.1x 1x 10x
	Alpha-cypermethrin	0.1x 1x
	Lambda-cyhalothrin	0.1x 1x
Formulations	Vivando	0.1x 1x 10x
	Quadris	0.1x 1x 10x
	Folicur 25 EW	0.1x 1x 10x
	Fusilade Max	0.1x 1x 10x
	Sivanto Prime	0.1x 1x 10x
	Fasthrin 10 EC	0.1x 1x 10x
Co-formulants	Naphtha	0.1x 1x 10x
	N,N-dimethyldecanamid	0.1x 1x

For over-spraying the earthworms, approximately three to four hours before this procedure, earthworms were washed with deionised water and placed in Petri dishes so they could void their gut contents. Aspersion of organisms was made by using the same equipment as that used for juveniles of *P. perezi*, at a rate of 40 ml/m² for all concentrations and controls. After aspersion, each earthworm was transferred to a Petri dish, filled with a filter paper of 85 g/m², 0.2 mm thick, and a diameter of 70 mm, moistened with 1 ml of deionised water. One organism was placed by Petri dish, which constituted a replicate. Five replicates were set per concentrations and control. The assay took place for 72 hours at 23 ± 1°C in total darkness. Mortality of organisms was checked each 24 h, and an earthworm was considered dead when it did not respond to a gentle mechanical stimulus for 15 seconds. At each 24 h deionised water was added to the filter paper, to avoid its dryness. At the end of the assay (72 h) mortality was registered and all alive earthworms were weighted (to the nearest 0.0001 g). The exposure procedures were adapted from the guideline Earthworm, Acute Toxicity Tests (OECD 1984).

5.1.4 Data analysis

Cumulative survival was compared among treatments using a long-rank test. Weight values were compared among treatments of the same compound and the respective controls by using univariate analysis of variance. Normal distribution and homoscedasticity of the data was checked with Kolmogorov-Smirnov and Bartlett's tests, respectively. Significance was set at $p < 0.05$.

5.2 Results

5.2.1 Mortality and external lesions

Considering all the performed controls, both negative and solvent ones, no significant mortality was registered in *E. andrei* exposed to these treatments.

Acetamiprid was among the most toxic tested active ingredients to *E. andrei*; the over-spray of earthworms with 0.1xAR resulted in 60 and 100% of mortality after 24 h and 48 h of exposure, respectively. Alterations in the skin (e.g. swelling, presence of structures filled with a liquid) were observed 24 h after the over-spay with acetamiprid (Figure 68). Given such a high mortality at 0.1xAR, the two other concentrations (1xAR and 10xAR) were not tested for this substance.

Figure 68: Images of adults of *Eisenia andrei*, 24 h after being over-sprayed with deionised water (a), and with 0.1xAR of acetamiprid (b and c)

Alterations observed in the skin of the organisms are indicated with yellow arrows. Cli: clitellum.

For all the other tested active ingredients and co-formulants, no mortality was registered, except for isoxaben and pirimicarb, where one organism died in each of the following treatments: 0.1xAR and 1xAR of isoxaben and 10xAR of pirimicarb. To note that although oxathiapiprolin induced no mortality in *E. andrei*, it caused skin lesions in the organisms at 10xAR (Figure 69).

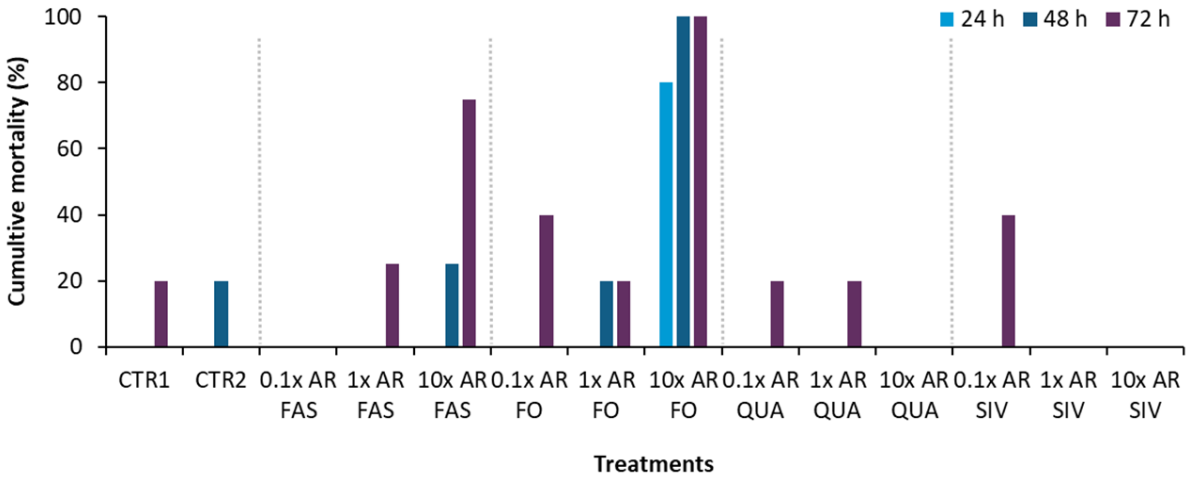
Figure 69: Images of adults of *Eisenia andrei*, 72 h after being over-sprayed with deionised water (a), and with 10xAR of oxathiapiprolin (b and c)

Alterations observed in the skin of the organisms are indicated with yellow arrows. Cli: clitellum.

As for the commercial formulations, over-spray with Fusilade Max and Vivando did not impair the survival of earthworms. As well, exposure to Quadris did not significantly impair the survival of earthworms, even though one organism died at 0.1xAR and another one at 1xAR (Figure 70; p = 0.550). Though, exposure to Fasthrin 10 EC, Folicur 25E W, and Sivanto Prime reduced the survival of *E. andrei* (p ≤ 1.25e-07), reaching mortality values of 75% for 10xAR of Fasthrin 10 EC, 100% at 10x AR of Folicur 25 EW, and 40% at 0.1xAR of Sivanto Prime (Figure 70).

Figure 70: Cumulative mortality of *Eisenia andrei*, 24, 48 and 72 hours after overspray with 0.1x, 1x and 10x of the application rate (AR) of four formulations

FAS: Fasthrin 10 EC, FO: Folicur 25 EW, QUA: Quadris, SIV: Sivanto Prime. Bars from light to dark blue show the cumulative mortality at 24, 48 and 72 hours of exposure, respectively. CTR1 corresponds to the control of the assay with FAS, FO and QUA, while CTR2 corresponds to the control of the assay done with SIV. Source: own illustration, [University of Aveiro].

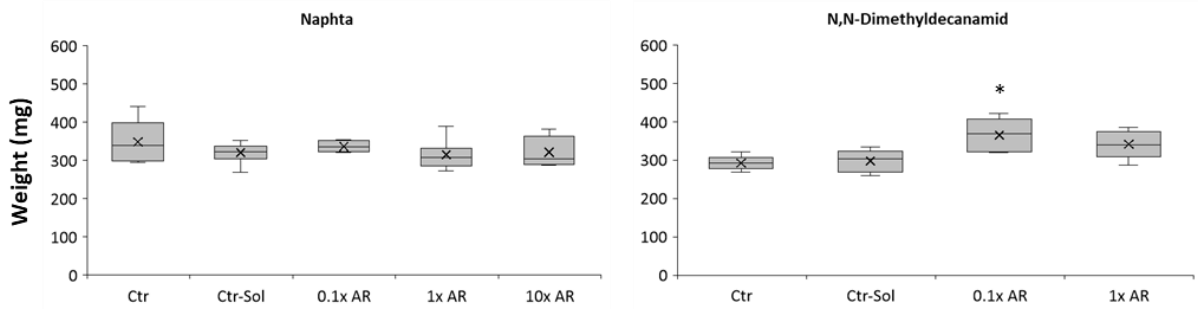


5.2.2 Weight

Among co-formulants, naphtha had no effects on *E. andrei* weight. Earthworms over-sprayed with 0.1xAR of N,N-dimethyldecanamid exhibited a higher body weight comparatively to those from the negative and control solvents ($p = 0.016$), but those exposed to 1xAR showed body weights not significantly different from the controls ($p = 0.138$) (Figure 71).

Figure 71: Weight of *Eisenia andrei* adults, 72 hours after overspray with 0.1x, 1x and/or 10x of the application rate (AR) of two co-formulants

Box plots show the mean (x), median (central line), 25th and 75th percentiles (bottom and top of boxes) and 1.5 interquartile range (bottom and top of line). Ctr: Control (deionised water); Ctr-Sol: Control solvent with 1% of acetone; Ctr-Sol-Trit: Control with 1% acetonitrile and 0.25% of Triton X. Asterisks indicate significant differences from Ctr-Sol (at p < 0.05). Source: own illustration, [University of Aveiro]..



Nine of the tested active ingredients (the fungicides metrafenone, azoxystrobin, benzovindiflupyr and oxathiapiprolin, the herbicides fluazifop-p-butyl, isoxaben, MCPA and metsulfuron-methyl, and the insecticide pirimicarb) caused no significant effects on the weight of *E. andrei* ($p \geq 0.073$). For the active ingredients that induced significant changes in weight of *E. andrei*, a clear dose-response pattern was not always observed. Specifically, lambda-cyhalothrin and pendimethalin caused a significant reduction in weight at 0.1xAR ($p \leq 0.027$) but induced no significant weight changes at 1xAR ($p \geq 0.057$), comparatively to their corresponding solvent controls (Figure 72).

A dose-response pattern was observed on the influence of alpha-cypermethrin and tebuconazole on the weight of *E. andrei*. Earthworms over-sprayed with 0.1xAR and 1xAR of each these two substances showed a significantly lower body weight comparatively to those exposed to the respective solvent controls ($p \leq 0.016$; Figure 73). Contrarily to the pattern observed for amphibians, where the effects of formulations were, in general, stronger than those of the active ingredients, it is interesting to notice that, despite those effects induced by the active ingredients alpha-cypermethrin and tebuconazole on the weight of earthworms, their commercial formulations (Fasthrin 10 EC and Folicur 25EW, respectively) caused no changes in exposed earthworms at 0.1xAR and 1xAR compared to the controls (Figure 73). Flupyradifurone induced a significant increase in body weight of *E. andrei* at 0.1xAR ($p = 0.035$), but no significant alterations in weight were observed at 1x and 10xAR ($p \geq 0.568$); this pattern was also observed in the weight of earthworms exposed to the flupyradifurone-based formulation Sivanto Prime, with significant effects at low exposure treatments (0.1xAR and 1xAR) but not at 10xAR among ($p \leq 0.004$; Figure 73).

Figure 72: Weight of *Eisenia andrei* adults, 72 hours after overspray with 0.1x, 1x and/or 10x of the application rate (AR) of nine pesticide active ingredients

Box plots show the mean (x), median (central line), 25th and 75th percentiles (bottom and top of boxes) and 1.5 interquartile range (bottom and top of line). Ctr: Control (deionised water); Ctr-Sol: Control solvent with 1% of acetone; Ctr-Sol-Trit: Control with 1% acetonitrile and 0.25% of Triton X. Asterisks indicate significant differences from Ctr-Sol (at p < 0.05). Source: own illustration, [University of Aveiro].

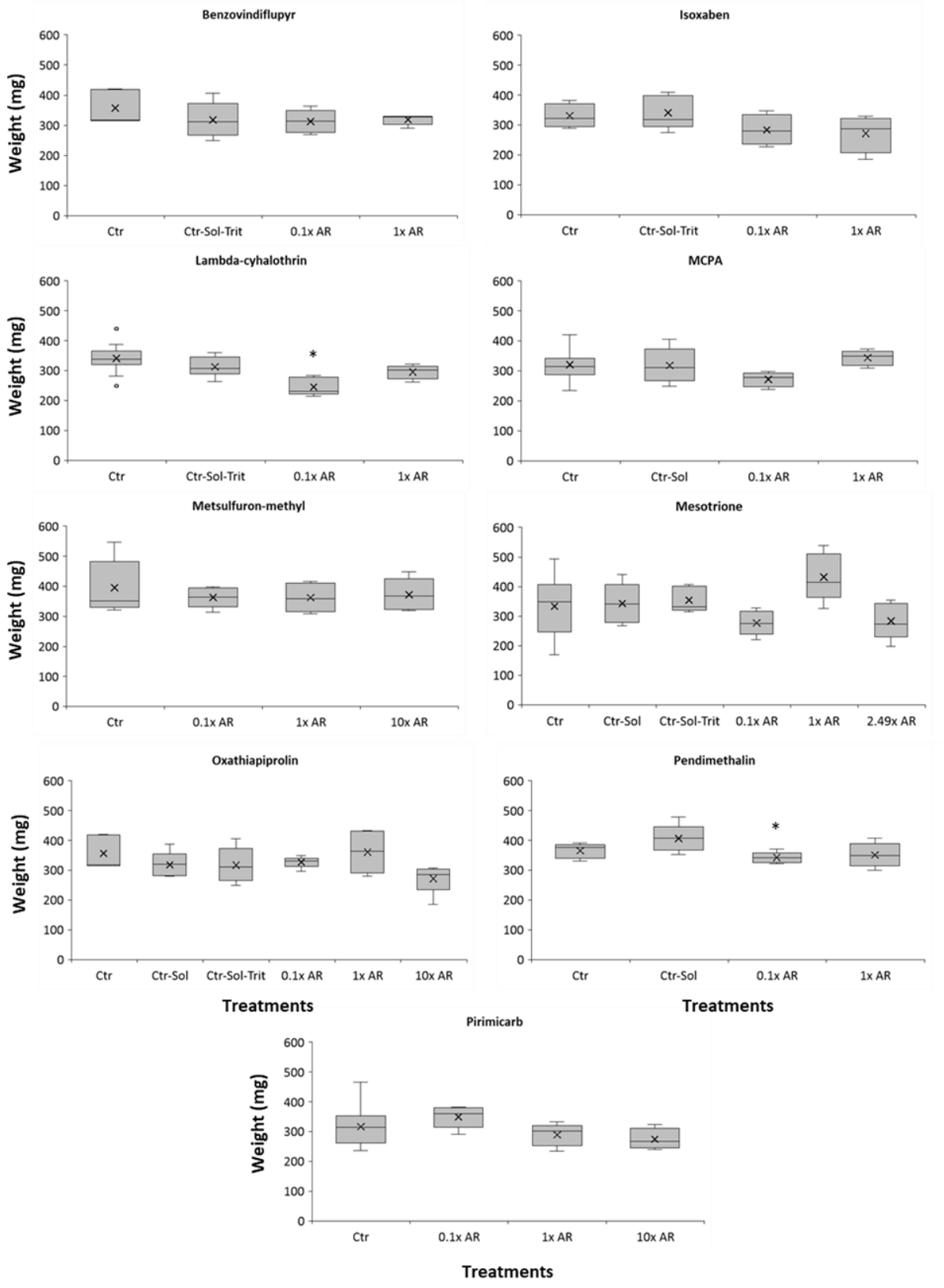
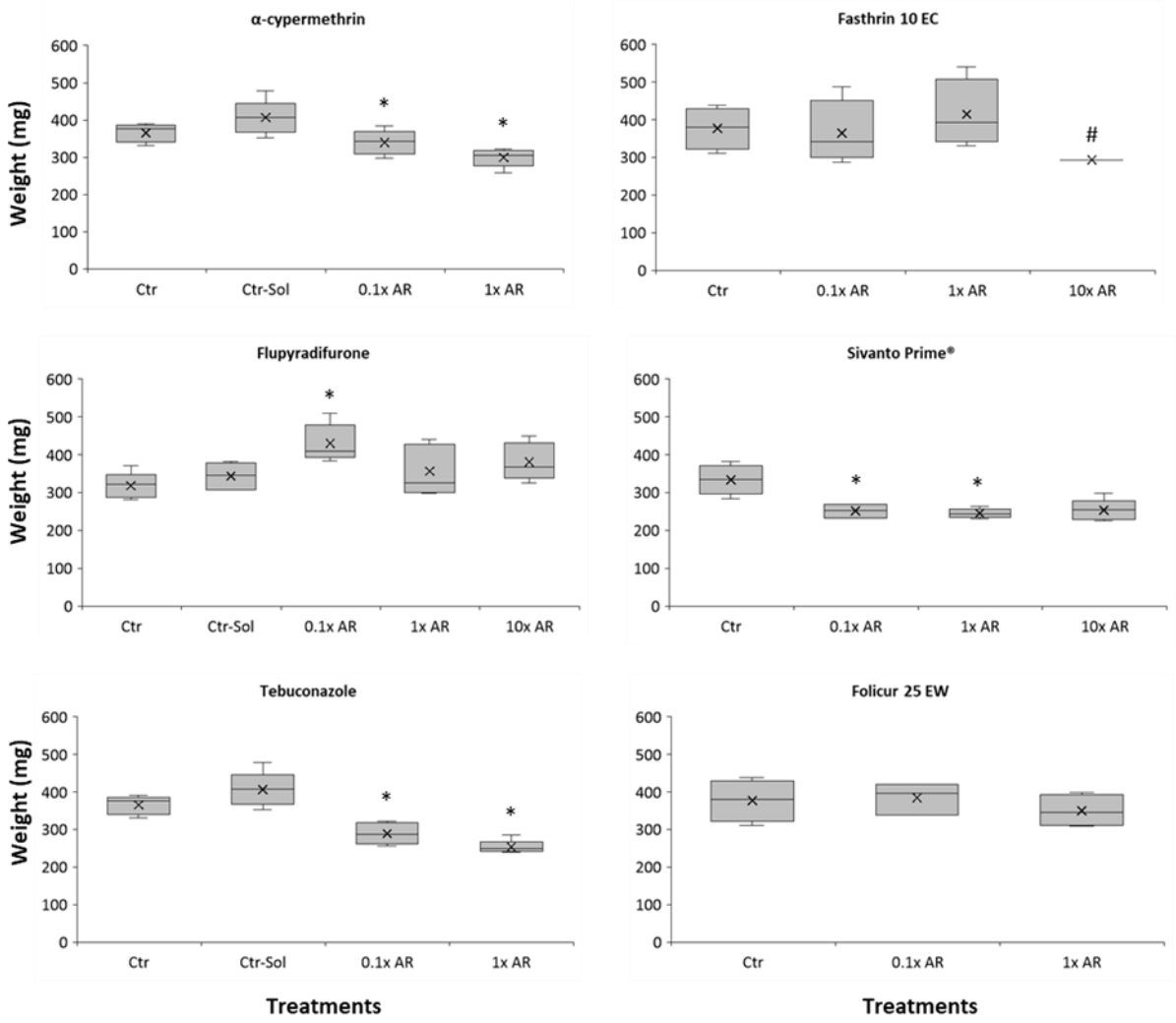


Figure 73: Weight of *Eisenia andrei* adults, 72 hours after overspray with 0.1x, 1x and/or 10x of the application rate (AR) of alpha cypermethrin, flupyradifurone, tebuconazole and their formulations Fasthrin 10 EC, Sivanto Prime and Folicur 25 EW, respectively

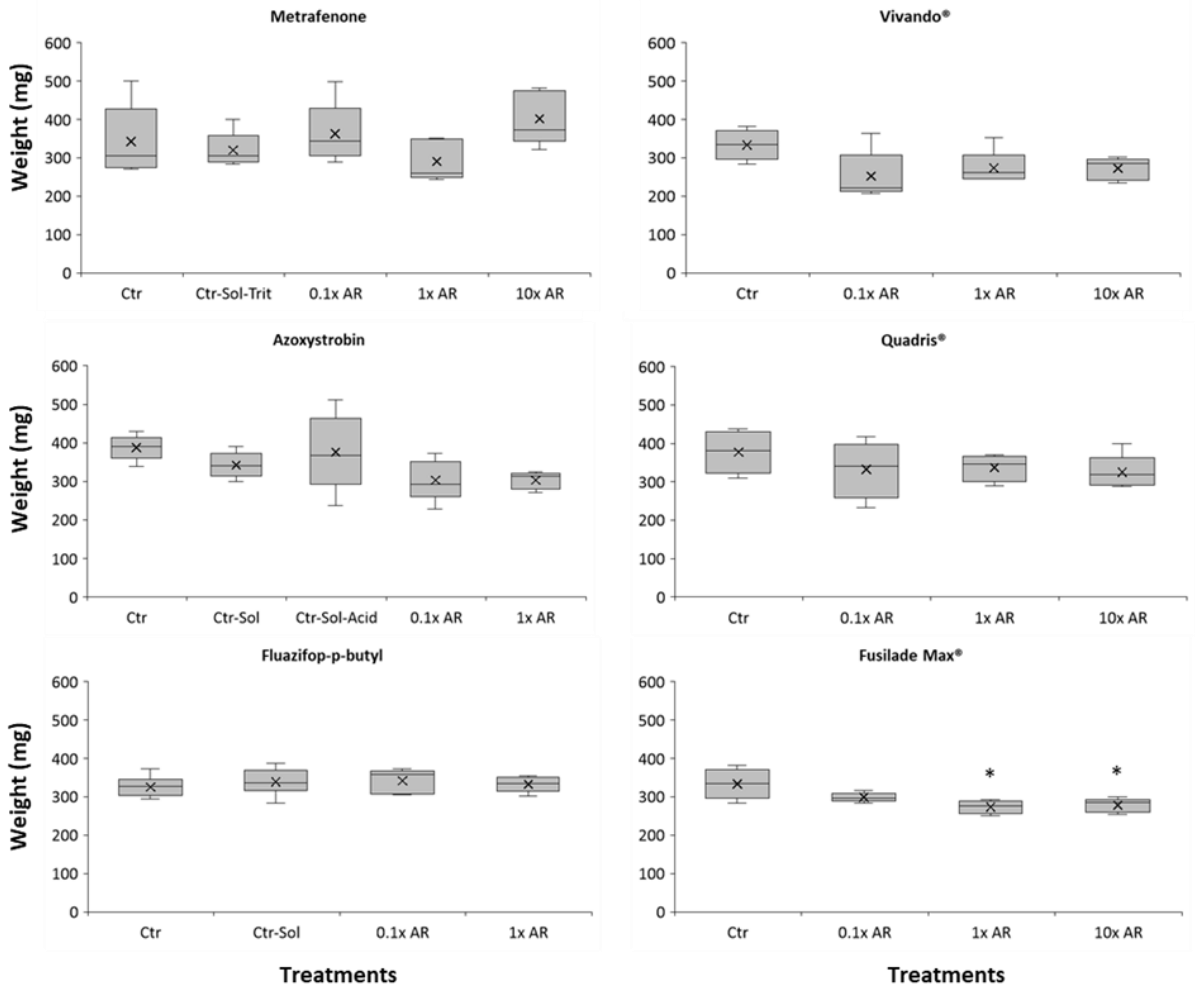
Box plots show the mean (x), median (central line), 25th and 75th percentiles (bottom and top of boxes) and 1.5 interquartile range (bottom and top of line). Ctr: Control (deionised water); Ctr-Sol: Control solvent with 1% of acetone. Asterisks indicate significant differences from Ctr-Sol (for active ingredients) or from Ctr (for formulations) (at p < 0.05). # indicates that the reported value refers to the length of only 1 organism). Source: own illustration, [University of Aveiro].



Regarding the weight of earthworms exposed to the rest of commercial formulations, Fusilade Max caused a significant reduction of this parameter for organisms over-sprayed with 1xAR and 10x AR ($p \leq 0.008$; Figure 74). Neither Vivando nor Quadris induced significant effects on the weight of exposed earthworms ($p \geq 0.602$).

Figure 74: Weight of *Eisenia andrei* adults, 72 hours after overspray with 0.1x, 1x and/or 10x of the application rate (AR) of metrafenone, axoxystrobin, fluazifop-p-butyl and their formulations Vivando, Quadris and Fusilade Max, respectively

Box plots show the mean (x), median (central line), 25th and 75th percentiles (bottom and top of boxes) and 1.5 interquartile range (bottom and top of line). Ctr: Control (deionised water); Ctr-Sol: Control solvent with 1% of acetone; Ctr-Sol-Trit: Control with 1% acetonitrile and 0.25% of Triton X; Ctr-Sol-Acid: Control with 1% acetonitrile and 0.1% orthophosphoric acid. Asterisks indicate significant differences from Ctrl (at p < 0.05). Source: own illustration, [University of Aveiro].



6 Synthesis and perspectives

6.1 Synthesis of experimental results

6.1.1 Inter-laboratory variability

One active substance and one formulated product were selected for inter-laboratory variability, metsulfuron-methyl and Folicur 25 EW. Metsulfuron-methyl increased mortality among frogs exposed to the highest overspray treatment (10xAR) in both laboratories, although in both cases the increase was not enough to reveal significant effects (Figure 15). Body condition was increased among frogs exposed to the herbicide in both laboratories, although the treatment causing such effect differed among sites (10xAR at UA and 1x AR at IREC). Folicur 25 EW reduced survival at both laboratories, despite some differences in the output across treatments (Figure 36). At UA, it was the combined exposure to the fungicide through both overspray and contaminated soil what caused a quick mortality of all exposed frogs, whereas at IREC, mortality at this combined treatment was higher than that of controls although in a non-significant manner. At IREC, however, the exposure to the same application rate as in the combined treatment by any of the single exposure routes significantly increased juvenile frog mortality. With regards to body condition of frogs exposed to Folicur 25 EW, the most noticeable effect was the increase in body condition of exposed animals on day 21 post-exposure.

Although some differences in the output of the compared treatments are noted between the two laboratories, there is a clear trend for repeatability of results. Metsulfuron-methyl increased mortality in a non-significant manner, whereas Folicur 25 EW was clearly lethal to frogs exposed to the application rates by either treatment in both labs, although small sample sizes can make the results to fall within or outside statistical significance because of small variations in the outcome of control or exposed treatments. Frogs exposed to both compared pesticides ended up with increased body conditions at both laboratories, probably because of a size-biased mortality towards smaller frogs in both cases (those with higher surface-to-volume ratio). In general, we believe that toxicity patterns are comparable between laboratories and that the combination of data generated from both sites is possible.

6.1.2 Apical endpoints

With a few exceptions, treatment of juvenile *P. perezi* with the different pesticides via overspray and/or dermal contact with treated surfaces did not cause lethal effects. Among active ingredients, significant mortality was found only in three out of the 16 tested substances: isoxaben, pirimicarb and lambda-cyhalothrin. It is noteworthy that a herbicide like isoxaben has caused lethal effects at a higher level than some of the expectedly more toxic insecticides like flupyradifurone, acetamiprid, or especially the pyrethroid alpha-cypermethrin. In the only study we are aware of that has evaluated the toxicity of isoxaben to amphibians Welch et al. (2010) did not find effects, when exposure happened via medium water, on developmental rate of on size and weight at metamorphosis in *Xenopus laevis*.

As explained above, pyrethroids have been flagged as being especially toxic to amphibians (Ortiz-Santaliestra et al. 2018), and in this context lambda-cyhalothrin is being revealed as one of the most toxic substances to juvenile *P. perezi*. However, the alpha-cypermethrin containing product Fasthrin 10 EC was the substance included in the present study having strongest lethal effects on frogs. A similar pattern is observed when comparing the effects of tebuconazole active ingredient and its formulation Folicur 25 EW, for which we observed a significant increase of

mortality when frogs were simultaneously exposed via overspray and through contact with treated soil. These findings support the importance of the formulations in determining toxicity. For the specific scenario of dermal uptake by amphibians, co-formulants favouring absorption are surely crucial in the determination of effects (Hedberg and Wallin 2010).

Despite these few examples of tested substances leading to increased mortality of juvenile *P. perezi*, our results on amphibian survival do not match, in general, with the severe damage that Belden et al. (2010) and Brühl et al. (2013) reported for certain pyraclostrobin-based formulations after overspray juveniles of *Anaxyrus cognatus* and *Rana temporaria*, respectively. Both studies found a quick, high mortality linked to the treatment; this magnitude of effects, either in time to death or in proportion of exposed animals who died, was not observed in our study even for the most toxoc substances. We have explored the possible sources of variation between our experiments and those reported in the referred papers in order to identify the reasons for the differential sensitivity:

- Species. Model species differ among the three experiments; whereas we used *P. perezi*, Brühl et al. (2013) used another Ranidae frog (*Rana temporaria*), while Belden et al. (2010) used a toad species (*Anaxyrus cognatus*) belonging to a different family (Bufonidae). We are not aware of patterns of differential toxicity to pollutants involving these species, but interspecific differences in sensitivity in amphibians are known to exist. If species were a source for the differential sensitivity, our conclusion should point to the necessity of determining a sensitive amphibian model for dermal toxicity testing, which, with the currently available information, is not possible. Actually, species sensitivity distributions created to compare tadpole sensitivity to waterborne pollutants did not show any phylogenetically-related trend in differential toxicity (Ortiz-Santaliestra et al. 2018), which supports the fact that an amphibian model of particular sensitivity to chemicals in general cannot be determined.

- Animals' body mass and age. Our individuals were tested 10 days after the end of the metamorphosis. They weighed between 0.5 and 2.5 g and measured between 12 and 27 mm , depending on the laboratory and the batch. Neither Belden et al. (2010) nor Brühl et al. (2013) provided information about the size or age of the animals they used. Belden et al. (2010) stated that they collected juveniles from the wild in July and tested them in September, which suggests that they were probably older than those used by us. However, bufonids use to reach metamorphosis with a smaller size than ranids, hence we cannot determine whether toadlets used by Belden et al. (2010) were different in size from our froglets. With regards to Brühl et al. (2013), they collected juveniles in August but did not specify anything about their size or the time they were maintained in captivity. Size could be a source of variation, with smaller animals suffering from stronger effects because of the increased surface-to-volume ratio. However, available information does not allow for determining the influence of this parameter in the different sensitivity.

- Animals' origin. Although we have all worked with wild-collected animals, there is an important difference between our study and the other ones. We collected animals as embryos an grew them in the lab, while Belden et al. (2010) and Brühl et al. (2013) collected animals from the wild when they were already in the juvenile stage. This can account for a reduced genetic diversity in our study, since our animals come from a few clutches whereas juveniles form the wild were probably coming from a larger number of cohorts (given the high natural mortality affecting amphibian pre-metamorphic stages). A reduced genetic diversity could determine a reduced plasticity to deal with contaminant exposure. On the other hand, the fact that our animals were grown in the lab reduces the chances of them

having suffered previous environmental stress, whereas wild-collected juveniles may have arrived to the experiment affected by current or recent infections, parasitisation, predatory stress or food shortage. In this context, at least Belden et al. (2010) maintained the animals in captivity for several weeks after collection before exposing them, whereas Brühl et al. (2013) apparently exposed frogs shortly after collection. The influence of external pressures having affected the populations of origin used by Belden et al. (2010) and Brühl et al. (2013) could result in collecting selected individuals that had survived to those pressures. Despite this option, we still believe that the degree of stress affecting wild-collected juveniles should make them more sensitive than if animals had been raised in the lab from their embryonic stage.

- Tested product. This is an obvious source of variation, and the main question to answer to determine why our results differ from those by the other studies. If the high acute toxicity observed by Belden et al. (2010) and Brühl et al. (2013) is something more or less exclusive of the tested pyraclostrobin-based formulation, it would be appropriate to test that formulation also in our model species, *P. perezi*. Therfore, this becomes a priority in order to contextualize the results of the present project and understand their applicability to the PPP regulatory framework.

- Test duration. Even if duration was different among studies, it should not be a source of variation. Belden et al. (2010) found 100% mortality after 72 h, whereas Brühl et al. (2013) did so on day 7 post-exposure. Should those temporal patterns have appeared in our study, we would have detected them.

- Application method. Brühl et al. (2013) used a laboratory track sprayer similar to what is used for simulation in agronomical or chemical industry. Belden et al. (2010) used an atomizer designed for medical use. Whereas none of these studies mentioned whether the application methods were calibrated, we think that the application methods used both in their studies and in ours are equally valid and reliable.

- Enclosures. Another important difference is where the animals were sprayed. For the overspray, we placed animals in petri dishes and then moved them to clean soil, whereas both Belden et al. (2010) and Brühl et al. (2013) treated animals and soils together. Therefore, their designs would compare to our soil+overspray treatments, which did not cause a significant mortality in the majority of cases but where we did not test the highest application rate. Nevertheless, mortality caused by pyraclostrobin in both studies happened also at the medium application rate, which is what we used in our soil+overspray treatments.

- Environmental conditions. The three studies were conducted under controlled conditions, which were very similar in all cases. The existing differences (e.g. temperature) are assumed to be consequence of the use of different species and in search of the optimal ranges in each case. The source water is very likely to be different, but even in this case it is difficult to assume that the differential sensitivity can be because of the source water, provided it was controlled for sanitization in all cases.

In summary, a follow-up study should focus on determining the toxicological sensitivity of our study models to the pyraclostrobin formulation Headline that killed the animals tested by Belden et al. (2010) and Brühl et al. (2013). This should serve us to better calibrate the representativeness for the whole amphibian group of the results obtained for the products tested in this study.

6.1.3 Histological analysis

The anatomical region of reference and the ROI used in the image analysis (see Figure 10) seem to include most of the parameters that should be evaluated by a histological analysis in a toxicological risk assessment of amphibian integument.

The clearest response to pesticides at the histological level is the increase in EI2 observed for seven out of the 16 analysed PPP. EI2 is the ratio between the epidermal area in the ROI and its perimeter, hence it is representative of either an epidermal thickness or of a loss of integrity that leads to irregularities in the structure (Figure 57). Increased skin thickness as a response to dermal contact with pesticides could be interpreted as a protective response to reduce absorption; it is understandable that skin resistance to absorption is directly proportional to its thickness, but whether organisms can respond to the presence of xenobiotics by increasing skin thickness remains unknown. It is noteworthy that only for Quadris the skin thickening came along with an increase of EI1 (i.e. decreased cellular density or increased average area per cell), suggesting hypertrophy, vacuolization, intracellular oedema/spongiosis or cell loss; in the rest of cases, the density of cells in the epidermal area (inverse to EI1) was unaffected by the eventual increase in the ratio area/perimeter (EI2), suggesting hyperplasia. Diffusion of contaminants through the epidermal layer can follow the transcellular (i.e. through the cells) or paracellular (i.e. between the cells) routes, apart from the localized absorption through pores or injuries. Paracellular absorption is easier as the number of barriers through which the chemicals must diffuse is lower than in the transcellular route, but when the epithelium is properly organized, the paracellular route may be restricted to small-sized molecules only (e.g. Horowitz et al. 2023). It is, therefore, a normal response to prevent dermal absorption of contaminants to increase skin thickness (increased EI2) by accumulating epithelial cells in the area (no changes in cell density, or in EI1).

The exposure of amphibian skin to the environment is particularly high if compared to other vertebrates that possess thicker or stronger protective structures. In addition, amphibian skin morphology must adapt to the external conditions, and so changes in the environment can result in modifications in skin thickness, pigmentation or gland morphology (Niu et al. 2024). Amphibian skin has therefore a plasticity, hence it is assumable that contact with certain chemical substances may also induce some of these changes. However, amphibian skin functions not only as barrier against the external medium, but also as an element involved in water balance, breathing or immunity (Çömden et al. 2023). These functions could be affected if the skin structure (e.g. thickness) is altered in response to chemical exposure to prevent from xenobiotic dermal absorption.

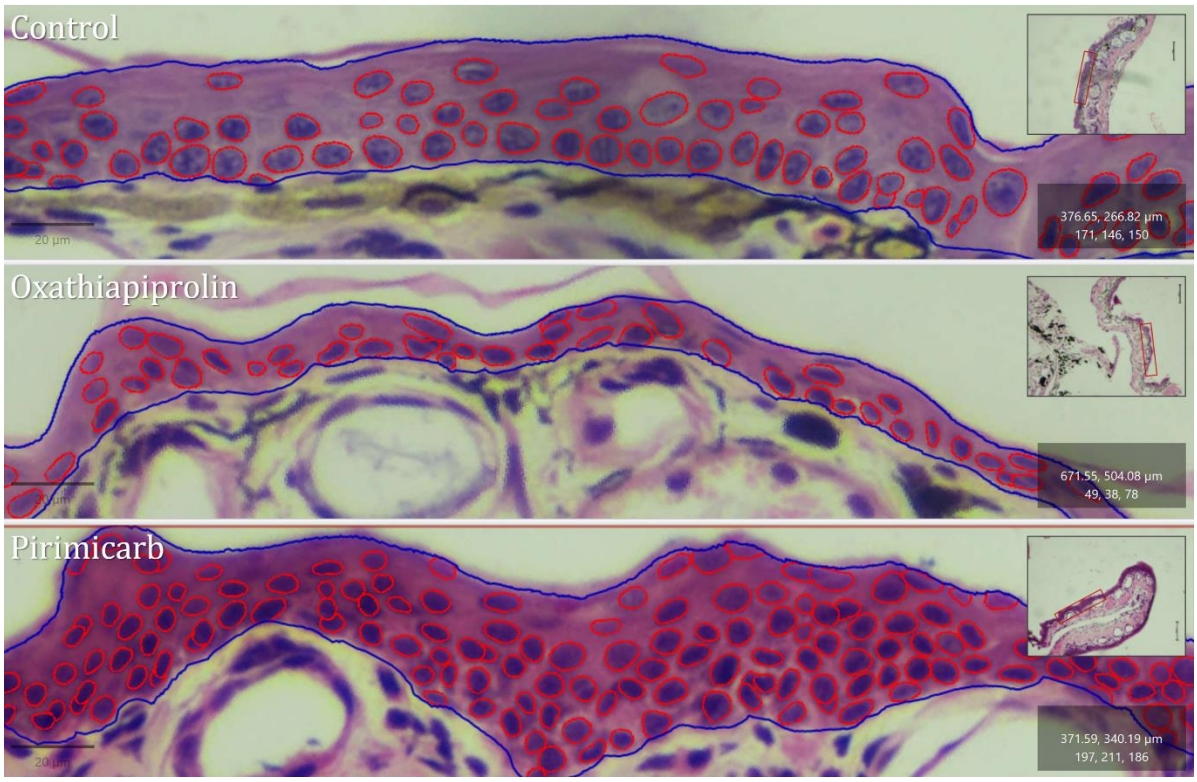
Another question to consider is the direct effect that substances, and in particular of co-formulants, have on the skin. Many formulations include substances to facilitate absorption. These substances are designed to act on teguments that are normally much less permeable than amphibian skins (e.g. plant surfaces, insect skins) and have efficient mechanisms to alter the skin structure and facilitate diffusion of chemicals through it (e.g. dimethylsulfoxide, or DMSO, can disorganize the structure of the cell membranes to open pores in the phospholipid bilayer; Marren 2011). In this context, frogs exposed to Fasthrin 10 EC showed a clear, dose-response increase in their thickness that was much stronger than what was noted in animals treated with the active ingredient only at the same concentration. Although pyrethroids have a high K_{ow}, which in principle facilitates the transit through the biological membranes, it has been observed

in mammalian skin that, even if they enter the skin, most of the applied substance remains in the outer layers (Hughes and Edwards 2010). Therefore, the role of co-formulants can be essential to help the active ingredients diffusing through the epidermis and reaching the more vascularized dermis. This would be consistent with the observation of a higher toxicity caused by Fasthrin 10 EC as compared to the active ingredient alpha-cypermethrin.

Finally, our observations regarding the EI1 have little consistency, with non-monotonic effects in some of the cases in which this index was affected. This index can be affected by para-physiological or pathological changes that alter the overall area occupied by cells. In addition, it may also be influenced by the area occupied by the epidermis; thus, changes in EI1 can be originated not only by modification in cell numbers but also by how the entire structure responds to this modification, leading to variations in EI1 value in one or another direction depending on how these two variables interact (Figure 75). A normalization of EI1 on the basis of the epidermal thickness (i.e. EI2) would help the interpretation of the index itself.

Figure 75: Comparison of epidermal area and cell count (StarDist method) between a control and two frogs exposed to 1xAR of oxathiapiprolin and pirimicarb

When compared to the control, the oxathiapiprolin-exposed individual has a similar number of cells, but these are smaller and occupy a thinner epidermis than the control. The pirimicarb-exposed individual, however, has an increased number of cells as compared to the control but without significantly enlarging the area occupied by these cells. In both treated frogs, the EI1 would be reduced relative to the control, but the origin of this change is a different response. The resolution of the QuPath viewer is matching, so all the images are represented at the same magnification to make them comparable.



In our experiments, exposure to flupyradifurone as active ingredient or as the formulation Sivanto Prime, as well as to oxathiapiprolin, seems to have generated and/or augmented pigmented melanomacrophage clusters at level of the dorsal area of the analysed anatomical region of reference. Histological analysis of pigmented melanomacrophage, melanocytes and melanine holds significant potential for assessing pesticide exposure risk in amphibians, as these

structures serve as biomarkers of immune function and environmental stress. These elements can be readily visualized and quantified due to their pigmentation, which makes them accessible for analysis via light microscopy, without the need for expensive reagents.

Studies have demonstrated that MMC metrics (e.g. aggregate size, pigmentation intensity, and number) respond to environmental stressors (Franco-Belussi et al. 2016), including pollutant exposure (Jantawongsri et al. 2015). However, these metrics can also vary because of other factors like sex, diet, season, and age (Steinel and Bolnick 2017), complicating the interpretation of results in unstandardized settings. Given this variability, eventual use of pigmented melanomacrophage in pesticide risk assessments for amphibians should carefully consider confounding factors and include appropriate positive controls to achieve a proper elucidation of the pesticide effects. These positive controls are essential to establish baseline histological responses and validate the sensitivity of the tests, and should include substances known to cause specific adverse effects, ensuring that the histological endpoints are functioning correctly. Taking into account integument toxicity, positive controls should adress specific types of damages like:

- Irritation: non-immunogenic local inflammatory reaction, which appears quickly after stimulation and disappears in principle after few days.
- Corrosion: skin tissue damage, including necrosis in the application site, usually irreversible.
- Sensitization: skin response of immunological nature after exposure to an active substance or a formulation.
- Induction: experimental exposure of a subject to the active substance tested with the aim of inducing a state of hypersensitivity.
- Challenge: Experimental exposure of a subject after an induction period to determine if a subject will exhibit a hypersensitivity reaction.

In this context, following (Llewelyn et al. 2019b), the following substances, known to have specific adverse effects on anuran skin, should be taken into consideration as positive controls:

- Propylene glycol: irritating, causing swelling of keratinocytes.
- Ethanol: corrosive and edemogen, causing loss of cellular outlines and separation of dermal fibrocytes.
- Lipopolysaccharides (LPS): immunogen molecules able to sensitize and provoke inflammation.

The assessment of amphibian skin glands, particularly mucous and granular glands, is a key aspect of histological analysis in pesticide exposure studies. These glands are crucial for functions such as cutaneous respiration, defense, and maintaining moisture. Histochemical techniques like haematoxylin-eosin staining, Alcian Blue (pH 2.5) for detecting sulphated and carboxylated glycosaminoglycans, and Periodic Acid-Schiff (PAS) staining for carbohydrates, allow researchers to analyze gland structure and function. In amphibians like *Pelophylax perezi*, evaluating the morphometric changes in these glands can reveal the impact of chemical exposures. Disruption in the production or secretion of mucus, for example, can indicate impaired skin function and increased susceptibility to environmental stressors. Thus, newly designed histological endpoints focused on gland morphology and secretion patterns should offer crucial insights into pesticides risk assessment of terrestrial amphibians.

Finally, it is important that quantitative metrics complement, rather than substitute, histopathological assessments. Quantitative and digital analyses can accelerate the evaluation process and provide valuable support in toxicological pathology, enhancing the interpretation and accuracy of findings when integrated with qualitative observations.

6.2 Pesticide properties driving amphibian risks

6.2.1 Evaluation based on sensitivity

In order to explore which properties of the tested active ingredients could be driving amphibian susceptibility, we started with a characterization of the observed effects that could help us standardizing the results obtained in the different experiments, and then including all the active ingredients in the same assessment. With this purpose, we assigned a toxicity level value to all tested substances: We included in this analysis also formulations and co-formulants because these will be used later in the comparison with earthworms (see section 6.4), but for the assessment of the properties driving toxicity of pesticide to amphibians we will use active ingredients only. The toxicity level values for *P. perezi* are shown in Table 12.

Using these toxicity level values, we explored the influence that the compiled physico-chemical and toxicological properties of pesticides (see Table 1 in section 3.2.1) could have on apical effects shown by amphibians. Formulations were excluded from this analysis. Within the properties dataset, there were four missing values in four different parameters; two of these parameters (DT_{50} in water-sediment systems for the whole system and 21-day NOEC in *Daphnia magna*) were excluded from the model, as including them would have completely excluded the active substances for which values were missing. For the other two, however, we managed to fill the blanks; the K_{foc} value for fluazifop-p-butyl was estimated from the K_{oc} for that substance, whereas a BCF for flupyradifurone was obtained from Huang et al. (2022) as an average of the calculated BCF for two aquatic arthropods. In addition, we also excluded the pKa from these models as its pseudo-constant distribution exerted a strong influence in the entire model output.

To avoid a model over-parameterization, we run a Principal Component Analysis (PCA) with the remaining 22 parameters, which were previously log-transformed. We extracted three principal components (PC), or dimensions that are explained by those original variables that associated among them. The correlation coefficients of each original variable with the extracted PC are shown in Table 13. The extracted PC explained 73.9% of the total variability of the system defined by the 22 original variables.

Table 12: Rank of tested substances according to the apical effects recorded in *Pelophylax perezi* juveniles

Each block is assigned a consecutive numerical value as a function of observed effects.

Substance	Effect	Toxicity level
Fasthrin 10 EC	Significant mortality at 0.1xAR after 21d	12
Pirimicarb	Significant mortality at 1xAR after 7d	11
Isoxaben Folicur 25 EW	Significant mortality under route combination (or less than significant if single route) after 7d	10
Lambda-cyhalothrin	Significant mortality under route combination after 21d (less than significant after 7d)	9
Quadris	Less than significant mortality at 1x AR after 21d	8
Alpha-cypermethrin Naphta	Less than significant mortality at 10x AR after 21d	7
Mesotrione Tebuconazole	Reduced body condition after 7d at 0.1x AR	6
Oxathiapiprolin Pendimethalin	Reduced body condition after 7d at 1x AR (either in soil or overspray)	5
Metrafenone	Reduced body condition at 0.1xAR after 21d	4
Fusilade Max	Reduced body condition at 1xAR after 21d	3
Azoxystrobin	Reduced body condition under route combination after 21d	2
Benzovindiflupyr MCPA Fluazifop-p-butyl Metsulfuron-methyl Acetamiprid* Flupyradifurone Vivando Sivanto Prime N,N-dimethyldecanamid	No effects or increased body condition	1

*A less than significant increase of mortality was found among frogs exposed to 0.1xAR acetamiprid. However, since none of the higher levels (1xAR or 10xAR) caused such response, we cannot consider it as a consistent effect.

This variable reduction permitted us to use those PC instead of the original variables, among some of which there was a strong collinearity that would have affected the model results. Thus, we ran a GzLM with a Poisson distribution of the amphibian toxicity index, that was used as the response variable. The model included as covariates the scores of the three extracted PC (i.e. the regression-calculated values that each active substance would have adopted in the "new" variable that represent each PC). The GzLM was run using a backwards selection procedure without including interactions between covariates. The best-fitted model was selected as the one with the lower Akaike's Information Criterion.

The selected model included the PC1 as the only covariate significantly explaining the variations in the amphibian toxicity level values (Table 14). As shown by the regression coefficient between PC1 and toxicity level value, the association between both variables was negative (i.e. increasing values of PC1 would associate with reduced toxicity to juvenile frogs). Thus, considering the original parameters that were associated with PC1 and the sign of their correlation coefficient (Table 13), toxicity to amphibians would increase with increasing values of K_{ow}, K_{foc}, and BCF, and with decreasing values of ADI, solubility in water, LC_{50} in fish, NOEC in fish, EC_{50} in *Daphnia*, contact LD_{50} in bees and inhalation LD_{50} in rats.

Table 13: Correlation coefficient of each physico-chemical or toxicological parameter with the principal components (PC) extracted from the Principal Component Analysis conducted on those parameters

Absolute values indicate the strength of the association between the original variable and PC, from no correlation (0.000) to full correlation (1.000). Signs indicate whether the association is direct (positive values, meaning that the variable would increase for increasing variables of the PC) or inverse (negative values). Variables with coefficients with absolute values above 0.6 are considered as represented by the PC.

Variable*	PC1	PC2	PC3
ADI	0.692	0.487	0.329
AOEL	0.325	0.630	0.168
Molecular mass	-0.499	0.599	-0.061
Solubility in water	0.838	-0.515	0.076
K_{ow}	-0.770	0.375	0.016
Soil DT_{50}	-0.199	0.463	0.826
Water DT_{50}	0.634	-0.069	0.683
K_{foc}	-0.929	0.226	-0.055
BCF	-0.800	0.281	-0.129
Oral LD_{50} mammals	0.572	0.689	-0.234
Oral LD_{50} birds	-0.333	0.681	-0.220
NOEC birds	0.489	0.585	-0.276
LC_{50} fish	0.906	-0.288	-0.127
NOEC fish	0.908	-0.207	-0.097
EC_{50} *Daphnia*	0.883	-0.090	-0.189
Contact LD_{50} bees	0.801	0.339	0.071
Oral LD_{50} bees	0.561	0.520	-0.061
LC_{50} earthworms	0.142	0.566	0.142
Dermal LD_{50} mammals	0.490	0.495	-0.105
Inhalation LD_{50} mammals	0.691	0.342	-0.307

*The variables were log-transformed prior to analysis

Table 14: Results of the best-fit generalized linear model to test the influence of the scores of the extracted principal components (PC) on amphibian toxicity level values

Correlation of the PC with the original variables are shown in Table 13. The dependent variable was adjusted to a Poisson distribution. The hypothesis contrast from the generalized linear model includes the information for the intercept and the PC1, selected as significantly explaining amphibian toxicity values. For each model term (intercept and PC1) the non-standardized regression coefficient (β) along with its standard error and 95% confidence intervals, is shown. The negative sign of the β for PC1 indicates that the association between PC1 scores and amphibian toxicity level is inverse (i.e. increasing PC1 scores indicate higher toxicity).

Source of variation	Hypothesis contrast			Regression parameter estimates		
	Wald's X^2	d.f.	p	β	Std. error	95% confidence interval
Intercept	137.930	1	<0.001	1.447	0.1232	1.206 – 1.689
PC1 scores	7.261	1	0.007	-0.287	0.1064	-0.495 – -0.078

Increased K_{ow} and BCF, and decreased water solubility are consistent parameters. Our results show that lipophilic substances would have higher potential to compromise juvenile frog survival and growth, which can be related with the higher potential of these substances to diffuse through body membranes. This result matches what has been reported by previous studies relative to the influence of K_{ow} and BCF in determining bioaccumulation of pesticides by terrestrial amphibians (Quaranta et al. 2009, Van Meter et al. 2015), hence the potential of these substances to cause toxic effects. The fact that substances with higher sorption coefficients (K_{foc}) would be more toxic to amphibians than those with the opposite characteristic, provided all soils used in the experiments had the same organic carbon content, would suggest that an important part of the toxicity could be related to the persistence of the substances after overspray. The influence of the sorption coefficient in determining absorption of chemical substances through amphibian skin has also been shown in previous studies (Van Meter et al. 2014, Van Meter et al. 2016). The rest of the selected parameters link the sensitivity shown by the *P. perezi* juveniles with that reported for other species. It must be stressed that, in general, sensitivity of aquatic species seems a better predictor than sensitivity of terrestrial ones, as the three considered toxicological parameters from aquatic species (LC_{50} in fish, NOEC in fish and EC_{50} in *Daphnia*) arose as potentially influential variables, while most of the toxicological parameters from terrestrial species did not. It is also noteworthy that the three selected parameters that are representative of toxicological sensitivity of terrestrial refer to different exposure routes: oral (ADI), dermal (contact LD_{50} in bees) and inhalation (inhalation LD_{50} in rats). ADI is a wide indicator of toxicity of a substance, while contact LD_{50} in bees could suggest that substances that are readily absorbed by the tegument of bees are also those easily diffusing through amphibian skin. Regarding inhalation, the susceptibility of terrestrial amphibians to substances that are toxic by this route has already been suggested (Adams et al. 2021); as skin acts as a respiratory organ in these animals (Burggren and Moallf 1984), overspray of substances that are toxic by inhalation could be especially detrimental to them.

In order to have a more quantitative approach of the influence of the different parameters on amphibian sensitivity to PPP, we ran a multiple linear regression including the physico-chemical and toxicological properties as explanatory variable. This regression was performed following a step by step forward selection procedure, so in this case it was not necessary to reduce the original variables; the step forward procedure introduces the variables one-by-one, considering

at each step the collinearity with the variables that have already entered the regression. This way, the selected variables are relatively independent from each other. The regression model was conducted on the log-transformed variables to minimize scale effects.

The EC_{50} in *Daphnia* was the only parameter selected in the multiple linear regression. The regression model returned the following equation:

$$\text{Toxicity reference value } = 4.367 - 0.962 \times \log(EC_{50} \text{ in } Daphnia)$$

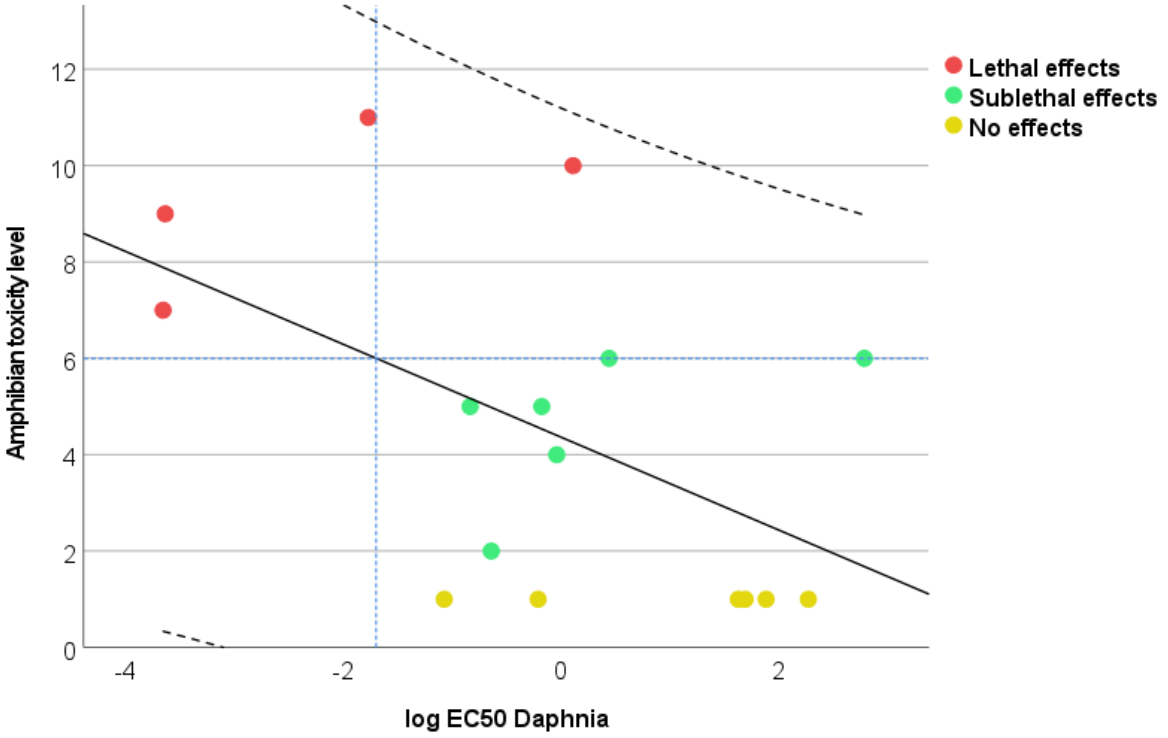
If we consider that lethal effects, according to the toxicity values defined in Table, correspond to values higher than 6, a threshold value of the EC_{50} in *Daphnia* could be inferred from the equation above to predict lethal effects of an active substance if applied on juvenile *P. perezi* via overspray, as follows:

$$\text{Threshold } EC_{50} \; in \; Daphnia = 10^{\frac{-1.633}{0.962}} = 0.02 \text{ mg/l}$$

Thus, special regard should be paid to substances with 48h-EC_{50} values for *Daphnia* ≤ 0.02 mg/l in what refers to their toxicity to terrestrial amphibian via overspray (Figure 76). If we return to the PPP database that we elaborated to select the substances to include in the present study (see section 3.2.1), 28 out of the 250 substances included therein would have fallen below this threshold (listed in the Appendix D). The majority of those substances are, as expected, insecticide, and it is also logical that three out of the four active ingredients that caused some mortality in our experiments appear in that list (pirimicarb, lambda-cyhalothrin and alpha-cypermethrin, with isoxaben as the only substance having caused mortality to frogs while having an EC50 for Daphnia above the calculated threshold). What is more interesting is that pyraclostrobin also appears in that list, which reinforces the idea that using this threshold could have avoided the marketing of the formulation Headline that caused strong and quick mortality to juvenile amphibian (Belden et al. 2010, Brühl et al. 2013).

Figure 76: Biplot representing the 16 tested active ingredients as a function of their toxicity level value for amphibians and 48-EC_{50} for *Daphnia*

Substances are classified depending on whether they caused lethal effects, sublethal effects or no apical effects. The black line represents the linear regression model resulting from the selection of EC_{50} in Daphnia *as a significant parameter to explain variations in the toxicity level value (see equation in the text), including its 95% confidence intervals (black dashed lines). The blue-dashed lines identify the thresholds for lethality, defined as values above 6 for the amphibian toxicity level and, concurrently, values below -1.7 of the log EC_{50} Daphnia (= 0.02 for the EC_{50} Daphnia, in mg/l). Source: own illustration, [IREC-CSIC].*



6.2.2 Evaluation based on pesticide accumulation

Another approach in investigating the properties of the substance driving effects to amphibians was based on accumulation of pesticides measured in exposed animals. We calculated accumulation ratios from the measured concentrations in liver and skin samples and the concentration in the sprayed product as follows:

$$Accumulation\ ratio\ from\ spray = \frac{Concentration\ measured\ in\ tissue\ (ng/g)}{Concentration\ in\ the\ sprayed\ broth\ (mg/l)}\ x\ 1000$$

When the difference between nominal and measured concentrations in sprayed broths (see section 4.2.3) was outside the range of the percentage recovery and its RSD calculated for each given substance (see section 4.1.4), we used the measured concentration instead of the nominal one to calculate the accumulation ratio.

Also, accumulation ratios from soils were also calculated considering the residues in soil samples measured on day 7 post-exposure:

$$Accumulation\ ratio\ from\ soil = \frac{Concentration\ measured\ in\ tissue\ (ng/g)}{Concentration\ in\ the\ soil\ (ng/g)}\ x\ 1000$$

These accumulation ratios are not meant to act as bioconcentration factors or similar, but to represent the likelihood of the different substances of being absorbed by amphibians and accumulated in tissues. Likewise, a higher detection of the substances in the tissues does not necessarily involve stronger effects, as metabolism also plays a role that cannot be determined with the approach used in the present study.

Accumulation ratios were correlated with the list of physico-chemical and toxicological properties of the active ingredients listed in Table 1 (Pearson's correlations, all variables log-transformed). These correlations were run considering active ingredients only. For animals exposed via overspray, we considered the accumulation ratios calculated on day 7 of experiment relative to the sprayed products, whereas for animals exposed via contact with soil we considered the accumulation ratios calculated on day 7 of experiment relative to the both the sprayed products and the soils. Animals combining both exposure routes were not included in this analysis to avoid confounding factors of the route combination.

Table 15: Results of Pearson's correlation between accumulation ratios and physico-chemical and toxicological properties of the analysed pesticide active ingredients

All variables were log-transformed prior to analysis. Results include the correlation coefficient (R), the number of compared substances (N) and the p-value (p). Only significant correlations (p<0.05) are displayed.

Accumulation ratio	Exposure route	Variable	Tissue	R	N	p
From sprayed broth	Overspray	Oral LD_{50} in mammals	Liver	-0.614	12	0.034
			Skin	-0.743	12	0.006
		Dermal LD_{50} in mammals	Skin	-0.612	12	0.035
		Bird NOEC	Skin	-0.656	12	0.021
		Oral LD_{50} in bees	Liver	-0.788	12	0.002
			Skin	-0.761	12	0.004
	Contact with soil	Oral LD_{50} in mammals	Liver	-0.769	8	0.026
			Skin	-0.794	8	0.019
		Bird NOEC	Liver	-0.728	8	0.041
		Oral LD_{50} in bees	Liver	-0.864	8	0.006
			Skin	-0.875	8	0.004
From soil	Contact with soil	Oral LD_{50} in mammals	Liver	-0.946	10	<0.001
			Skin	-0.946	10	<0.001
		Oral LD_{50} in birds	Liver	-0.777	10	0.008
			Skin	-0.730	10	0.017

All the selected variables being significantly correlated with accumulation ratios referred to toxicological properties affecting terrestrial organisms, always with a negative sign, indicating that substances causing higher toxicity were those that accumulated at a higher rate in

amphibians exposed either by overspray or through contact with contaminated substrates (Table 15). In particular, accumulation ratios in frogs exposed via overspray (relative to concentrations in sprayed broths) were negatively correlated with the oral and dermal LD_{50} in mammals (the latter only for accumulation ratios calculated in skin), the avian NOEC (only for accumulation ratios calculated in skin) and the oral LD_{50} in bees (Figure 77). Accumulation ratios relative to the sprayed product concentration in frogs exposed via soil contact were negatively correlated with oral LD_{50} in mammals and in bees, and, for accumulation ratios calculated in the liver, also with the avian NOEC (Figure 78). Finally, accumulation ratios relative to the concentrations measured in soils were negatively correlated with the oral LD_{50} values for birds and mammals (Figure 79).

As reviewed in section 2.1, dermal absorption of substances in amphibians has been suggested to be influenced mostly by the K_{ow} and the K_{foc}/K_{oc}, although the information is limited and not always consistent among studies. Our results point no effect of any of these properties in the accumulation of the analysed pesticides in frog livers or skins. However, it is important to remember that pesticide residue analyses happen 7 or 21 days after spraying frogs or soils, and that there may have been a decline in these residues because of metabolism and excretion in animals or because degradation in soils.

Figure 77: Biplots representing significant associations between toxicological properties and accumulation ratios relating pesticide concentrations in sprayed broths with livers or skins of frogs exposed via overspray

The lines represent the adjustment of the linear regression model for each tissue and their 95% confidence intervals. Data correspond to tissue concentrations measured on day 7. To improve visualization, axes are depicted in logarithmic scale. Source: own illustration, [IREC-CSIC].

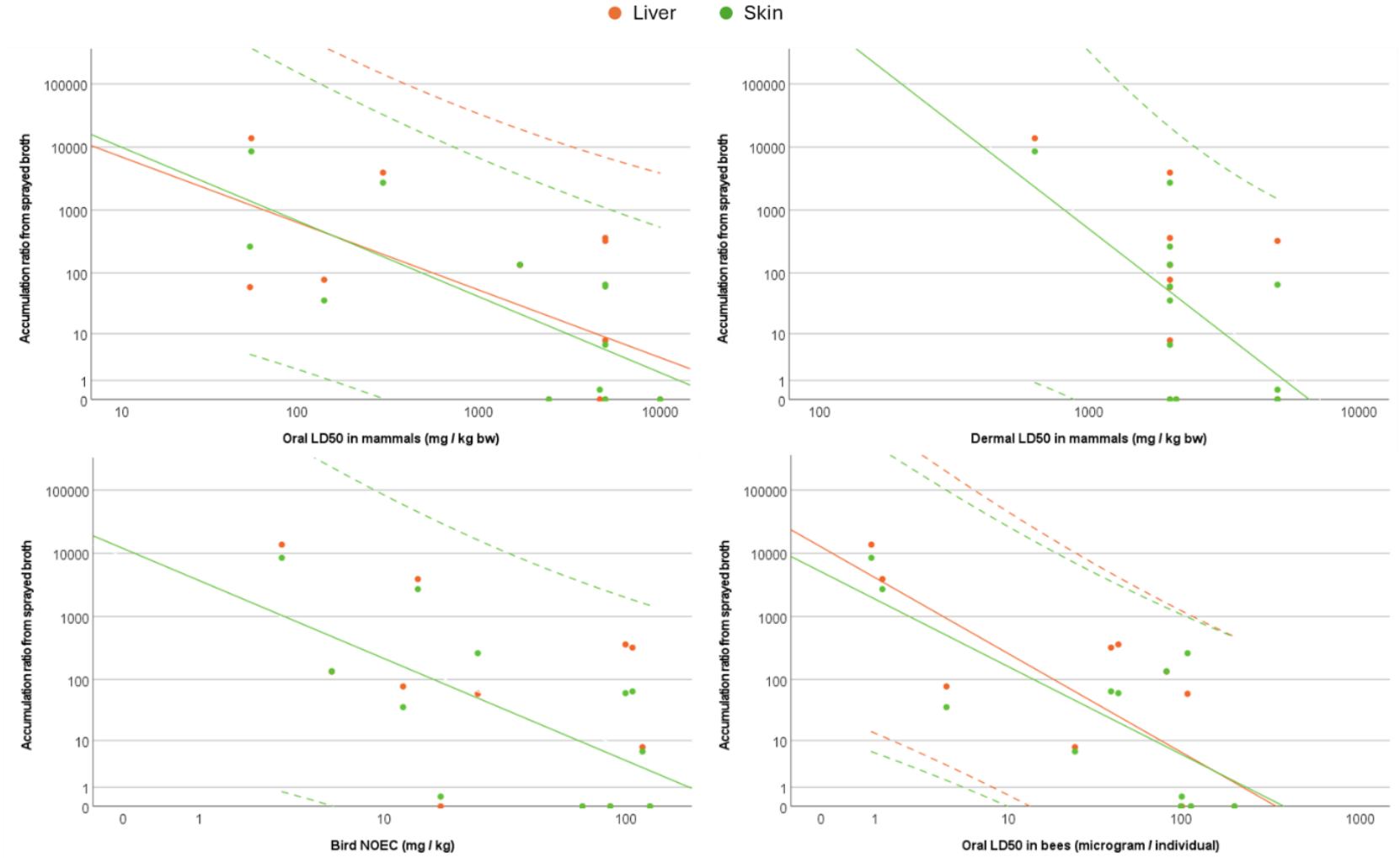


Figure 78: Biplots representing significant associations between toxicological properties and accumulation ratios relating pesticide concentrations in sprayed broths with livers or skins of frogs exposed via contact with treated soil

The lines represent the adjustment of the linear regression model for each tissue and their 95% confidence intervals. Data correspond to tissue concentrations measured on day 7. To improve visualization, some axes are depicted in logarithmic scale. Source: own illustration, [IREC-CSIC].

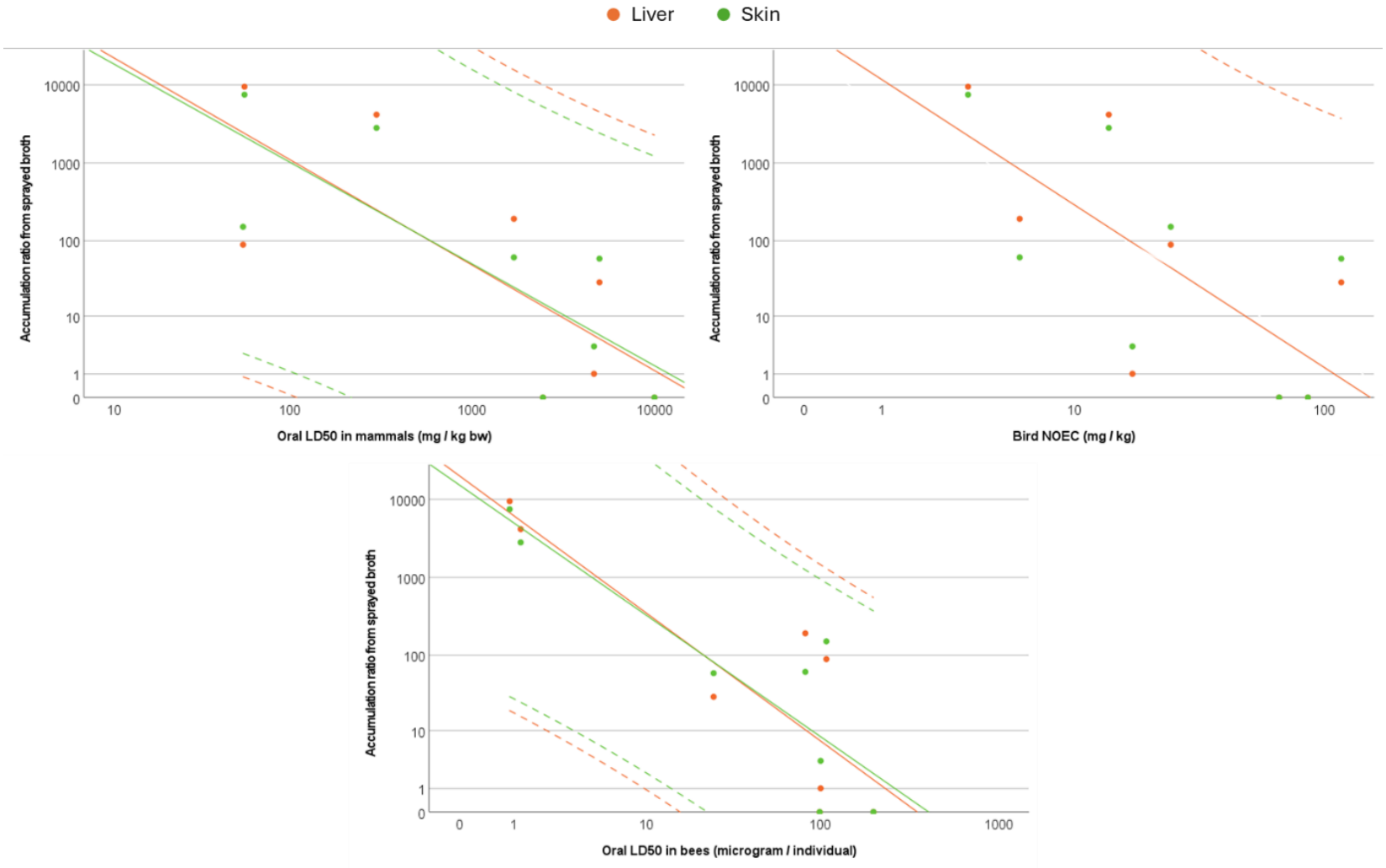
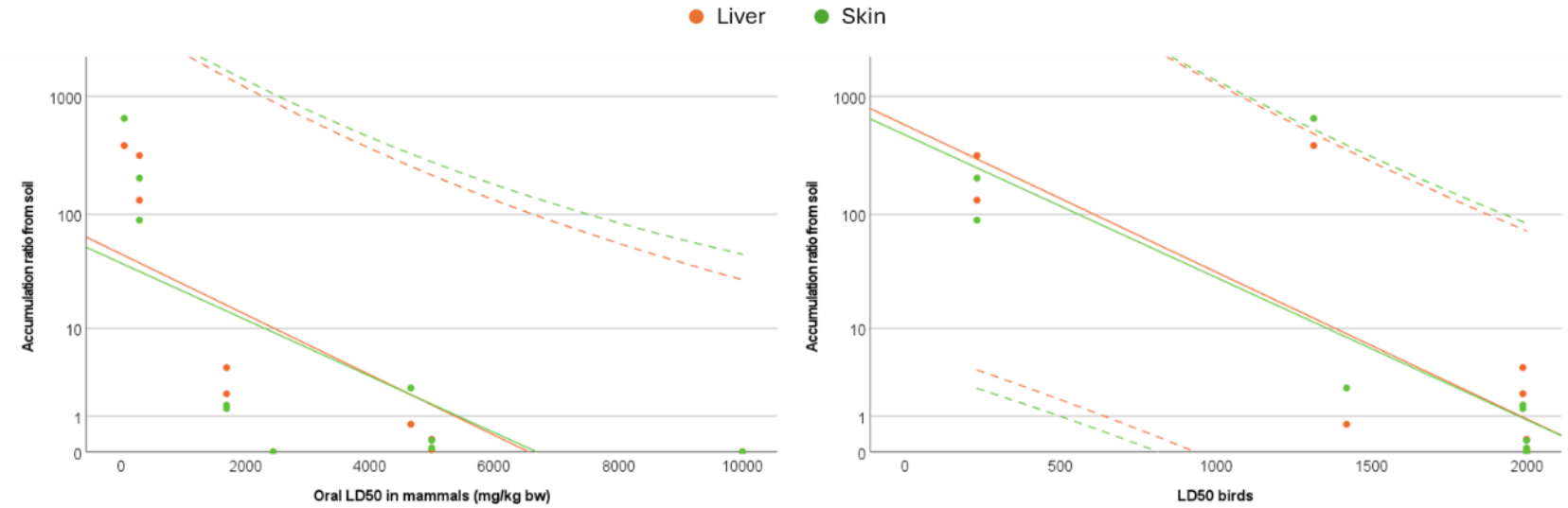


Figure 79: Biplots representing significant associations between toxicological properties and accumulation ratios relating pesticide concentrations in sprayed soils with livers or skins of frogs exposed via contact with treated soil

The lines represent the adjustment of the linear regression model for each tissue and their 95% confidence intervals. Data correspond to soil and tissue concentrations measured on day 7. To improve visualization, some axes are depicted in logarithmic scale. Source: own illustration, [IREC-CSIC].



6.3 Calculation of threshold values

The second approach to explore which properties of the active ingredients could determine amphibian toxicological sensitivity consisted of calculating toxicity threshold value to further link them to the physico-chemical and toxicological characteristics of the substances. For this analysis we considered only overspray exposures. We ran probit models with the data from the experiments; in particular, for each tested active ingredient, formulation or co-formulant, we ran three variations of the probit models: (i) including the actual data obtained in the experiments; (ii) simulating the absence of mortality in controls, to maximize the chances of finding significant dose-response effects; and (iii) removing the 10x application rate (or those replacing it for experiments with limited substance solubility) from the experiments in which this application rate did not result in a dose-response effect when combined with the lower application rates. Probit models were run using mortality rates recorded on days 7 or 21. When a significant model was obtained, we calculated the median application rate causing mortality from the probit model parameters. This median application rate was converted into the actual spray concentration (in kg/ha) of each product to obtain a median lethal overspray concentration (overspray-LC_{50}). As done with the accumulation ratios described above, when the difference between nominal and measured concentrations in sprayed broths (see section 4.2.3) was outside the range of the percentage recovery and its RSD calculated for each given substance (see section 4.1.4), we used the measured concentration instead of the nominal one to transform the application rate into the overspray-LC_{50}. Further, these overspray-LC_{50} values were converted into a dermal median lethal dose (overspray-LD_{50}), for which we took the same assumptions as used in the EFSA Scientific Opinion that half of the body surface receives the full application rate and that 100% of the product is absorbed via skin (EFSA PPR Panel et al. 2018). The body surface was calculated using the allometric equation for frogs, which is indicated in the USEPA Wildlife exposure factors handbook (USEPA 1993):

$$\text{Body surface } (\text{cm}^2) = 1.131 \times \text{Body weight } (\text{g})^{0.579}$$

A total of 156 probit models were run, out of which we found 13 significant models, plus the model on results of the naphtha experiment on day 21 using the real data was close to the statistical significance (p=0.064). We used the model results to estimate $LC50_{os}$ values for all those substances (Table 16). Application rate of naphtha as part of Fasthrin 10 EC (120 ml/ha, see Table 6 in section 3.5.2) was transformed in a weight-based value using the naphtha density of 0.86 g/ml, which resulted in an application rate of 103.2 g/ha. The obtained values were compared with LD_{50} values estimated for amphibians following the method proposed by Weltje et al. (2017). Briefly, those authors proposed a protocol to estimate a fish LD_{50} from the 96h-LC_{50} value for rainbow trout, usually included in the PPP application dossiers, using the BCF. Then, they used an inter-species correlation to derive a regression equation that would serve to convert the estimated fish LD_{50} in an amphibian LD_{50}. The calculated values according to this method are also included in Table 16.

Table 16: Summary of the significant Probit models obtained with mortality data in overspray treatments

The table displays the median lethal concentrations calculated, using the Probit models, as a function of the spray application rate (overspray-LC_{50}) and resulting median lethal dose (overspray-LD_{50}), together with the LD_{50} estimated according to Weltje et al. (2017) (fish-based-LD_{50}). When data correspond to formulations, all parameters are given in units relative to the active ingredient.

Substance	Lab	Experiment day	Model variation	Median effect application rate (xAR)	Overspray-LC_{50} (mg/ha)	Overspray-LD_{50} (mg/kg bw)	Fish-based-LD_{50} (mg/kg bw)*
Quadris	IREC	21	Without highest AR	0.614	0.153	0.740	30.22
Folicur 25 EW	IREC	7	Without highest AR	1.354	0.270	1.460	243.38
	UA	7	Original data	0.591	0.118	1.181	
	IREC	21	Without highest AR	0.764	0.153	0.738	
Metsulfuron-methyl	UA	7	No control mortality	11.373	0.068	0.552	85.11
	IREC	21	Without highest AR	1.115	0.007	0.030	
	UA	21	No control mortality	8.224	0.049	0.372	
Pirimicarb	UA	7	Without highest AR	0.671	0.035	0.339	1044.04
	UA	21	Original data	5.713	0.297	2.227	
	UA	21	No control mortality	7.315	0.380	2.851	
	UA	21	Without highest AR	0.811	0.042	0.314	
Fasthrin 10 EC	IREC	7	Original data	11.519	0.172	0.844	0.16
	IREC	21	No control mortality	7.845	0.118	0.530	
Naphtha	UA	21	Original data	11.922	1.230	11.346	Not available

*Always referred to the active ingredient alone

As reflected in the table, the estimated overspray LD_{50} values according to the result of the present study are generally much lower than the values estimated from fish data, which were proposed by Weltje et al. (2017) as a method to characterize acute dermal toxicity for amphibians. We must be cautious with the interpretation of these results, as both approaches to estimate LD_{50} values involve a high degree of extrapolation, and the estimates resulting from our overspray tests are anyway based on a few treatment levels. This low number of treatment levels can exert a high variability in the output of the Probit model; in fact, removing the highest treatment level caused a strong reduction of the estimated benchmark values (for those substances in which methods could be compared), so this is probably an over-conservative method. However, also the estimates made from original data are resulting in estimated LD_{50} values that are much lower than those calculated from fish data, with the exception of alpha-cypermethrin. It must be noticed also that, in some cases, we are comparing data from our study that were obtained from formulations with data extrapolated from fish that refer to active ingredients. In any case, the differences are in some cases large enough to consider further investigations regarding how exposure scenario may affect dermal toxicity of pesticides to amphibians. According to these estimations, overspray is apparently supposing a scenario of special risk linked to dermal exposure of amphibians to PPP.

6.4 Role of earthworms as surrogates for evaluation dermal toxicity of pesticides to amphibians

In order to find possible parallelism between toxicity to amphibians and earthworms, the effects of the different compounds, including both active substances, formulations and co-formulants, were ranked according to the effects noted on earthworms following the same approach as for amphibians (see section 6.2). The toxicity reference values for earthworms are shown in Table 17.

The potential of earthworms to predict amphibian toxicity was checked by running a Spearman correlation between the toxicity level values of both groups. The correlation was weak but significant, showing that there is some parallelism in the mechanisms driving the occurrence of apical effects in both groups ($R_s = 0.449$, $p = 0.028$). To explore this data more in deep, we obtained a linear regression model (Figure 80) that, although its adjustment was only close to statistical significance ($p = 0.093$) allowed us to obtain the residuals. The absolute value of those residuals (i.e. the distance from each dot in the figure relative to the regression line) was considered as an inverse measure of the goodness whom which the amphibian toxicological sensitivity could be predicted from the earthworm sensitivity. Then, we ran Pearson correlations of those residual absolute values with the different physico-chemical and toxicological properties of the pesticides (log-transformed). The purpose of this analysis was to inquire which properties would be most influential in preventing a good prediction.

The results showed two significant correlations; on the one hand, substances with higher molecular mass were negatively correlated to the residual absolute values ($R = -0.499$, $p = 0.049$), meaning that toxicity of large molecules to amphibians would be easier to predict from earthworm data than toxicity of small molecules. The other significant correlation was found with the avian LD_{50}, and its sign was also negative ($R = -0.651$, $p = 0.006$), meaning that a better prediction from earthworms to amphibians can be achieved for substances that are more toxic to birds. We must notice that these correlations were run using active ingredients only, as the dataset for physico-chemistry or toxicology of formulations and co-formulants is far from being complete. These results do not mean that molecular mass or avian LD_{50} play a significant role in determining the toxicity of pesticides on amphibians or earthworms but provide some

indications about the type of molecules for which predictability using earthworm as surrogates is more or less plausible. It is important to remind that a significant Spearman correlation between toxicity level values was found, showing an interesting potential for earthworms to act as surrogates for amphibian dermal toxicity that should to be explored more deeply by extending the number of compared substances.

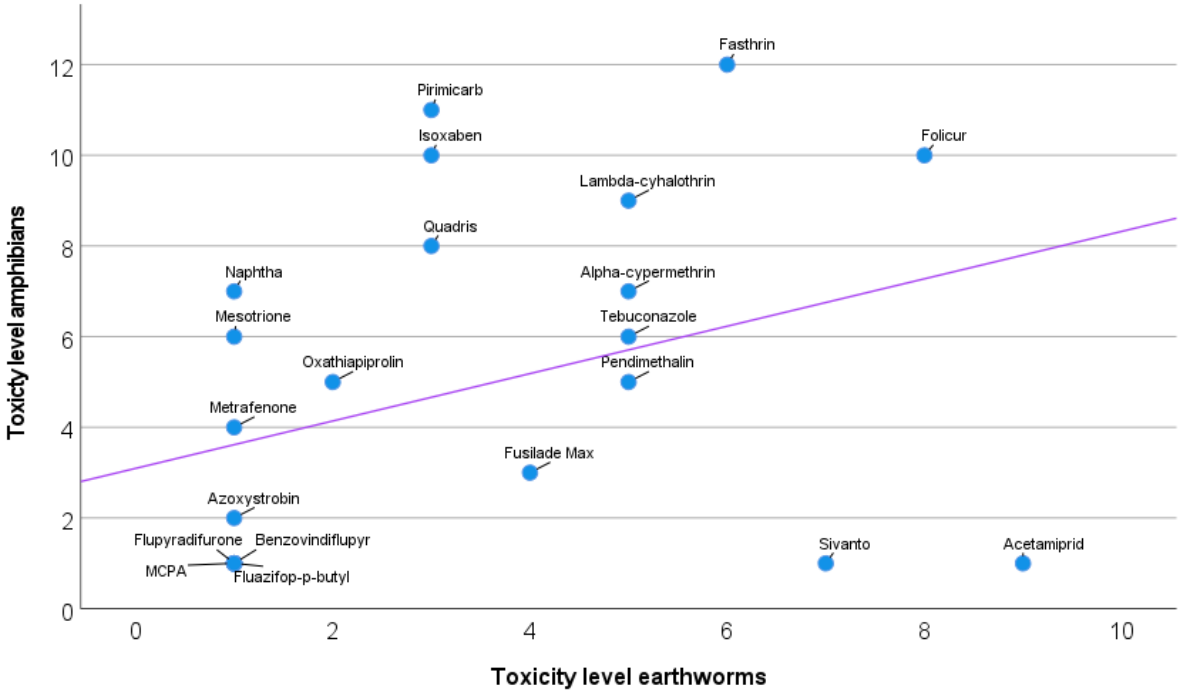
Table 17: Rank of tested substances according to the apical effects recorded in *Eisenia andrei* adults

Each block is assigned a consecutive numerical value as a function of observed effects.

Substance	Effect	Toxicity level
Acetamiprid	Significant mortality after 24h at 0.1xAR	9
Folicur 25 EW	Significant mortality after 24h at 10xAR	8
Sivanto Prime	Significant mortality after 72h at 0.1xAR	7
Fasthrin 10 EC	Significant mortality after 72h at 10xAR	6
Pendimethalin Tebuconazole Alpha-cypermethrin Lambda-cyhalothrin	Reduced body mass after 72 h at 0.1xAR	5
Fusilade Max	Reduced body mass after 72 h at 1xAR	4
Isoxaben Pirimicarb Quadris	Isolated mortality (non-significant) cases	3
Oxathiapiprolin	Skin lesions at 10xAR	2
Metrafenone Benzovindiflupyr Azoxystrobin MCPA Fluazifop-p-butyl Metsulfuron-methyl Mesotrione Flupyradifurone Vivando Naphta N,N-dimethyldecanamid	No effects or increased body mass	1

Figure 80: Biplot representing the tested active ingredients, formulations and co-formulants as a function of their toxicity level value for amphibians and earthworms

The purple line represents the adjustment of the linear regression model (y = 3.094 + 0.523x). To improve visualization, metsulfuron-methyl, Vivando and N,N'-dimethyldecanamid are not labelled, their point being the same as for MCPA. Source: own illustration, [IREC-CSIC].



7 List of references

Adams, E.; Gerstle, V.; Schmitt, T.; Brühl, C.A. (2021): Co-formulants and adjuvants affect the acute aquatic and terrestrial toxicity of a cycloxydim herbicide formulation to European common frogs (*Rana temporaria*). Science of the Total Environment 789: 147865. https://doi.org/10.1016/j.scitotenv.2021.147865

AERU (2020): PPDB: Pesticide Properties DataBase. https://sitem.herts.ac.uk/aeru/ppdb/ (08.10.2020)

Agius, C.M. (1979): The role of melano-macrophage centres in iron storage in normal and diseased fish. Journal of Fish Diseases 2: 337–343. https://doi.org/10.1111/j.1365-2761.1979.tb00175.x

Agius, C. (1980): Phylogenetic development of melano–macrophage centres in fish. Journal of Zoology 191: 11–31. https://doi.org/10.1111/j.1469-7998.1980.tb01446.x

Agius, C.; Roberts, R.J. (2003): Melano-macrophage centres and their role in fish pathology. Journal of Fish Diseases 26: 499–509. https://doi.org/10.1046/j.1365-2761.2003.00485.x

Agostini, M.G.; Natale, G.S.; Ronco, A.E. (2010): Lethal and sublethal effects of cypermethrin to *Hypsiboas pulchellus* tadpoles. Ecotoxicology 19: 1545–1550. https://doi.org/10.1007/s10646-010-0539-3

Annett, R.; Habibi, H.R.; Hontela, A. (2014): Impact of glyphosate and glyphosate-based herbicides on the freshwater environment. Journal of Applied Toxicology 34: 458–479. https://doi.org/10.1002/jat.2997

Armbrust, K.L.; Peeler, H.B. (2002): Effects of formulation on the run-off of imidacloprid from turf. Pest Management Science 58: 702–706. https://doi.org/10.1002/ps.518

Aronzon, C.M.; Sandoval, M.T.; Herkovits, J.; Perez-Coll, C.S. (2011): Stage-dependent toxicity of 2,4-dichlorophenoxyacetic on the embryonic development of a South American toad, *Rhinella arenarum*. Environmental Toxicology 26: 373–381. https://doi.org/10.1002/tox.20564

Aronzon, C.M.; Svartz, G.V.; Perez-Coll, C.S. (2016): Synergy between diazinon and nonylphenol in toxicity during the early development of the *Rhinella arenarum* toad. Water Air and Soil Pollution 227: 139. https://doi.org/10.1007/s11270-016-2799-x

ASTM (2002): Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians, ASTM test E729-96. American Society for Testing and Materials, West Conshohocken, Pennsilvania. https://doi.org/10.1520/E0729-96R14

Babalola, O.O.; Truter, J.C.; Van Wyk, J.H. (2021): Lethal and teratogenic impacts of imazapyr, diquat dibromide, and glufosinate ammonium herbicide formulations using Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). Archives of Environmental Contamination and Toxicology 80: 708–716. https://doi.org/10.1007/s00244-020-00756-5

Balba, H. (2007): Review of strobilurin fungicide chemicals. Journal of Environmental Science and Health B 42: 441–451. https://doi.org/10.1080/03601230701316465

Bankhead, P.; Loughrey, M.B.; Fernández, J.A.; Dombrowski, Y.; McArt, D.G.; Dunne, P.D.; McQuaid, S.; Gray, R.T.; Murray, L.J.; Coleman, H.G.; James, J.A.; Salto-Tellez, M.; Hamilton, P.W. (2017): QuPath: Open source software for digital pathology image analysis. Scientific Reports 7: 16878. https://doi.org/10.1038/s41598-017-17204-5

Baynes, R.E.; Riviere, J.E. (1998): Influence of inert ingredients in pesticide formulations on dermal absorption of carbaryl. American Journal of Veterinary Research 59: 168–175. https://doi.org/10.2460/ajvr.1998.59.02.168

Baynes, R.E.; Brooks, J.D.; Mumtaz, M.; Riviere, J.E. (2002): Effect of chemical interactions in pentachlorophenol mixtures on skin and membrane transport. Toxicological Sciences 69: 295–305. https://doi.org/10.1093/toxsci/69.2.295

Belden, J.; McMurry, S.; Smith, L.; Reilley, P. (2010): Acute toxicity of fungicide formulations to amphibians at environmentally relevant concentrations. Environmental Toxicology and Chemistry 29: 2477–2480. https://doi.org/10.1002/etc.297

Bernal, M.H.; Solomon, K.R.; Carrasquilla, G. (2009): Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. Journal of Toxicology and Environmental Health A 72: 966–973. https://doi.org/10.1080/15287390902929717

Berrill, M.; Bertram, S.; Wilson, S.; Louis, S.; Brigham, D.; Stromberg, C. (1993): Lethal and sublethal impacts of pyretrhroid insecticides on amphibian embryos and tadpoles. Environmental Toxicology and Chemistry 12: 525–539. https://doi.org/10.1002/etc.5620120313

Bloch, D.; Marx-Stoelting, P.; Martin, S. (2020): Towards a tiered test strategy for plant protection products to address mixture toxicity by alternative approaches in human health assessment. Pest Management Science 76: 3326–3332. https://doi.org/10.1002/ps.6034

Boone, M.D. (2008): Examining the single and interactive effects of three insecticides on amphibian metamorphosis. Environmental Toxicology and Chemistry 27:1561–1568. https://doi.org/10.1897/07-520.1

Brodman, R.; Newman, W.D.; Laurie, K.; Osterfeld, S.; Lenzo, N. (2010): Interaction of an aquatic herbicide and predatory salamander density on wetland communities. Journal of Herpetology 44: 69–82. https://doi.org/10.1670/08-320.1

Brühl, C.A.; Schmidt, T.; Pieper, S.; Alscher, A. (2013): Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? Scientific Reports 3: 1135. https://doi.org/10.1038/srep01135

Burggren, W.; Moallf, R. (1984): 'Active' regulation of cutaneous exchange by capillary recruitment in amphibians: Experimental evidence and a revised model for skin respiration. Respiration Physiology 55: 379–392. https://doi.org/10.1016/0034-5687(84)90059-8

Carvalho, W.F.; de Arcaute, C.R.; Perez-Iglesias, J.M.; Laborde, M.R.R.; Soloneski, S.; Larramendy, M.L. (2019): DNA damage exerted by mixtures of commercial formulations of glyphosate and imazethapyr herbicides in *Rhinella arenarum* (Anura, Bufonidae) tadpoles. Ecotoxicology 28: 367–377. https://doi.org/10.1007/s10646-019-02029-x

Cattani, D.; de Liz Oliveira Cavalli, V.L.; Heinz Rieg, C.E.; Domingues, J.T.; Dal-Cim, T.; Tasca, C.I.; Mena Barreto Silva, F.R.; Zamoner, A. (2014): Mechanisms underlying the neurotoxicity induced by glyphosate-based herbicide in immature rat hippocampus: Involvement of glutamate excitotoxicity. Toxicology 320: 34–45. https://doi.org/10.1016/j.tox.2014.03.001

Chen, C.Y.; Hathaway, K.M.; Folt, C.L. (2004): Multiple stress effects of Vision (R) herbicide, pH, and food on zooplankton and larval amphibian species from forest wetlands. Environmental Toxicology and Chemistry 23: 823–831. https://doi.org/10.1897/03-108

Chen, C.Y.; Hathaway, K.M.; Thompson, D.G.; Folt, C.L. (2008): Multiple stressor effects of herbicide, pH, and food on wetland zooplankton and a larval amphibian. Ecotoxicology and Environmental Safety 71: 209–218. https://doi.org/10.1016/j.ecoenv.2007.08.007

Çömden, E.A.; Yenmiş, M.; Çakır, B. (2023): The complex bridge between aquatic and terrestrial life: skin changes during development of amphibians. Journal of Developmental Biology 11: 6. https://doi.org/10.3390/jdb11010006

Cox, C.; Surgan, M. (2006): Unidentified inert ingredients in pesticides: Implications for human and environmental health. Environmental Health Perspectives 114: 1803–1806. https://doi.org/10.1289/ehp.9374

Crane, M.; Finnegan, M.; Weltje, L.; Kosmala-Grzechnik, S.; Gross, M.; Wheeler, J.R. (2016): Acute oral toxicity of chemicals in terrestrial life stages of amphibians: Comparisons to birds and mammals. Regulatory Toxicology and Pharmacology 80: 335–341. https://doi.org/10.1016/j.yrtph.2016.05.004

Crochet, P.-A.; Dubois, A.; Tunner, H. (1995): *Rana (Pelophylax) ridibunda* Pallas, 1771, *Rana (Pelophylax) perezi* Seoane, 1885 and their associated klepton (Amphibia, Anura): morphological diagnoses and description of a new taxon. Bulletin du Museum National d'Histoire Naturelle A 17: 11–30. https://doi.org/10.5962/p.290311

Cusaac, J.P.W.; Morrison, S.A.; Belden, J.B.; Smith, L.M.; McMurry, S.T. (2016): Acute toxicity of Headline® fungicide to Blanchard's cricket frogs (*Acris blanchardi*). Ecotoxicology 25: 447–455. https://doi.org/10.1007/s10646-015-1602-x

da Silva, M.B.; Fraga, R.E.; Nishiyama, P.B.; Siqueira da Silva, I.S.; Badaro Costa, N.L.; Alves de Oliveira, L.A.; Rocha, M.A.; Junca, F.A. (2020): Leukocyte profiles in *Odontophrynus carvalhoi* (Amphibia: Odontophrynidae) tadpoles exposed to organophosphate chlorpyrifos pesticides. Water Air and Soil Pollution 231: 372. https://doi.org/10.1007/s11270-020-04726-4

Daam, M.A.; Moutinho, M.F.; Espíndola, E.L.G.; Schiesari, L. (2019): Lethal toxicity of the herbicides acetochlor, ametryn, glyphosate and metribuzin to tropical frog larvae. Ecotoxicology 28: 707–715. https://doi.org/10.1007/s10646-019-02067-5

David, M.; Marigoudar, S.R.; Patil, V.K.; Halappa, R. (2012): Behavioral, morphological deformities and biomarkers of oxidative damage as indicators of sublethal cypermethrin intoxication on the tadpoles of *D. melanostictus* (Schneider, 1799). Pesticide Biochemistry and Physiology 103: 127–134. https://doi.org/10.1016/j.pestbp.2012.04.009

Dearden, J.C. (1990): Physico-chemical descriptors. In: Karcher, W.; Devillers, J. [eds.]: Practical applications of quantitative structure-activity relationships (QSAR) in environmental chemistry and toxicology. Kluwer, Brussels – Luxembourg, p. 25–59

Dick, I.P.; Blain, P.G.; Williams, F.M. (1997a): The percutaneous absorption and skin distribution of lindane in man: I. In vivo studies. Human and Experimental Toxicology 16: 645–651. https://doi.org/10.1177/096032719701601103

Dick, I.P.; Blain, P.G.; Williams, F.M. (1997b): The percutaneous absorption and skin distribution of lindane in man: II. In vitro studies. Human and Experimental Toxicology 16: 652–657. https://doi.org/10.1177/096032719701601104

Dinehart, S.K.; Smith, L.M.; McMurry, S.T.; Anderson, T.A.; Smith, P.N.; Haukos, D.A. (2009): Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Science of the Total Environment 407: 1065–1071. https://doi.org/10.1016/j.scitotenv.2008.10.010

Dinehart, S.K.; Smith, L.M.; McMurry, S.T.; Smith, P.N.; Anderson, T.A.; Haukos, D.A. (2010): Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval *Spea multiplicata* and *S. bombifrons* from the Southern High Plains, USA. Environmental Pollution 158: 2610–2617. https://doi.org/10.1016/j.envpol.2010.05.006

Domenech, C.E.; Machado de Domenech, E.E.; Balegno, H.F.; de Mendoza, D.; Farías, R.N. (1977): Pesticide action and membrane fluidity allosteric behavior of rat erythrocyte membrane-bound acetylcholinesterase in the presence of organophosphorous compounds. FEBS Letters 74: 243–246. https://doi.org/10.1016/0014-5793(77)80855-7

ECHA (2020): Solvent naphtha (petroleum), heavy arom. https://echa.europa.eu/substance-information/-/substanceinfo/100.059.253#:~:text=Solvent%20naphtha%20%28petroleum%29%2C%20heavy%20arom.%20A%20complex%20combination,of%20approximately%20165%C2%B0C%20to%20290%C2%B0C%20%28330%C2%B0F%20to%20554%C2%B0F%29 (25.07.2021)

Edge, C.B.; Gahl, M.K.; Pauli, BD.; Thompson, D.G.; Houlahan, J.E. (2011): Exposure of juvenile green frogs (*Lithobates clamitans*) in littoral enclosures to a glyphosate-based herbicide. Ecotoxicology and Environmental Safety 74: 1363–1369. https://doi.org/10.1016/j.ecoenv.2011.04.020

Edge, C.B.; Gahl, M.K.; Thompson, D.G.; Houlahan, J.E. (2013): Laboratory and field exposure of two species of juvenile amphibians to a glyphosate-based herbicide and *Batrachochytrium dendrobatidis*. Science of the Total Environment 444: 145–152. https://doi.org/10.1016/j.scitotenv.2012.11.045

Edginton, A.N.; Sheridan, P.M.; Stephenson, G.R.; Thompson, D.G.; Boermans, H.J (2004): Comparative effects of pH and Vision (R) herbicide on two life stages of four anuran amphibian species. Environmental Toxicology and Chemistry 23: 815–822ç. https://doi.org/10.1897/03-115

Edwards, C.A.; Bohlen, P.J. (1996): Biology and ecology of earthworms (4th edition). Springer Science and Business Media, New York. https://doi.org/10.1007/978-0-387-74943-3

EFSA PPR Panel; Ockleford, C.P.; Adriaanse, P.; Berny, P.; Brock, T.; Duquesne, S.; Grilli, S.; Hernandez-Jerez, A.F.; Bennekou, S.H.; Klein, M.; Kuhl, T.; Laskowski, R.; Machera, K.; Pelkonen, O.; Pieper, S.; Stemmer, M.; Sundh, I.; Teodorovic, I.; Tiktak, A.; Topping, C.J.; Wolterink, G.; Aldrich, A.; Berg, C.; Ortiz-Santaliestra, M.E.; Weir, S.; Streissl, F.; Smith, R.H. (2018): Scientific Opinion on the state of the science on pesticide risk assessment for amphibians and reptiles. EFSA Journal 16: e05125. https://doi.org/10.2903/j.efsa.2018.5125

Egea-Serrano, A. (2009): Rana común – *Pelophylax perezi* (López Seoane, 1885). https://vertebradosibericos.org/anfibios/pelper.html (02.05.2020)

Evard, H.; Kruve, A.; Leito, I. (2016): Tutorial on estimating the limit of detection using LC-MS analysis, part I: Theoretical review. Analytica Chimica Acta 942: 23–39. https://doi.org/10.1016/j.aca.2016.08.043

Franco-Belussi, L.; Sköld, H.N.; de Oliveira, C. (2016): Internal pigment cells respond to external UV radiation in frogs. Journal of Experimental Biology 219: 1378–1383. https://doi.org/10.1242/jeb.134973

Fründ, H.-C.; Butt, K.; Capowiez, Y.; Eisenhauer, N.; Emmerling, C.; Ernst, G.; Potthoff, M.; Schädler, M.; Schrader, S. (2010): Using earthworms as model organisms in the laboratory: Recommendations for experimental implementations. Pedobiologia 53: 119–125. https://doi.org/10.1016/j.pedobi.2009.07.002

Fulop, G.M.; McMillan, D.B. (1984): Phagocytosis in the spleen of the sunfish *Lepomis* spp. Journal of Morphology 179: 175–195. https://doi.org/10.1002/jmor.1051790205

Giesy, J.P.; Dobson, S.; Solomon, K.R. (2000): Ecotoxicological Risk Assessment for Roundup® Herbicide. Reviews of Environmental Contamination and Toxicology 167: 35–120. https://doi.org/10.1007/978-1-4612-1156-3_2

Gosner, K.L. (1960): A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16: 183–190

Goutner, V.: Frigis, K.; Konstantinou, I.K.; Sakellarides, T.M.; Albanis, T.A. (2012): Organochlorine pesticide residue concentrations and accumulation patterns in waterbirds and in their prey at Lake Kerkini, a Ramsar wetland, Greece. Journal of Biological Research 17: 154–168

Grajeda-Cota, P.; Ramírez-Mares, M.V.; González de Mejía, E. (2004): Vitamin C protects against in vitro cytotoxicity of cypermethrin in rat hepatocytes. Toxicology in Vitro 18: 13–19. https://doi.org/10.1016/S0887-2333(03)00077-8

Güngördü, A.; Uçkun, M. (2015): Comparative assessment of in vitro and in vivo toxicity of azinphos methyl and its commercial formulation. Environmental Toxicology 30: 1091–1101. https://doi.org/10.1002/tox.21982

Harris, M.L.; Bishop, C.A.; Struger, J.; Ripley, B.; Bogart, J.P. (1998): The functional integrity of northern leopard frog (*Rana pipiens*) and green frog (*Rana clamitans*) populations in orchard wetlands. II. Effects of pesticides

and eutrophic conditions on early life stage development. Environmental Toxicology and Chemistry 17: 1351–1363. https://doi.org/10.1002/etc.5620170720

Haya, K. (1989): Toxicity of pyrethroid insecticides to fish. Environmental Toxicology and Chemistry 8: 381–391. https://doi.org/10.1002/etc.5620080504

Hedberg, D.; Wallin, M. (2010): Effects of Roundup and glyphosate formulations on intracellular transport, microtubules and actin filaments in *Xenopus laevis* melanophores. Toxicology in Vitro 24: 795–802. https://doi.org/10.1016/j.tiv.2009.12.020

Henson-Ramsey, H.; Kennedy-Stoskopf, S.; Levine, J.F.; Taylor, S.K.; Shea, D.; Stoskopf, M.K. (2008): Acute toxicity and tissue distributions of malathion in *Ambystoma tigrinum*. Archives of Environmental Contamination and Toxicology 55: 481–487. https://doi.org/10.1007/s00244-007-9091-4

Herráez, M.P.; Zapata, A.G. (1986): Structure and function of the melano-macrophage centres of the goldfish *Carassius auratus*. Veterinary Immunology and Immunopathology 12: 117–126. https://doi.org/10.1016/0165-2427(86)90116-9

HESI (2020): EnviroTox Database & Tools. https://envirotoxdatabase.org/ (11.10.2020)

Hewitt, A.J.; Solomon, K.R.; Marshall, E.J.P. (2009): Spray droplet size, drift potential, and risks to nontarget organisms from aerially applied glyphosate for coca control in colombia. Journal of Toxicology and Environmental Health A 72: 921–929. https://doi.org/10.1080/15287390902929667

Hooser, E.A.; Belden, J.B.; Smith, L.M.; McMurry, S.T. (2012): Acute toxicity of three strobilurin fungicide formulations and their active ingredients to tadpoles. Ecotoxicology 21: 1458–1464. https://doi.org/10.1007/s10646-012-0899-y

Horowitz, A.; Chanez-Paredes, S.D.; Haest, X.; Turner, J.R. (2023): Paracellular permeability and tight junction regulation in gut health and disease. Nature Reviews Gastroenterology & Hepatology 20: 417–432ç. https://doi.org/10.1038/s41575-023-00766-3

Hourdry, J.; L'Hermite, A.; Ferrand, R. (1996): Changes in the digestive tract and feeding behavior of anuran amphibians during metamorphosis. Physiological Zoology 69: 219–251. https://doi.org/10.1086/physzool.69.2.30164181

Howe, C.M.; Berrill, M.; Pauli, B.D:; Helbing, C.C.; Werry, K.; Veldhoen, N. (2004): Toxicity of glyphosate-based pesticides to four North American frog species. Environmental Toxicology and Chemistry 23: 1928–1938. https://doi.org/10.1897/03-71

Huang, A.; Mangold-Döring, A.; Focks, A.; Zhang, C.; Van den Brink, P.J. (2022): Comparing the acute and chronic toxicity of flupyradifurone and imidacloprid to non-target aquatic arthropod species. Ecotoxicology and Environmental Safety 243: 113977. https://doi.org/10.1016/j.ecoenv.2022.113977

Hughes, M.F.; Edwards, B.C. (2010): In vitro dermal absorption of pyrethroid pesticides in human and rat skin. Toxicology and Applied Pharmacology 246: 29–37. https://doi.org/10.1016/j.taap.2010.04.003

IUCN (2024): The IUCN Red List of Threatened Species. Version 2024-1. The International Union for Conservation of Nature, Gland, Switzerland.

Jacques, M.T.; Bornhorst, J.; Soares, M.V.; Schwerdtle, T.; Garcia, S.; Ávila, D.S. (2019): Reprotoxicity of glyphosate-based formulation in *Caenorhabditis elegans* is not due to the active ingredient only. Environmental Pollution 252: 1854–1862. https://doi.org/10.1016/j.envpol.2019.06.099

Jantawongsri , K.; Thammachoti, P.; Kitana, J.; Khonsue, W.; Varanusupakul, P.; Kitana, N. (2015): Altered immune response of the rice frog *Fejervarya limnocharis* living in agricultural area with intensive herbicide utilization at Nan province, Thailand. EnvironmentAsia 8: 68–74. https://doi.org/10.14456/ea.2015.9

Kaufmann, K.; Dohmen, P. (2016): Adaption of a dermal in vitro method to investigate the uptake of chemicals across amphibian skin. Environmental Sciences Europe 28: 10. https://doi.org/10.1186/s12302-016-0080-y

Koyama, K.; Koyama, K.; Goto, K. (1997): Cardiovascular effects of a herbicide containing glufosinate and a surfactant: in vitro and in vivo analyses in rats. Toxicology and Applied Pharmacology 145: 409–414. https://doi.org/10.1006/taap.1997.8196

Krynak, K.L.; Burke, D.J.; Benard, M.F. (2017): Rodeo(TM) herbicide negatively affects Blanchard's Cricket Frogs (*Acris blanchardi*) survival and alters the skin-associated bacterial community. Journal of Herpetology 51: 402–410. https://doi.org/10.1670/16-092

Lajmanovich, R.C.; Cabagna-Zenklusen, M.C.; Attademo, A.M.; Junges, C.M.; Peltzer, P.M.; Basso, A.; Lorenzatti, E. (2014): Induction of micronuclei and nuclear abnormalities in tadpoles of the common toad (*Rhinella arenarum*) treated with the herbicides Liberty (R) and glufosinate-ammonium. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 769: 7–12. https://doi.org/10.1016/j.mrgentox.2014.04.009

Lapidus, R.G.; Sokolove, P.M. (1993): Spermine Inhibition of the permeability transition of isolated rat liver mitochondria: an investigation of mechanism. Archives of Biochemistry and Biophysics 306: 246–253. https://doi.org/10.1006/abbi.1993.1507

Larsen, H.A.; Austbø, L.; Mørkøre, T.; Thorsen, J.; Hordvik, I.; Fischer, U.; Jirillo, E.; Rimstad, E.; Koppang, E.O. (2012): Pigment-producing granulomatous myopathy in Atlantic salmon: a novel inflammatory response. Fish and Shellfish Immunology 33: 277–285. https://doi.org/10.1016/j.fsi.2012.05.012

Laverack, M.S. (1963): The Physiology of Earthworms. Pergamon Press, Oxford. https://doi.org/10.1016/C2013-0-01700-8

Lee, B.-T.; Shin, K.-H.; Kim, J.-Y.; Kim, K.-W. (2008): Progress in Earthworm Ecotoxicology. In: Kim, Y.J.; Platt, U. [eds.]: Advanced Environmental Monitoring. Springer Netherlands, Dordrecht, p. 248–258. https://doi.org/10.1007/978-1-4020-6364-0_19

Levis, N.A.; Schooler, M.L.; Johnson, J.R.; Collyer, M.L. (2016): Non-adaptive phenotypic plasticity: the effects of terrestrial and aquatic herbicides on larval salamander morphology and swim speed. Biological Journal of the Linnean Society 118: 569–581. https://doi.org/10.1111/bij.12761

Llewelyn, V.K.; Berger, L.; Glass, B.D. (2019a): Effects of skin region and relative lipophilicity on percutaneous absorption in the toad *Rhinella marina*. Environmental Toxicology and Chemistry 38: 361–367. https://doi.org/10.1002/etc.4302

Llewelyn, V.K.; Berger, L.; Glass, B.D. (2019b): Permeability of frog skin to chemicals: effect of penetration enhancers. Heliyon 5: e02127. https://doi.org/10.1016/j.heliyon.2019.e02127

Llewelyn, V.K.; Berger, L.; Glass, B.D. (2020): Percutaneous absorption in frog species: variability in skin may influence delivery of therapeutics. Journal of Veterinary Pharmacology and Therapeutics 43: 91–95. https://doi.org/10.1111/jvp.12824

Majumder, R.; Kaviraj, A. (2015): Variation in acute toxicity between technical grade and commercial formulation of cypermethrin to some non-target freshwater organisms. International Journal of Current Research 7: 16755–16759

Malone, R.W.; Shipitalo, M.J.; Wauchope, R.D.; Sumner, H. (2004): Residual and contact herbicide transport through field lysimeters via preferential flow. Journal of Environmental Quality 33: 2141–2148. https://doi.org/10.2134/jeq2004.2141

Mann, R.M.; Bidwell. J.R. (1999): The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. Archives of Environmental Contamination and Toxicology 36: 193–199. https://doi.org/10.1007/s002449900460

Marren, K. (2011): Dimethyl sulfoxide: an effective penetration enhancer for topical administration of NSAIDs. The Physician and Sports Medicine 39: 75–82. https://doi.org/10.3810/psm.2011.09.1923

Martinuzzi, C.S.; Attademo, A.M.; Peltzer, P.M.; Mac Loughlin, T.M.; Marino, D.J.G.; Lajmanovich, R.C. (2020): Comparative toxicity of two different dimethoate formulations in the common toad (*Rhinella arenarum*) tadpoles. Bulletin of Environmental Contamination and Toxicology 104: 35–40. https://doi.org/10.1007/s00128-019-02741-8

Mathews, C.K.; van Holde, K.E. (1990): Biochemistry. Benjamin/Cummings Publishing Co, Redwood City, California

Mathieu-Denoncourt, J.; Wallace, S.J.; de Solla, S.R.; Langlois, V.S. (2016): Influence of lipophilicity on the toxicity of bisphenol A and phthalates to aquatic organisms. Bulletin of Environmental Contamination and Toxicology 97: 4–10. https://doi.org/10.1007/s00128-016-1812-9

Miglani, R.; Bisht, S.S. (2019): World of earthworms with pesticides and insecticides. Interdisciplinary Toxicology 12: 71–82. https://doi.org/10.2478/intox-2019-0008

Montemurro, N.; Grieco, F.; Lacertosa, G.; Visconti, A. (2002): Chlorpyrifos decline curves and residue levels from different commercial formulations applied to oranges. Journal of Agricultural and Food Chemistry 50: 5975–5980. https://doi.org/10.1021/jf0256687

Moutinho, M.F.; de Almeida, E.A.; Espindola, E.L.G.; Daam, M.A.; Schiesari, L. (2020): Herbicides employed in sugarcane plantations have lethal and sublethal effects to larval *Boana pardalis* (Amphibia, Hylidae). Ecotoxicology 29: 1043–1051. https://doi.org/10.1007/s10646-020-02226-z

Nagy, K.A.; Girard, I.A.; Brown, T.K. (1999): Energetics of free-ranging mammals, reptiles, and birds. Annual Review of Nutrition 19: 247–277. https://doi.org/10.1146/annurev.nutr.19.1.247

Niu, Y.; Zhang, X.; Zhang, H.; Men, S.; Xu, T.; Ding, L.; Li, X.; Wang, L.; Wang, H.; Storey, K.B.; Chen, Q. (2024): Ecological adaptations of amphibians to environmental changes along an altitudinal gradient (Case Study: *Bufo gargarizans*) from phenotypic and genetic perspectives. BMC Biology 22: 231. https://doi.org/10.1186/s12915-024-02033-6

OECD (1984): Test No. 207: Earthworm, Acute Toxicity Tests. OECD Publishing, Paris. https://doi.org/10.1787/20745761

OECD (2004): Test No. 222: Earthworm Reproduction Test (*Eisenia fetida*/*Eisenia andrei*). OECD Publishing, Paris. https://doi.org/10.1787/20745761

OECD (2009): Test No. 231: Amphibian Metamorphosis Assay. OECD Publishing, Paris. https://doi.org/10.1787/20745761

Ortiz-Santaliestra, M.E.; Maia, J.P.; Egea-Serrano, A.; Lopes, I. (2018): Validity of fish, birds and mammals as surrogates for amphibians and reptiles in pesticide toxicity assessment. Ecotoxicology 27: 819–833. https://doi.org/10.1007/s10646-018-1911-y

Partearroyo, M.A.; Pilling, S.J.; Jones, M.N. (1991): The lysis of isolated fish (*Oncorhynchus mykiss*) gill epithelial cells by surfactants. Comparative Biochemistry and Physiology C 100: 381–388. https://doi.org/10.1016/0742-8413(91)90012-I

Peig, J.; Green, A.J. (2009): New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos 118: 1883–1891. https://doi.org/10.1111/j.1600-0706.2009.17643.x

Perkins, P.J.; Boermans, H.J.; Stephenson, G.R. (2000): Toxicity of glyphosate and triclopyr using the Frog Embryo Teratogenesis Assay-*Xenopus*. Environmental Toxicology and Chemistry 19: 940–945. https://doi.org/10.1002/etc.5620190422

Pline, W.A.; Hatzios, K.K.; Hagood, E.S. (2000): Weed and herbicide-resistant soybean (*Glycine max*) response to glufosinate and glyphosate plus ammonium sulfate and pelargonic acid. Weed Technology 14: 667–674. https://doi.org/10.1614/0890-037X(2000)014[0667:WAHRSG]2.0.CO;2

Plonka, P.M.; Michalczyk, D.; Popik, M.; Handjiski, B.; Slominski, A.; Paus, R. (2005): Splenic eumelanin differs from hair eumelanin in C57BL/6 mice. Acta Biochimica Polonica 52: 433–441

Porro, L.B.; Richards, C.T. (2017): Digital dissection of the model organism *Xenopus laevis* using contrast-enhanced computed tomography. Journal of Anatomy 231: 169–191. https://doi.org/10.1111/joa.12625

Puglis, H.J.; Boone, M.D. (2011): Effects of technical-grade active ingredient vs. commercial formulation of seven pesticides in the presence or absence of uv radiation on survival of green frog tadpoles. Archives of Environmental Contamination and Toxicology 60: 145–155. https://doi.org/10.1007/s00244-010-9528-z

Quaranta, A.; Bellantuono, V.; Cassano, G.; Lippe, C. (2009): Why amphibians are more sensitive than mammals to xenobiotics. Plos One 4: e7699. https://doi.org/10.1371/journal.pone.0007699

Relyea, R.A. (2005a): The lethal impact of Roundup on aquatic and terrestrial amphibians. Ecological Applications 15: 1118–1124. https://doi.org/10.1890/04-1291

Relyea, R.A. (2005b): The lethal impacts of Roundup and predatory stress on six species of North American tadpoles. Archives of Environmental Contamination and Toxicology 48: 351–357. https://doi.org/10.1007/s00244-004-0086-0

Relyea, R.A. (2011): Amphibians are not ready for Roundup®. In: Elliott, J.; Bishop, C.A.; Morrissey, C. [eds.]: Wildlife Ecotoxicology. Springer, New York, p. 267–300. https://doi.org/10.1007/978-0-387-89432-4_9

Relyea, R.A.; Jones, D.K. (2009): The toxicity of Roundup Original Max (R) to 13 species of larval amphibians. Environmental Toxicology and Chemistry 28: 2004–2008. https://doi.org/10.1897/09-021.1

Relyea, R.A.; Schoeppner, N.M.; Hoverman, J.T. (2005): Pesticides and amphibians: The importance of community context. Ecological Applications 15: 1125–1134. https://doi.org/10.1890/04-0559

Roshon, R.D.; McCann, J.H.; Thompson, D.G.; Stephenson, G.R. (1999): Effects of seven forestry management herbicides on *Myriophyllum sibiricum*, as compared with other nontarget aquatic organisms. Canadian Journal of Forest Research 29: 1158–1169. https://doi.org/10.1139/x99-093

Sams, C.; Mason, H.J.; Rawbone, R. (2000): Evidence for the activation of organophosphate pesticides by cytochromes P450 3A4 and 2D6 in human liver microsomes. Toxicology Letters 116: 217–221. https://doi.org/10.1016/S0378-4274(00)00221-6

Sánchez-Montes, G.; Martínez-Solano, I. (2011): Population size, habitat use and movement patterns during the breeding season in a population of Perez's frog (*Pelophylax perezi*) in central Spain. Basic and Applied Herpetology 25: 81–96

Schmidt, B.R.; Feldmann, R.; Schaub, M. (2005): Demographic processes underlying population growth and decline in *Salamandra salamandra*. Conservation Biology 19: 1149–1156. https://doi.org/10.1111/j.1523-1739.2005.00164.x

Schmidt, U.; Weigert, M.; Broaddus, C.; Myers, G. (2018): Cell detection with star-convex polygons. In: Frangi, A.; Schnabel, J.; Davatzikos, C.; Alberola-López, C.; Fichtinger, G. [eds.]: Medical Image Computing and Computer Assisted Intervention – MICCAI 2018. Springer International Publishing, Cham, p. 265–273. https://doi.org/10.1007/978-3-030-00934-2_30

Secombes, C.J. (1982): Histological changes in lymphoid organs of carp following injection of soluble or particulate antigens. Developmental and Comparative Immunology 6: 53–58

Sparling, D.W.; Lowe, T.P.; Pinkney, A.E. (1997): Toxicity of Abate(R) to green frog tadpoles. Bulletin of Environmental Contamination and Toxicology 58: 475–481. https://doi.org/10.1007/s001289900359

Steinel, N.C.; Bolnick, D.I. (2017): Melanomacrophage centers as a histological indicator of immune function in fish and other poikilotherms. Frontiers in Immunology 8: 827. https://doi.org/10.3389/fimmu.2017.00827

Svartz, G.V.; Pérez–Coll, C.S. (2013): Comparative toxicity of cypermethrin and a commercial formulation on *Rhinella arenarum* larval development (Anura: Bufonidae). International Journal of Environment and Health 6: 320–329. https://doi.org/10.1504/ijenvh.2013.056973

Swann, J.M.; Schultz, T.W.; Kennedy, J.R. (1996): The effects of the organophosphorous insecticides Dursban(TM) and Lorsban(TM) on the ciliated epithelium of the frog palate in vitro. Archives of Environmental Contamination and Toxicology 30: 188–194. https://doi.org/10.1007/BF00215797

Talent, L.G. (2005): Effect of temperature on toxicity of a natural pyrethrin pesticide to green anole lizards (*Anolis carolinensis*). Environmental Toxicology and Chemistry 24: 3113–3116. https://doi.org/10.1897/05-053R.1

Trumbo, J. (2005): An assessment of the hazard of a mixture of the herbicide Rodeo and the non-ionic surfactant R-11 to aquatic invertebrates and larval amphibians. California Fish and Game 91: 38–46

Tsui, M.T.K.; Chu, L.M. (2003): Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52: 1189–1197. https://doi.org/10.1016/S0045-6535(03)00306-0

Turhan, D.Ö.; Güngördü, A.; Ozmen, M. (2020): Developmental and lethal effects of glyphosate and a glyphosate-based product on *Xenopus laevis* embryos and tadpoles. Bulletin of Environmental Contamination and Toxicology 104: 173–179. https://doi.org/10.1007/s00128-019-02774-z

Ujszegi, J.; Gál, Z.; Mikó, Z.; Hettyey, A. (2015): No observable effect of a glyphosate-based herbicide on two top predators of temporal water bodies. Environmental Toxicology and Chemistry 34: 307–313. https://doi.org/10.1002/etc.2798

Ujszegi, J.; Gál, Z.; Mikó, Z.; Hettyey, A. (2016): No effect of a glyphosate-based herbicide on larval dragonflies (*Aeshna cyanea*) and adult newts (*Lissotriton vulgaris*) in a laboratory-based experiment. Acta Zoologica Academiae Scientiarum Hungaricae 62: 355–367. https://doi.org/10.17109/azh.62.4.355.2016

USEPA (1993): Wildlife Exposure Factors Handbook. United States Environmental Protection Agency, Washington, DC

USEPA (2000): Pelargonic Acid (217500) Fact Sheet. United States Environmental Protection Agency, Washington, DC

Van Meter, R.J.; Glinski, D.A.; Hong, T.; Cyterski, M.; Henderson, W.M.; Purucker, S.T. (2014): Estimating terrestrial amphibian pesticide body burden through dermal exposure. Environmental Pollution 193: 262–268. https://doi.org/10.1016/j.envpol.2014.07.003

Van Meter, R.J.; Glinski, D.A.; Henderson, W.M.; Garrison, A.W.; Cyterski, M.; Purucker, S.T. (2015): Pesticide uptake across the amphibian dermis through soil and overspray exposures. Archives of Environmental Contamination and Toxicology 69: 545–556. https://doi.org/10.1007/s00244-015-0183-2

Van Meter, R.J.; Glinski, D.A.; Henderson, W.M.; Purucker, S.T. (2016): Soil organic matter content effects on dermal pesticide bioconcentration in American toads (*Bufo americanus*). Environmental Toxicology and Chemistry 35: 2734–2741. https://doi.org/10.1002/etc.3439

Vincent, K.; Davidson, C. (2015): The toxicity of glyphosate alone and glyphosate-surfactant mixtures to western toad (*Anaxyrus boreas*) tadpoles. Environmental Toxicology and Chemistry 34: 2791–2795. https://doi.org/10.1002/etc.3118

Wagner, N.; Lötters, S. (2013): Effects of water contamination on site selection by amphibians: experiences from an arena approach with European frogs and newts. Archives of Environmental Contamination and Toxicology 65: 98–104. https://doi.org/10.1007/s00244-013-9873-9

Wagner, N.; Lötters, S.; Veith, M.; Viertel, B. (2015): Acute toxic effects of the herbicide formulation and the active ingredient used in cycloxydim-tolerant maize cultivation on embryos and larvae of the African clawed frog, *Xenopus laevis*. Bulletin of Environmental Contamination and Toxicology 94: 412–418. https://doi.org/10.1007/s00128-015-1474-z

Wauchope, R.D.; Yeh, S.; Linders, J.B.H.J.; Kloskowski, R.; Tanaka, K.; Rubin, B.; Katayama, A.; Kördel, W.; Gerstl, Z.; Lane, M.; Unsworth, J.B. (2002): Pesticide soil sorption parameters: theory, measurement, uses, limitations and reliability. Pest Management Science 58: 419–445. https://doi.org/10.1002/ps.489

Weir, S.M.; Suski, J.G.; Salice, C.J. (2010): Ecological risk of anthropogenic pollutants to reptiles: Evaluating assumptions of sensitivity and exposure. Environmental Pollution 158: 3596–3606. https://doi.org/10.1016/j.envpol.2010.08.011

Weir, S.M.; Talent, L.G.; Anderson, T.A.; Salice, C.J. (2014): Unraveling the relative importance of oral and dermal contaminant exposure in reptiles: insights from studies using the western fence lizard (*Sceloporus occidentalis*). Plos One 9: e99666. https://doi.org/10.1371/journal.pone.0099666

Weir, S.M.; Yu, S.; Talent, L.G.; Maul, J.D.; Anderson, T.A.; Salice, C.J. (2015): Improving reptile ecological risk assessment: Oral and dermal toxicity of pesticides to a common lizard species (*Sceloporus occidentalis*). Environmental Toxicology and Chemistry 34: 1778–1786. https://doi.org/10.1002/etc.2975

Welch, W.; Ross, S.; Steinle, A.C.; Schneegurt, M.A. (2010): Effects of sublethal concentrations of isoxaben on the development of the African clawed frog, *Xenopus laevis*. Toxicological and Environmental Chemistry 92: 1895–1899. https://doi.org/10.1080/02772248.2010.482844

Weltje, L.; Janz, P.; Sowig, P. (2017): An interspecies correlation model to predict acute dermal toxicity of plant protection products to terrestrial life stages of amphibians using fish acute toxicity and bioconcentration data. Chemosphere 189: 619–626. https://doi.org/10.1016/j.chemosphere.2017.09.047

Wilkinson, C.F.; Murray, M.; Marcus, C.B. (1984): Interaction of methylenedioxyphenyl compounds with cytochrome P-450 and microsomal oxidation. Reviews in Biochemical Toxicology 6: 27–63

Williams, B.K.; Semlitsch, R.D. (2010): Larval responses of three Midwestern anurans to chronic, low-dose exposures of four herbicides. Archives of Environmental Contamination and Toxicology 58: 819–827. https://doi.org/10.1007/s00244-009-9390-z

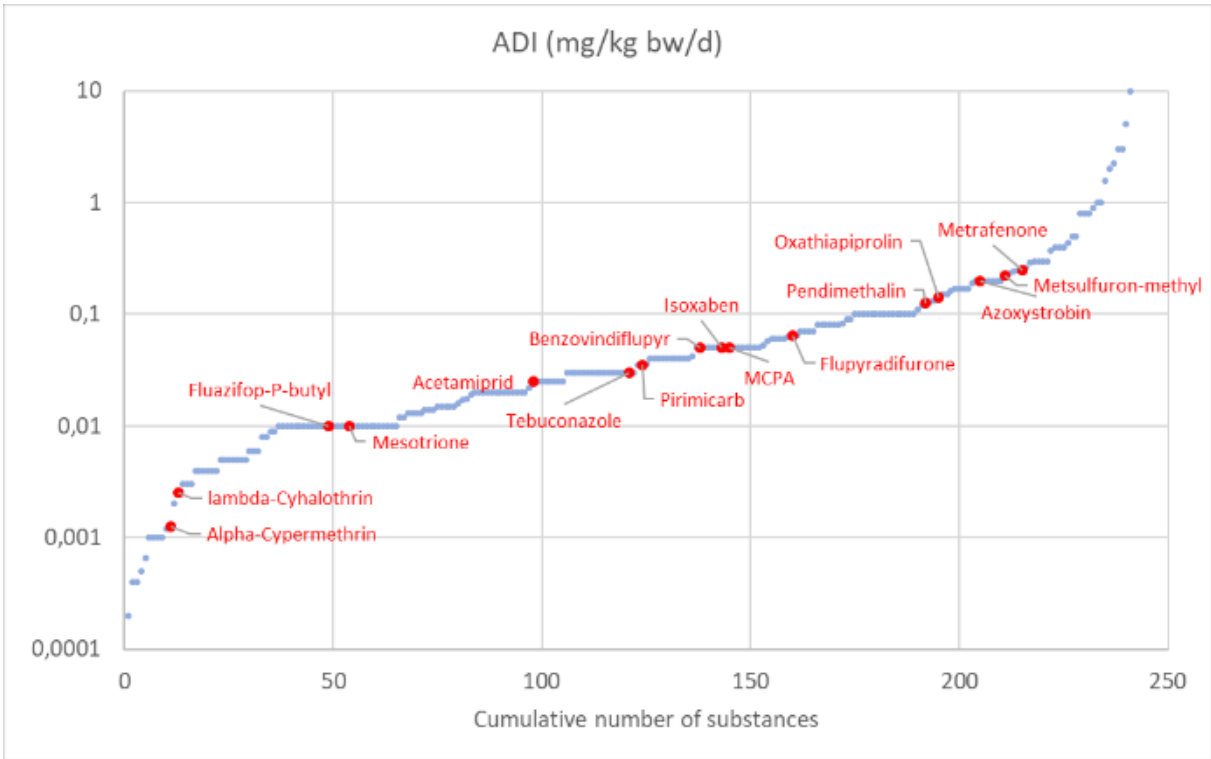
Wilson, P.C.; Whitwell, T.; Riley, M.B. (1995): Effects of ground cover and formulation on herbicides in runoff water from miniature nursery sites. Weed Science 43: 671–677. https://doi.org/10.1017/s0043174500081819

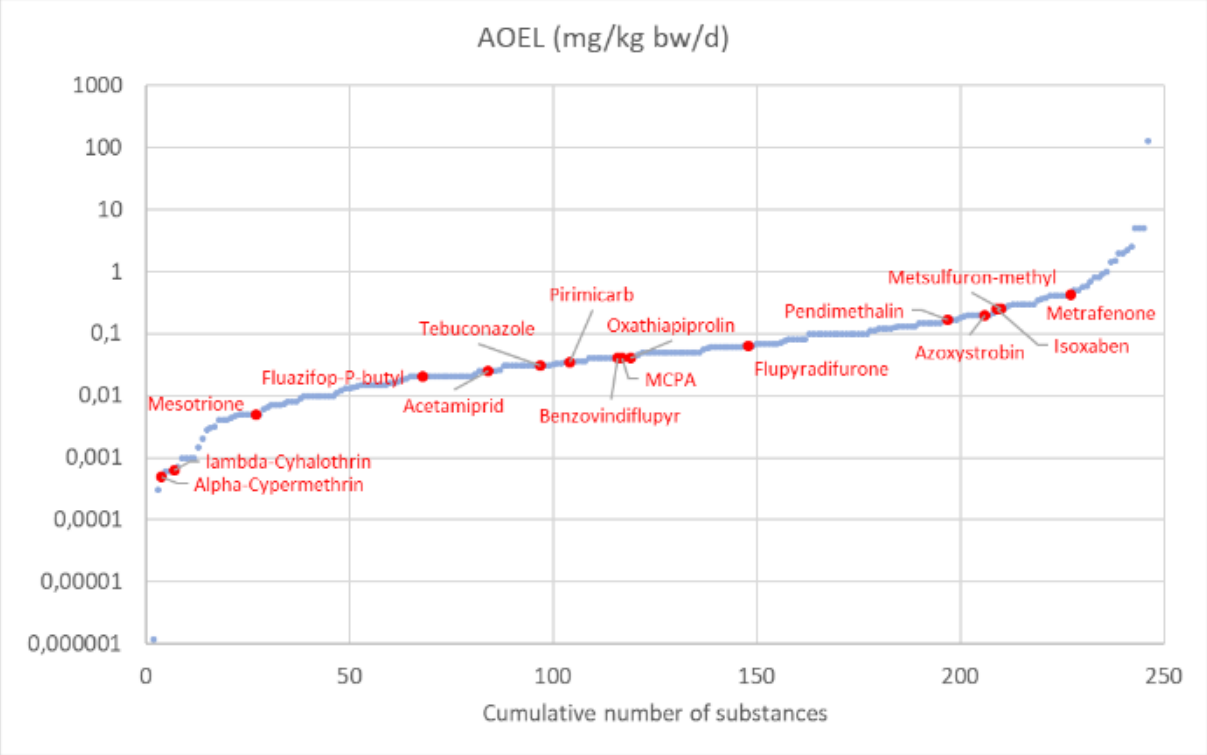
Wojtaszek, B.F.; Buscarini, T.M.; Chartrand, D.T.; Stephenson, G.R.; Thompson, D.G. (2005): Effect of Release (R) herbicide on mortality, avoidance response, and growth of amphibian larvae in two forest wetlands. Environmental Toxicology and Chemistry 24: 2533–2544. https://doi.org/10.1897/04-377r.1

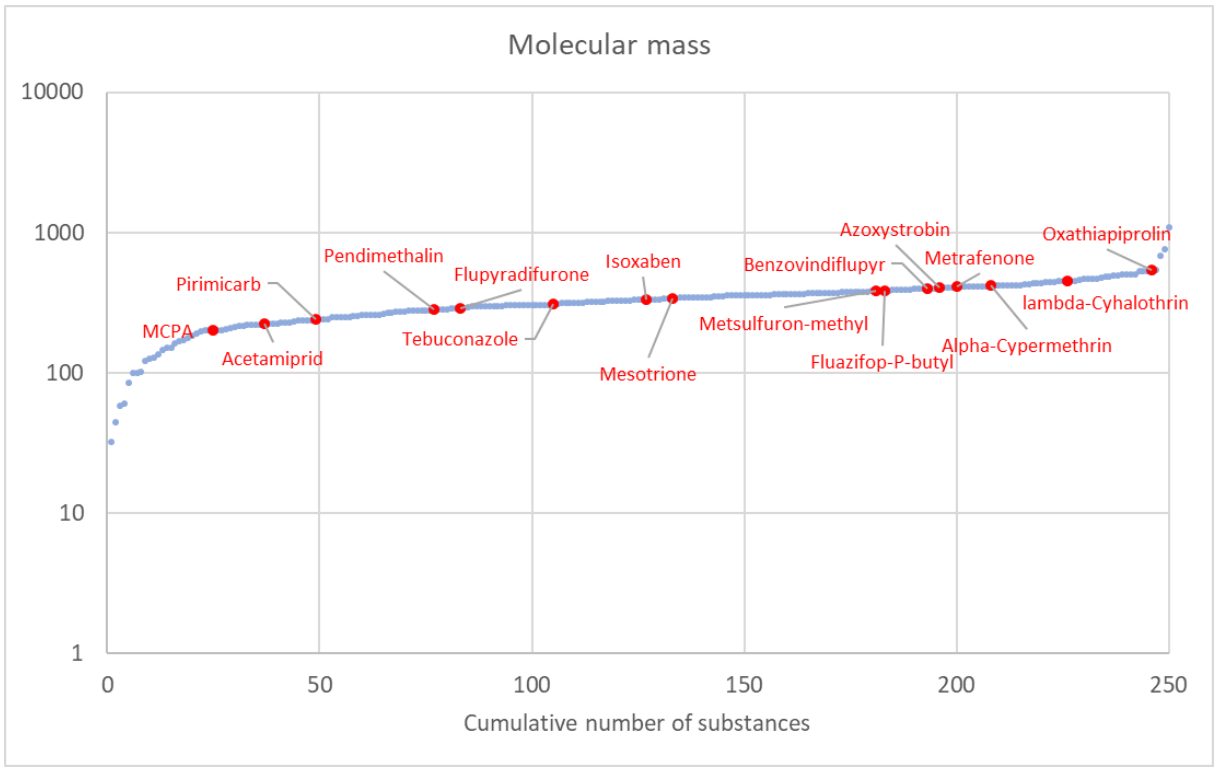
Wright, K.M.; Whitaker, B.R. (2001). Amphibian Medicine and Captive Husbandry. Krieger Publishing Company, Malabar, Florida.

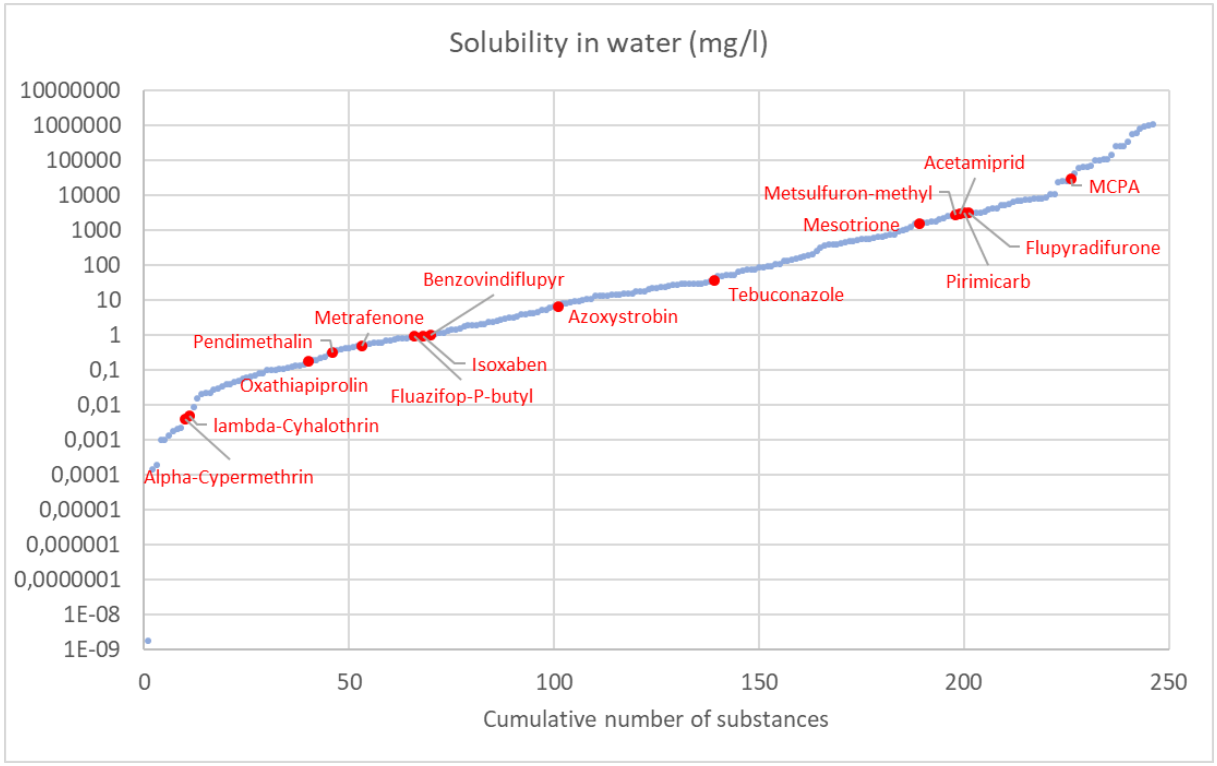
A Appendix. Distribution of the selected active ingredients along gradients of the physico-chemical and toxicological properties considered

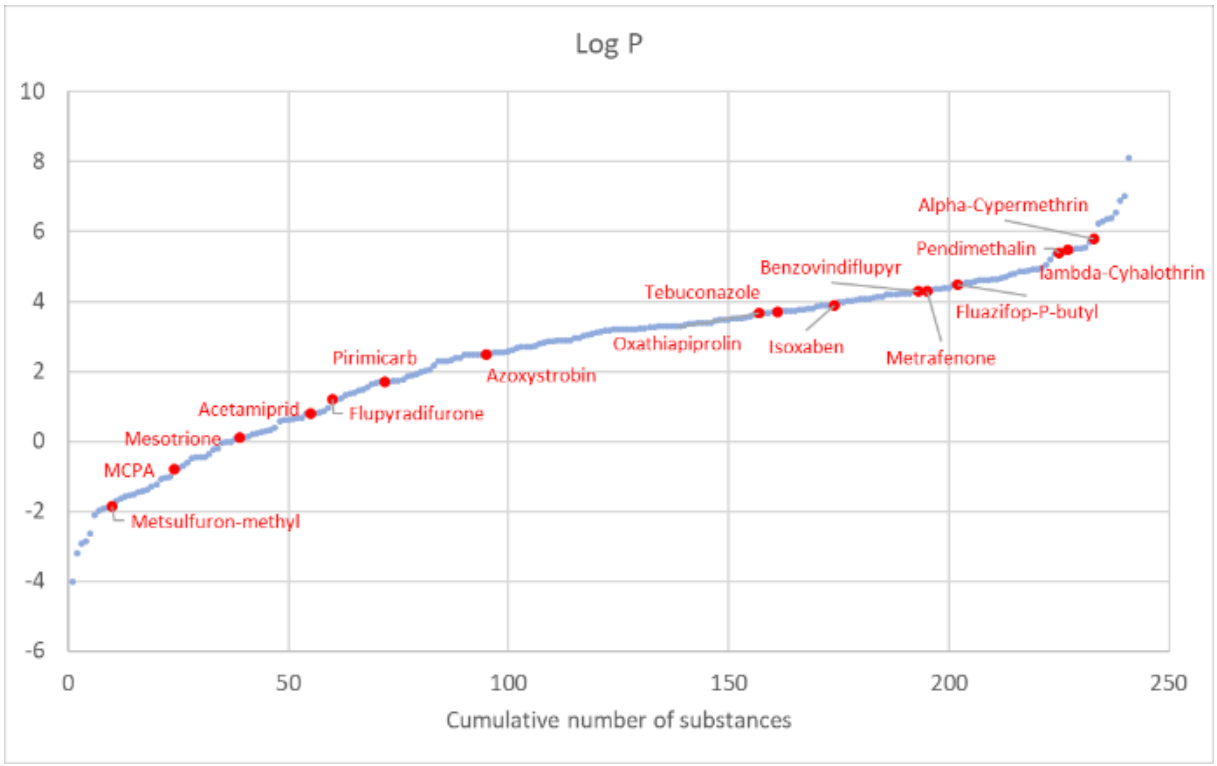
Source: own illustrations, [IREC-CSIC].






Molecular mass
10000
1000
100
10
1
0
50
100
150
200
250
Cumulative number of substances
Pirimicarb
Pendimethalin
Flupyradifurone
Isoxaben
Azoxystrobin
Benzovindiflupyr
Metrafenone
Oxathiapiprolin
MCPA
Acetamiprid
Tebuconazole
Mesotrione
Metsulfuron-methyl
Fluazifop-P-butyl
Alpha-Cypermethrin
lambda-Cyhalothrin


Solubility in water (mg/l)
10000000
1000000
100000
10000
1000
100
10
1
0,1
0,01
0,001
0,0001
0,00001
0,000001
0,0000001
1E-08
1E-09
0
50
100
150
200
250
Cumulative number of substances
Acetamiprid
MCPA
Metsulfuron-methyl
Mesotrione
Flupyradifurone
Pirimicarb
Benzovindiflupyr
Tebuconazole
Metrafenone
Azoxystrobin
Pendimethalin
Isoxaben
Oxathiapiprolin
Fluazifop-P-butyl
lambda-Cyhalothrin
Alpha-Cypermethrin


Log P
10
8
6
4
2
0
-2
-4
-6
0
50
100
150
200
250
Cumulative number of substances
Alpha-Cypermethrin
Pendimethalin
lambda-Cyhalothrin
Benzovindiflupyr
Tebuconazole
Fluazifop-P-butyl
Oxathiapiprolin
Isoxaben
Metrafenone
Pirimicarb
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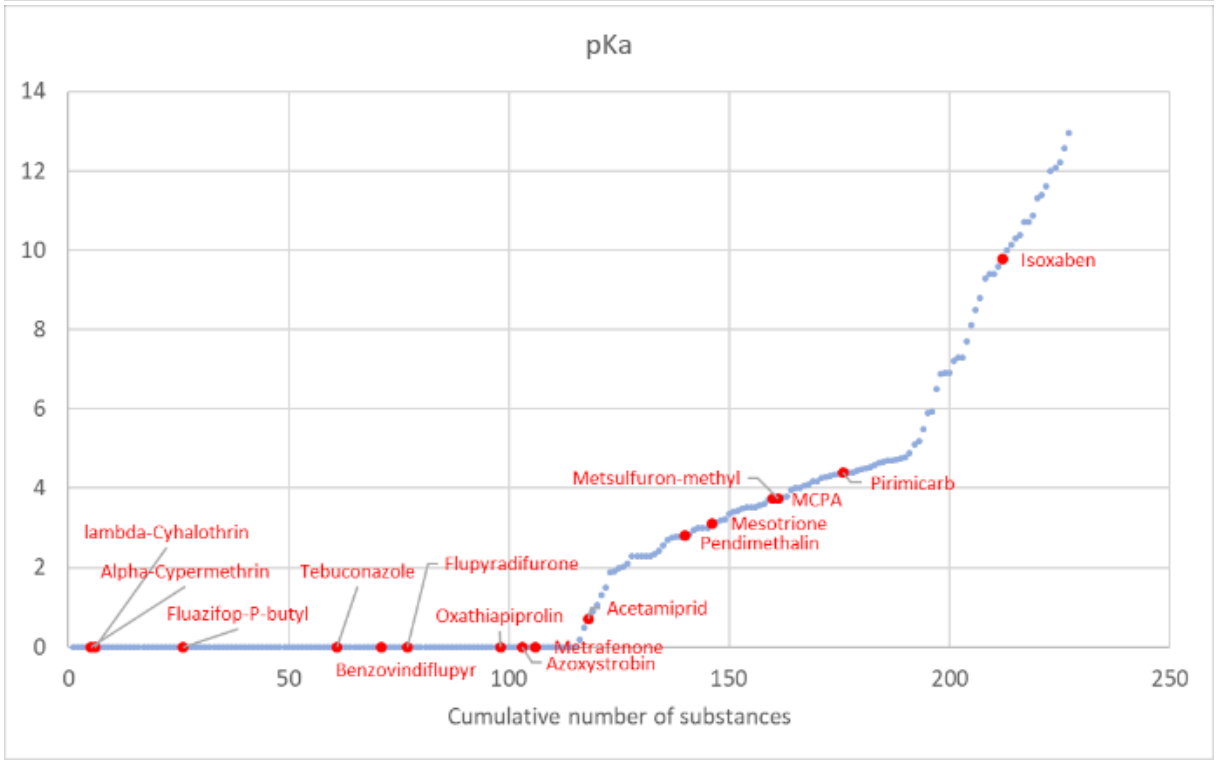
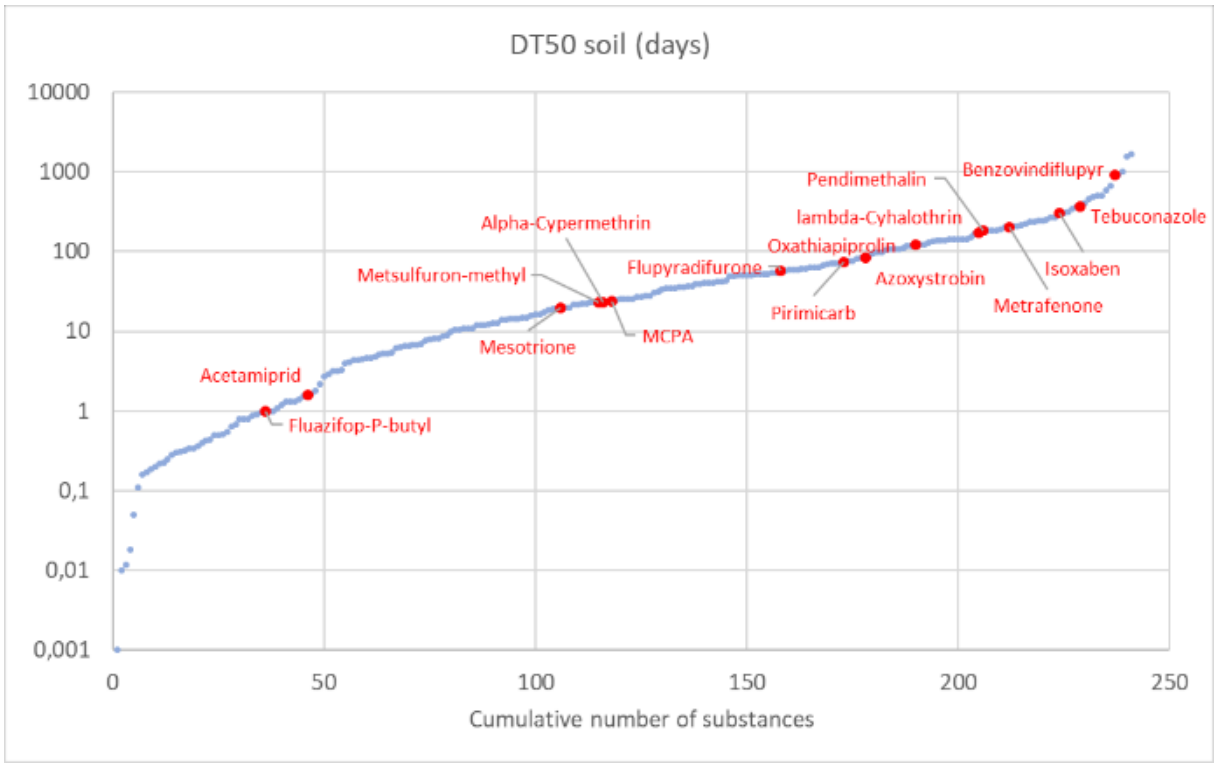
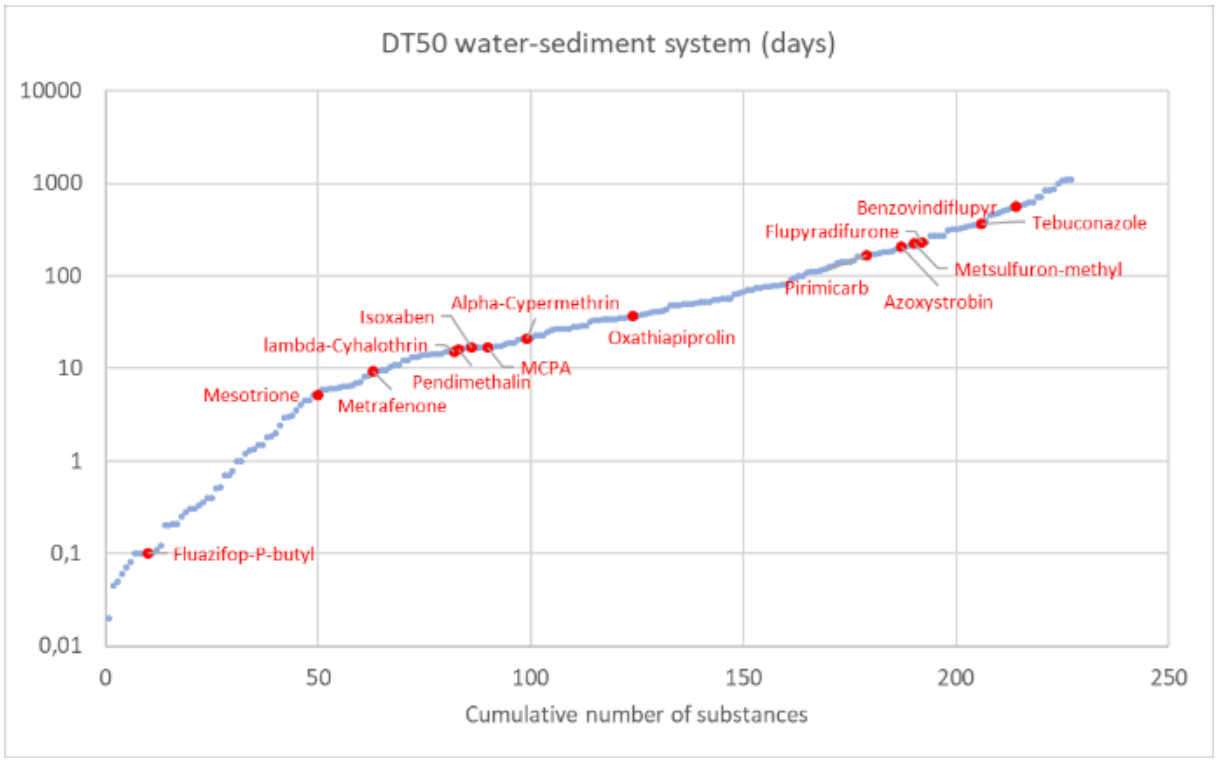
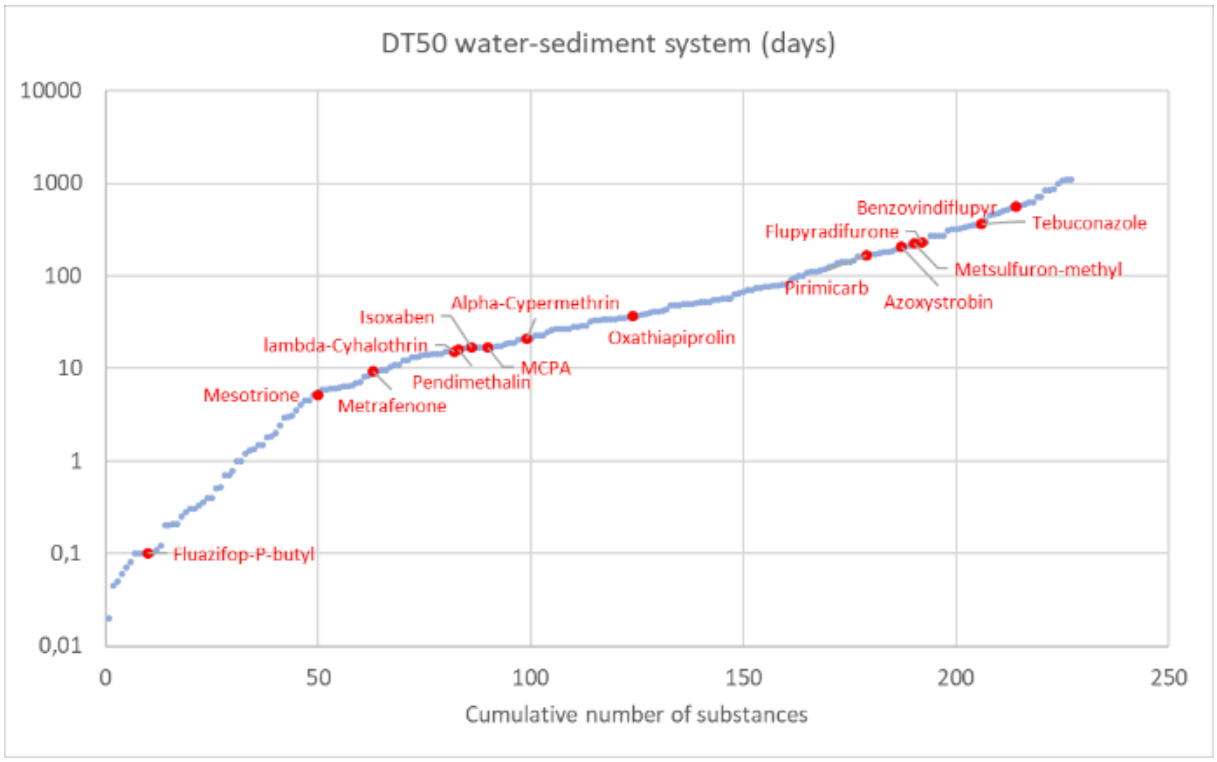
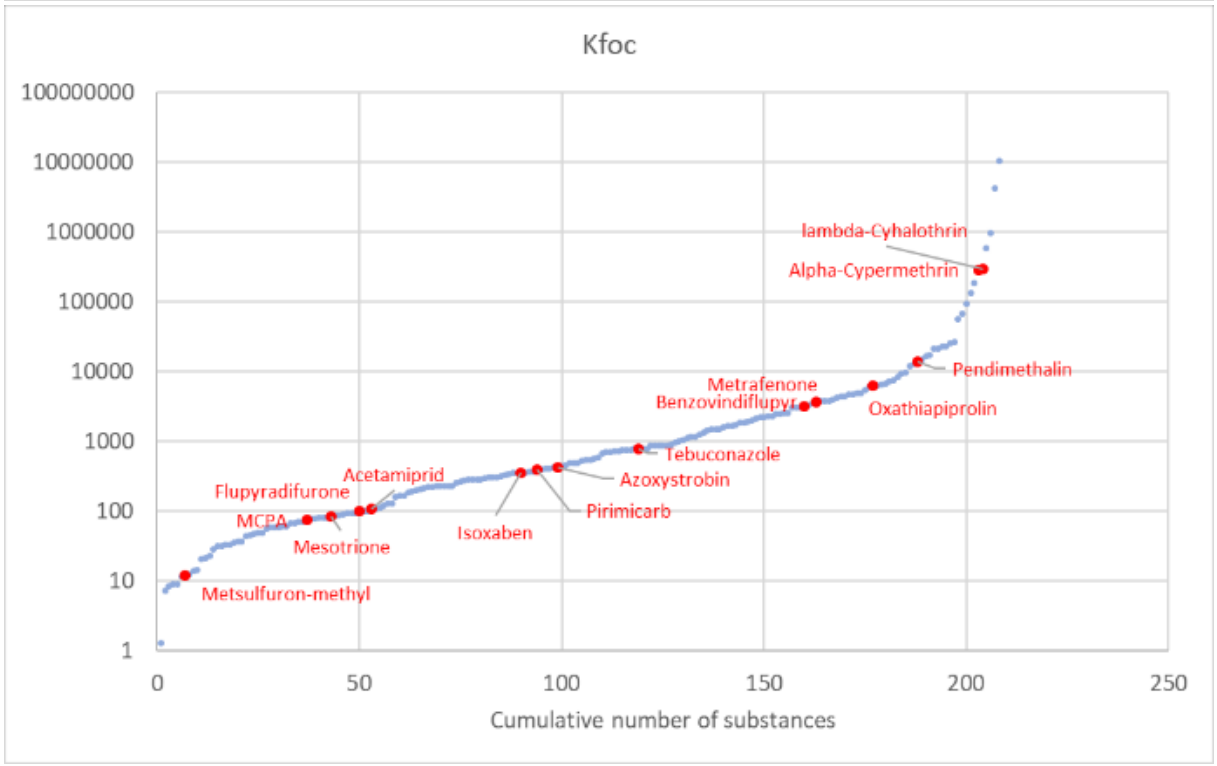
Acetamiprid
Flupyradifurone
Mesotrione
MCPA
Metsulfuron-methyl

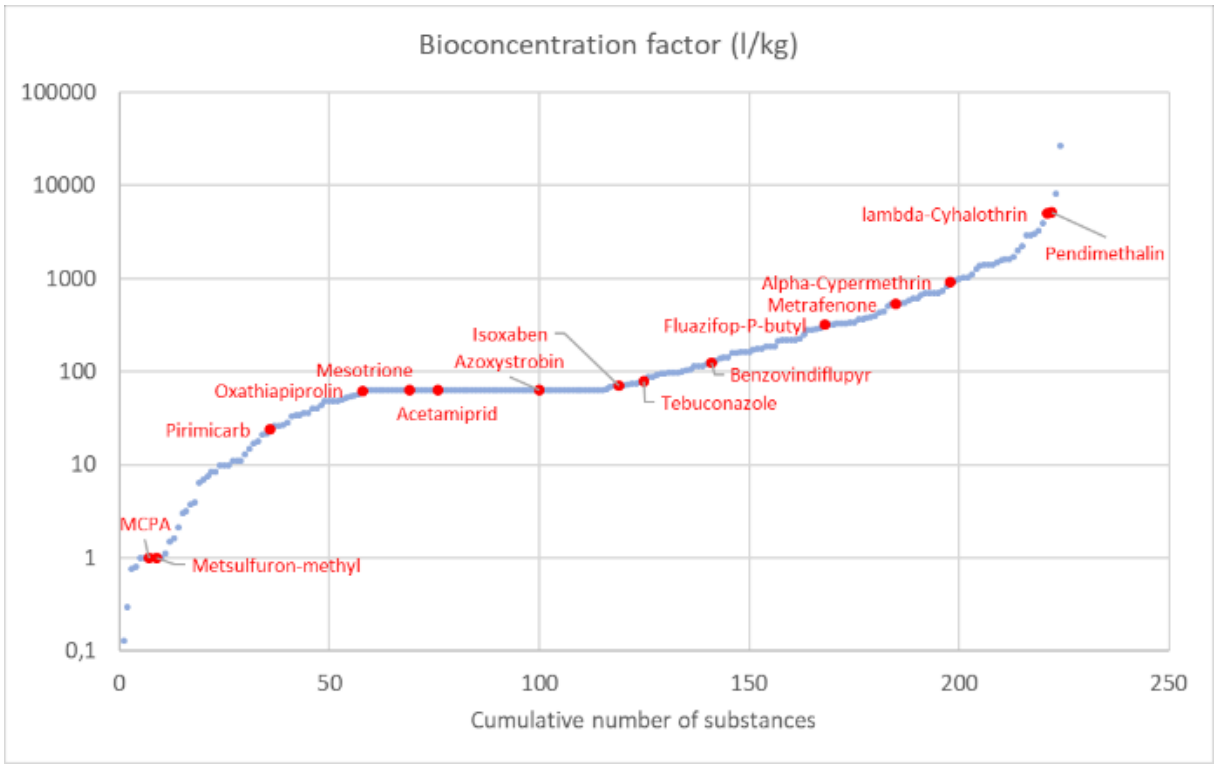

pKa
14
12
10
8
6
4
2
0
0
50
100
150
200
250
Cumulative number of substances
Isoxaben
Metsulfuron-methyl
Pirimicarb
MCPA
Mesotrione
Pendimethalin
lambda-Cyhalothrin
Alpha-Cypermethrin
Tebuconazole
Flupyradifurone
Fluazifop-P-butyl
Oxathiapiprolin
Acetamiprid
Metrafenone
Azoxystrobin
Benzovindiflupyr


DT50 soil (days)
10000
1000
100
10
1
0,1
0,01
0,001
0
50
100
150
200
250
Cumulative number of substances
Pendimethalin
Benzovindiflupyr
Alpha-Cypermethrin
lambda-Cyhalothrin
Tebuconazole
Oxathiapiprolin
Metsulfuron-methyl
Flupyradifurone
Azoxystrobin
Isoxaben
Pirimicarb
Metrafenone
MCPA
Mesotrione
Acetamiprid
Fluazifop-P-butyl


DT50 water-sediment system (days)
10000
1000
100
10
1
0,1
0,01
0
50
100
150
200
250
Cumulative number of substances
Benzovindiflupyr
Tebuconazole
Flupyradifurone
Metsulfuron-methyl
Pirimicarb
Azoxystrobin
Alpha-Cypermethrin
Isoxaben
Oxathiapiprolin
lambda-Cyhalothrin
MCPA
Pendimethalin
Mesotrione
Metrafenone
Fluazifop-P-butyl


DT50 water-sediment system (days)
10000
1000
100
10
1
0,1
0,01
0
50
100
150
200
250
Cumulative number of substances
Benzovindiflupyr
Tebuconazole
Flupyradifurone
Metsulfuron-methyl
Pirimicarb
Azoxystrobin
Alpha-Cypermethrin
Isoxaben
Oxathiapiprolin
lambda-Cyhalothrin
MCPA
Pendimethalin
Mesotrione
Metrafenone
Fluazifop-P-butyl


Kfoc
100000000
10000000
1000000
100000
10000
1000
100
10
1
0
50
100
150
200
250
Cumulative number of substances
lambda-Cyhalothrin
Alpha-Cypermethrin
Pendimethalin
Metrafenone
Benzovindiflupyr
Oxathiapiprolin
Tebuconazole
Acetamiprid
Azoxystrobin
Flupyradifurone
Pirimicarb
MCPA
Isoxaben
Mesotrione
Metsulfuron-methyl


Bioconcentration factor (l/kg)
100000
10000
1000
100
10
1
0,1
0
50
100
150
200
250
Cumulative number of substances
lambda-Cyhalothrin
Pendimethalin
Alpha-Cypermethrin
Metrafenone
Fluazifop-P-butyl
Isoxaben
Azoxystrobin
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Mesotrione
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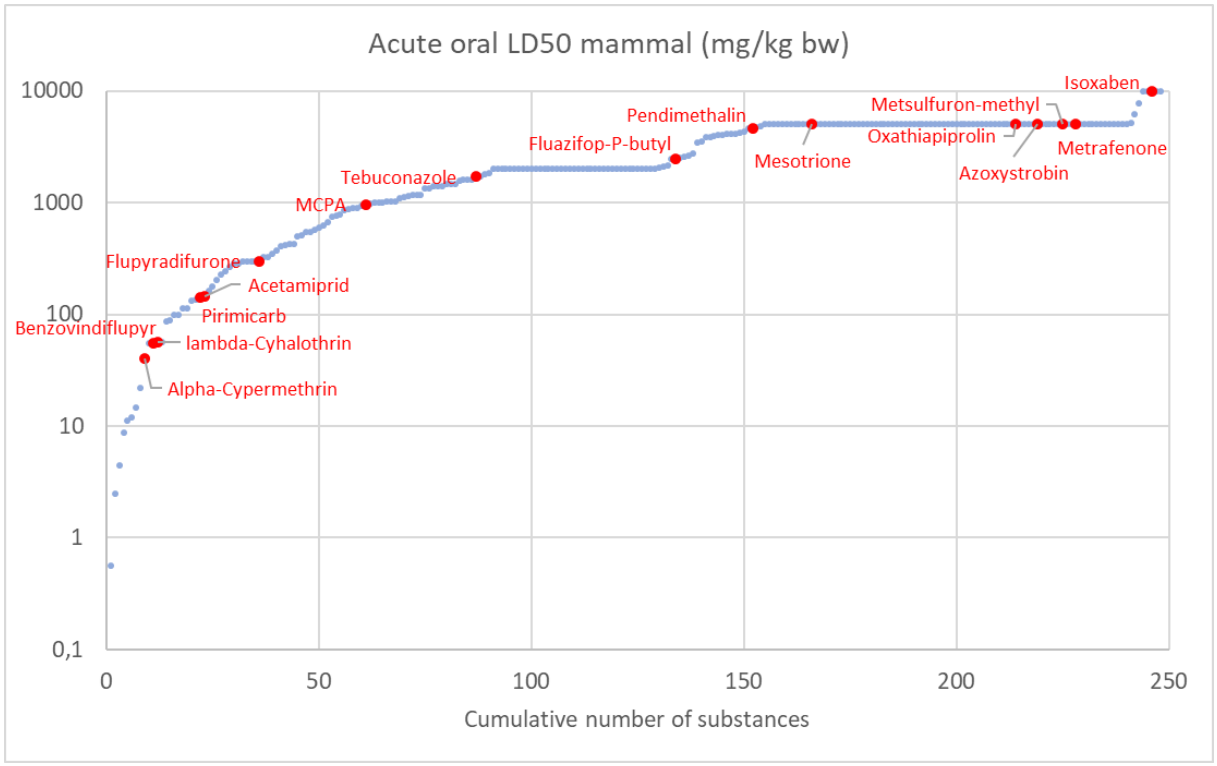
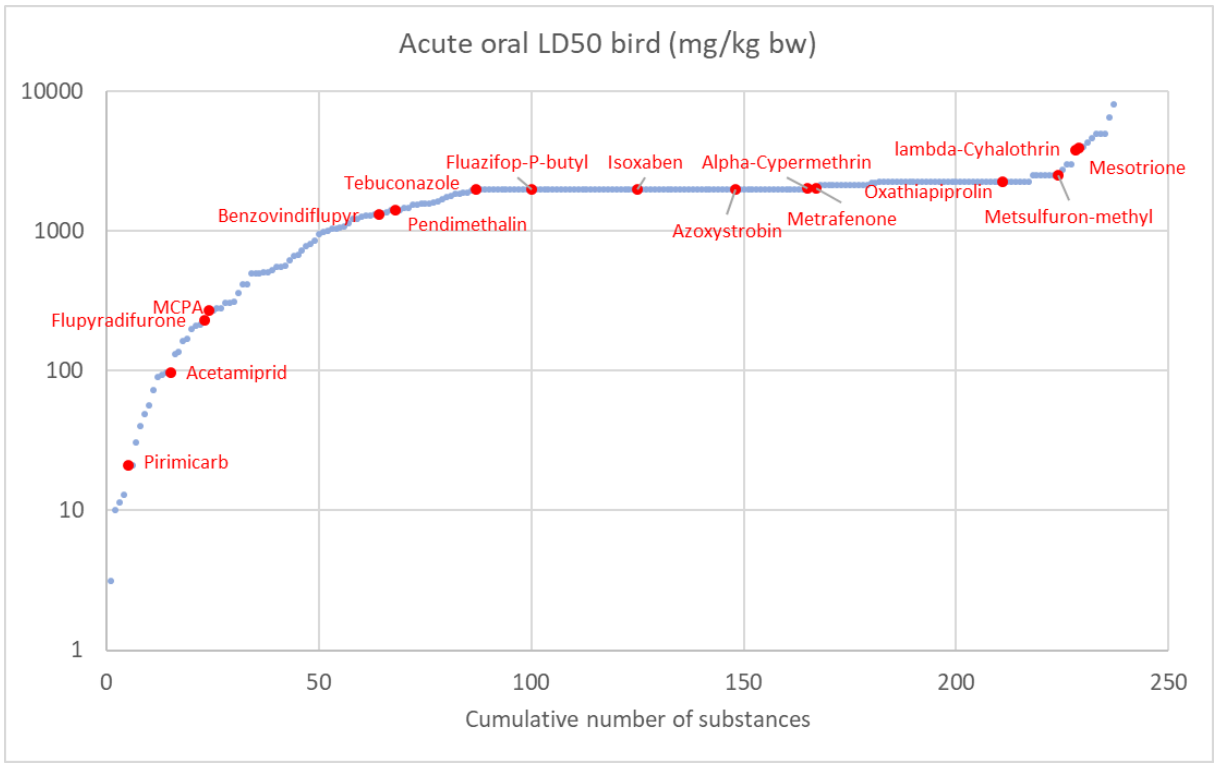
Tebuconazole
Acetamiprid
Pirimicarb
MCPA
Metsulfuron-methyl


Acute oral LD50 mammal (mg/kg bw)
10000
1000
100
10
1
0,1
0
50
100
150
200
250
Cumulative number of substances
Isoxaben
Metsulfuron-methyl
Pendimethalin
Oxathiapiprolin
Fluazifop-P-butyl
Metrafenone
Mesotrione
Azoxystrobin
Tebuconazole
MCPA
Flupyradifurone
Acetamiprid
Pirimicarb
Benzovindiflupyr
lambda-Cyhalothrin
Alpha-Cypermethrin


Acute oral LD50 bird (mg/kg bw)
10000
1000
100
10
1
0
50
100
150
200
250
Cumulative number of substances
Fluazifop-P-butyl
Isoxaben
Alpha-Cypermethrin
lambda-Cyhalothrin
Mesotrione
Tebuconazole
Oxathiapiprolin
Benzovindiflupyr
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Azoxystrobin
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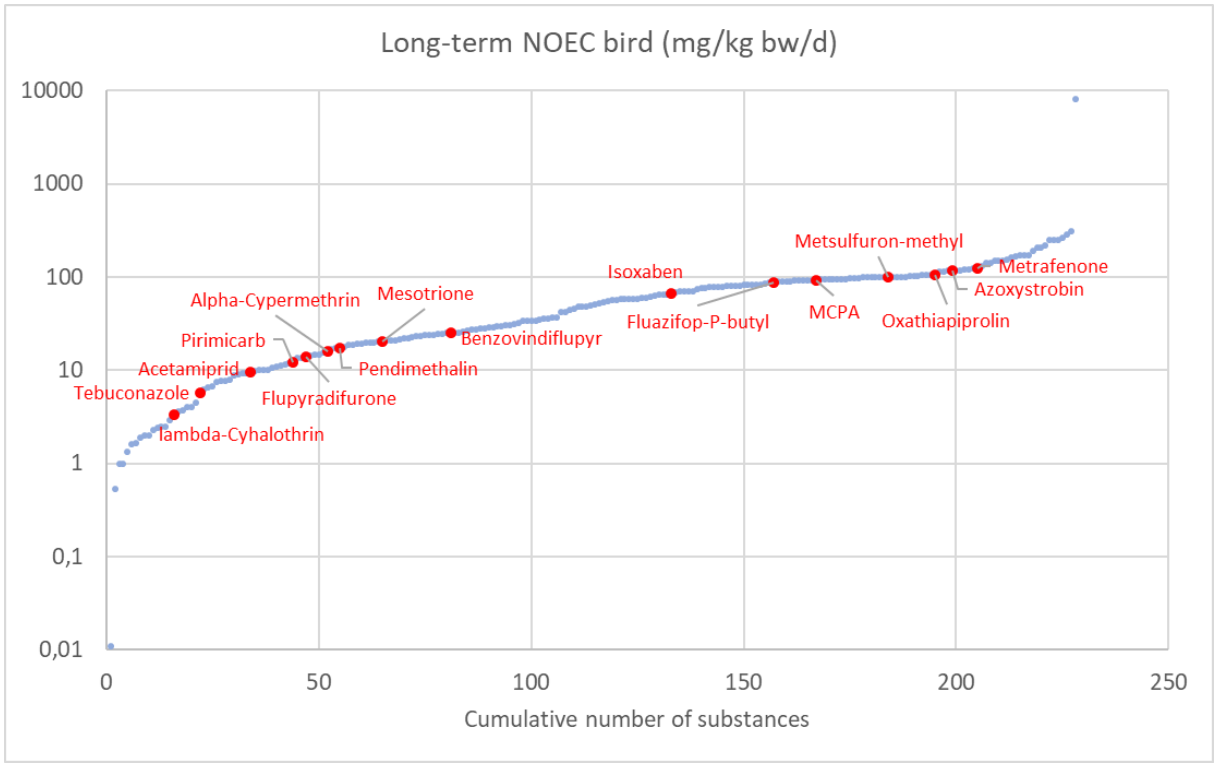
MCPA
Flupyradifurone
Acetamiprid
Pirimicarb


Long-term NOEC bird (mg/kg bw/d)
10000
1000
100
10
1
0,1
0,01
0
50
100
150
200
250
Cumulative number of substances
Metsulfuron-methyl
Isoxaben
Metrafenone
Azoxystrobin
Alpha-Cypermethrin
Mesotrione
MCPA
Fluazifop-P-butyl
Oxathiapiprolin
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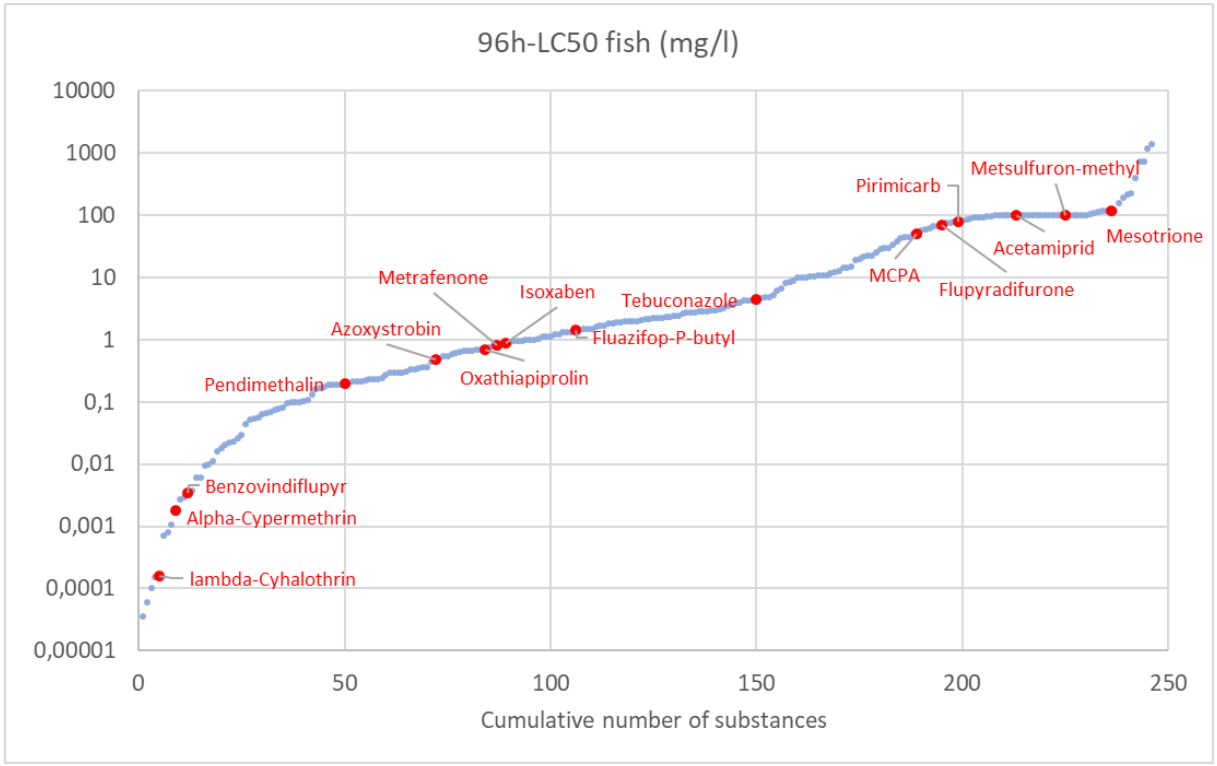
Tebuconazole
Flupyradifurone
lambda-Cyhalothrin


96h-LC50 fish (mg/l)
10000
1000
100
10
1
0,1
0,01
0,001
0,0001
0,00001
0
50
100
150
200
250
Cumulative number of substances
Pirimicarb
Metsulfuron-methyl
Mesotrione
Acetamiprid
MCPA
Flupyradifurone
Metrafenone
Isoxaben
Tebuconazole
Azoxystrobin
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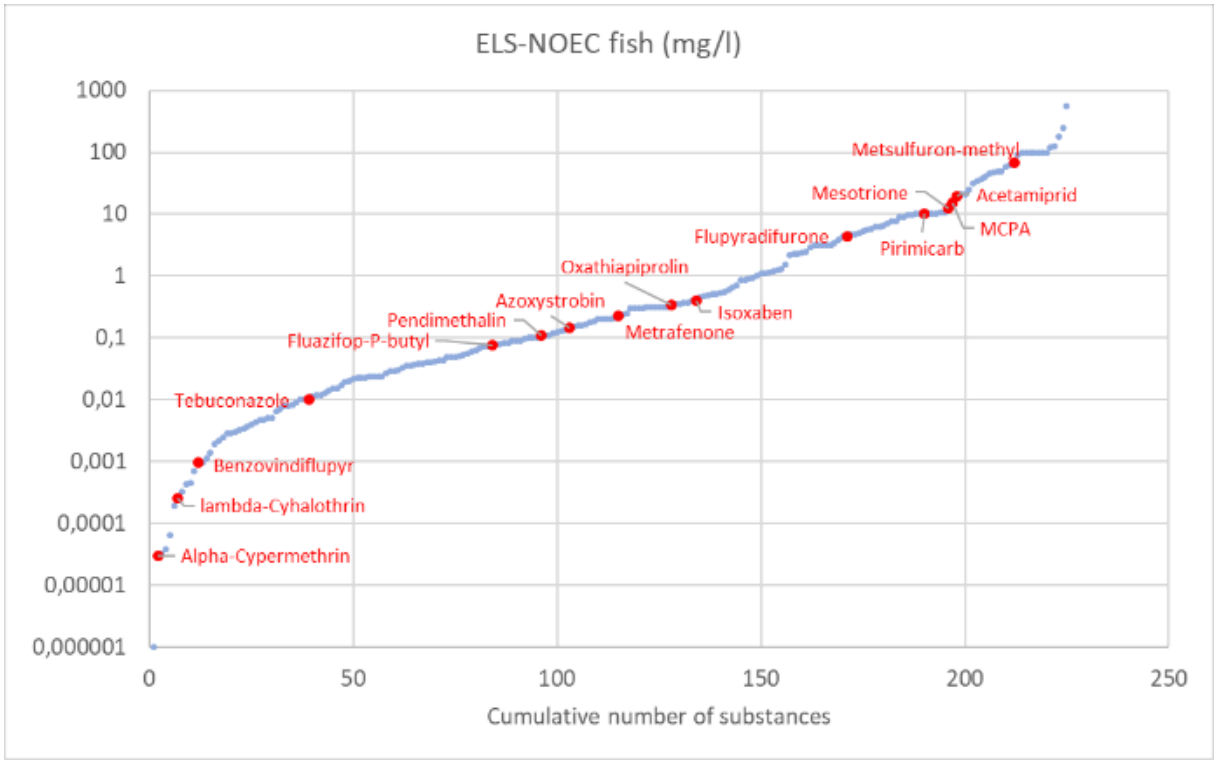
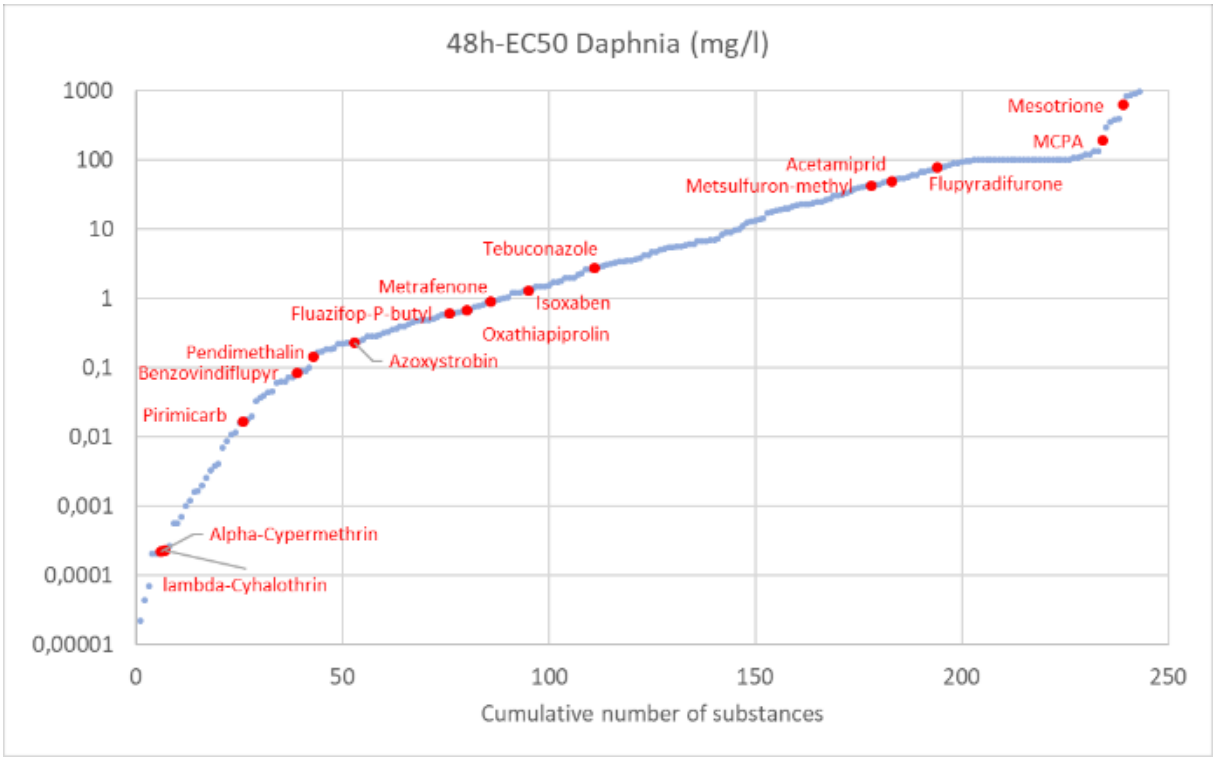
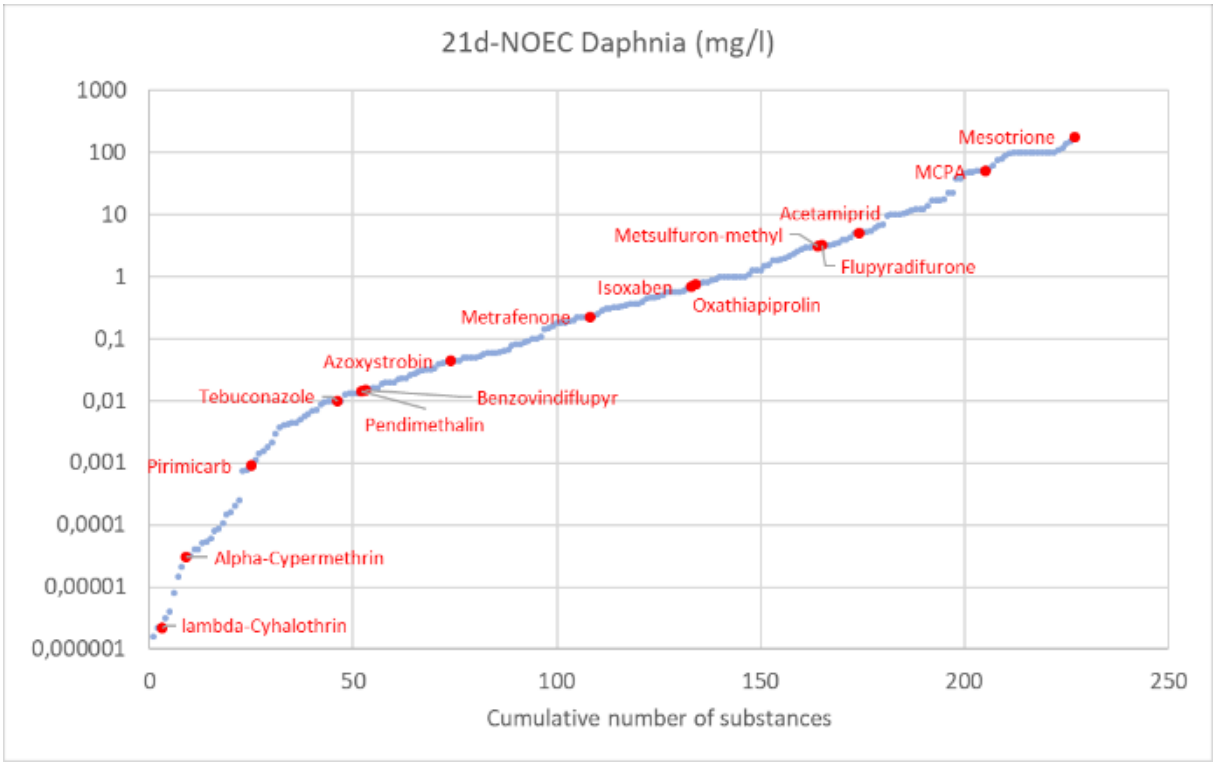
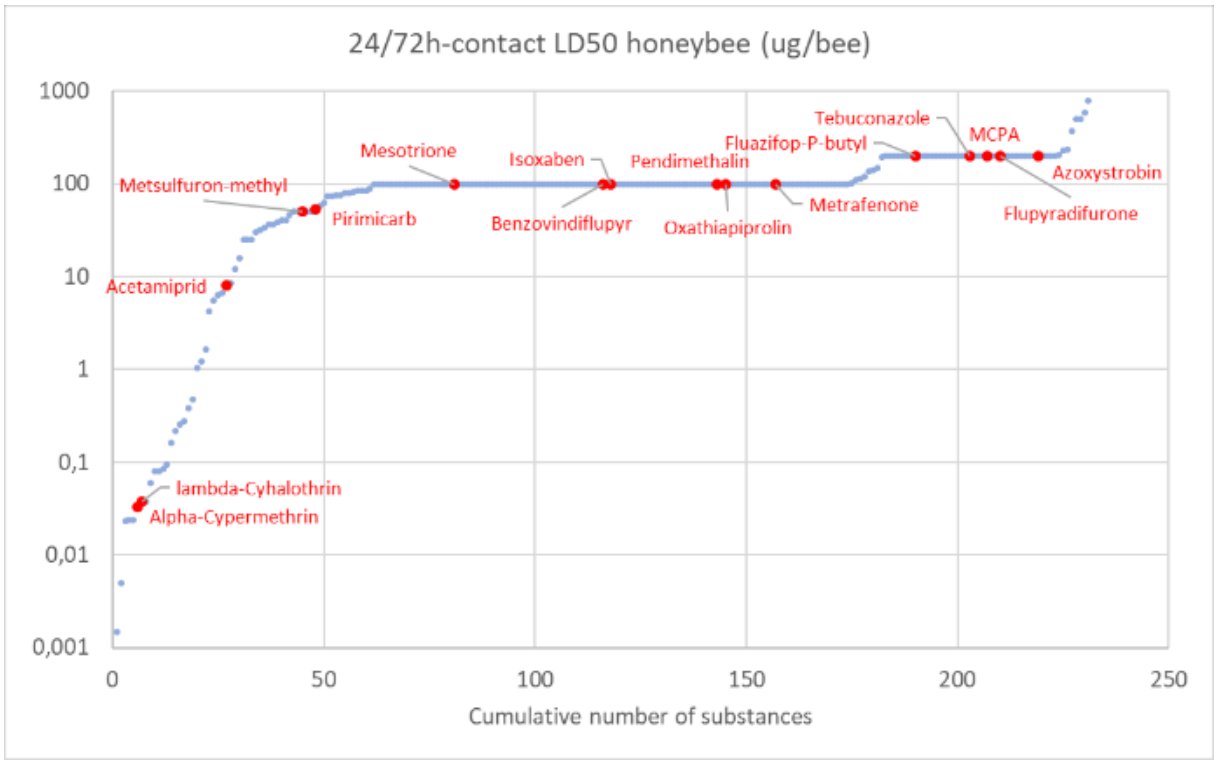
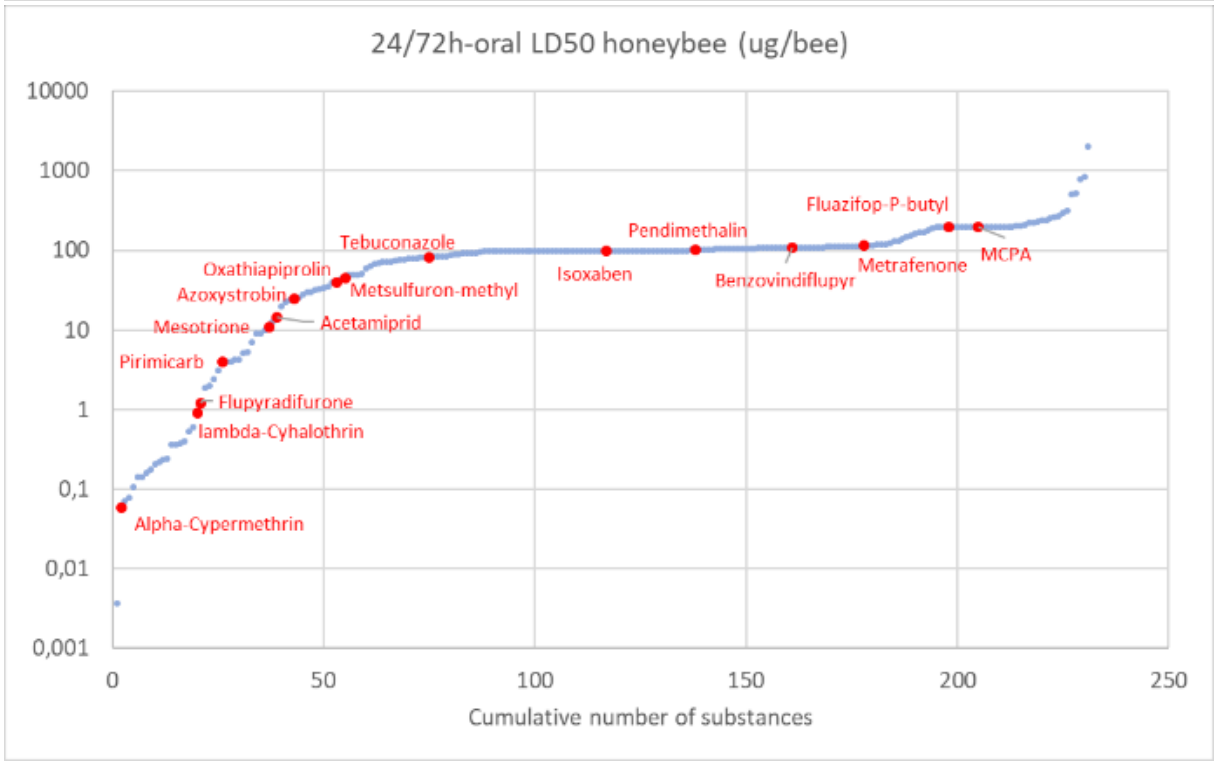
Pendimethalin
Benzovindiflupyr
Alpha-Cypermethrin
lambda-Cyhalothrin

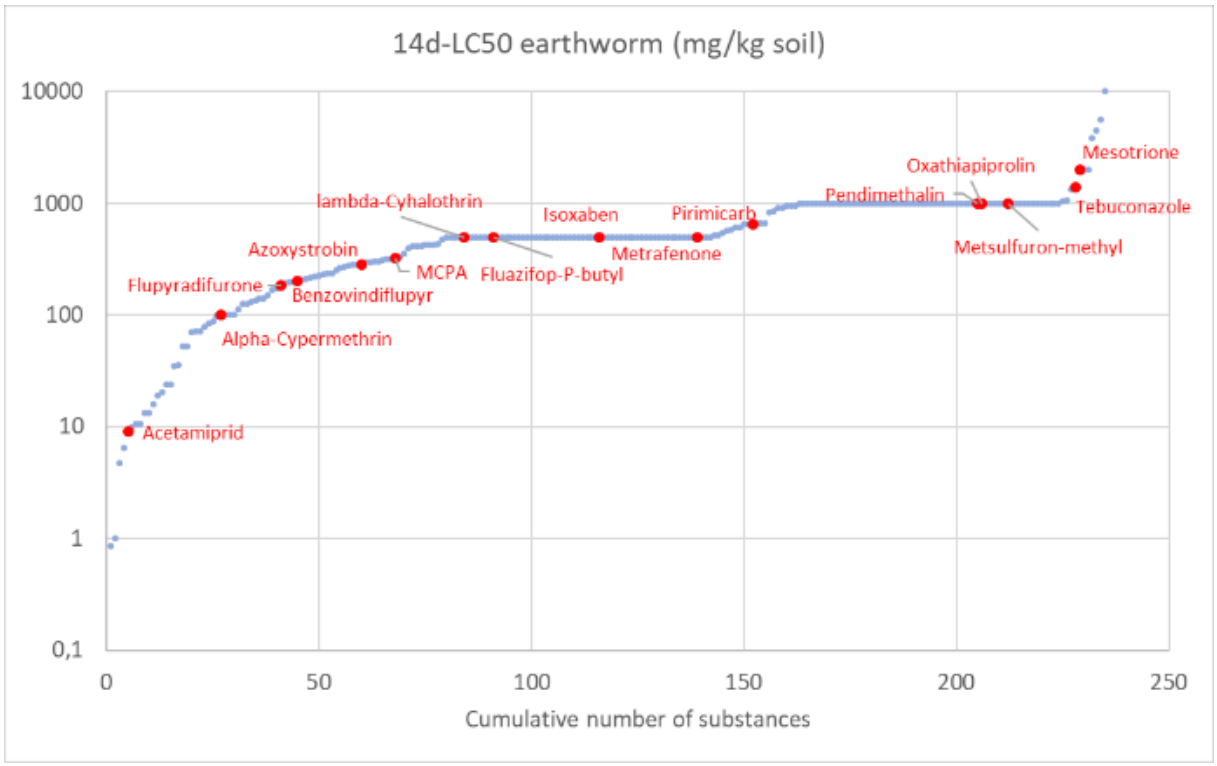

ELS-NOEC fish (mg/l)
1000
100
10
1
0,1
0,01
0,001
0,0001
0,00001
0,000001
0
50
100
150
200
250
Cumulative number of substances
Metsulfuron-methyl
Mesotrione
Acetamiprid
MCPA
Flupyradifurone
Pirimicarb
Oxathiapiprolin
Azoxystrobin
Isoxaben
Pendimethalin
Metrafenone
Fluazifop-P-butyl
Tebuconazole
Benzovindiflupyr
lambda-Cyhalothrin
Alpha-Cypermethrin

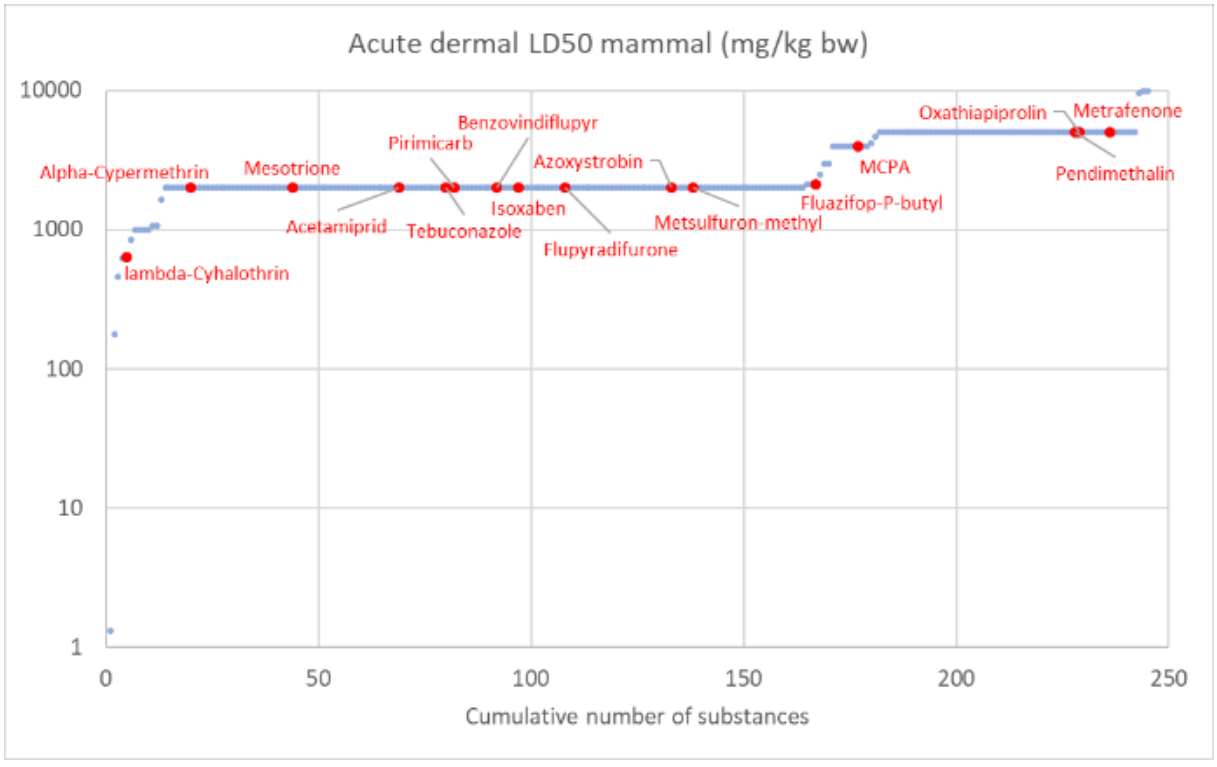

48h-EC50 Daphnia (mg/l)
1000
100
10
1
0,1
0,01
0,001
0,0001
0,00001
0
50
100
150
200
250
Cumulative number of substances
Mesotrione
MCPA
Acetamiprid
Metsulfuron-methyl
Flupyradifurone
Tebuconazole
Metrafenone
Isoxaben
Fluazifop-P-butyl
Oxathiapiprolin
Pendimethalin
Azoxystrobin
Benzovindiflupyr
Pirimicarb
Alpha-Cypermethrin
lambda-Cyhalothrin

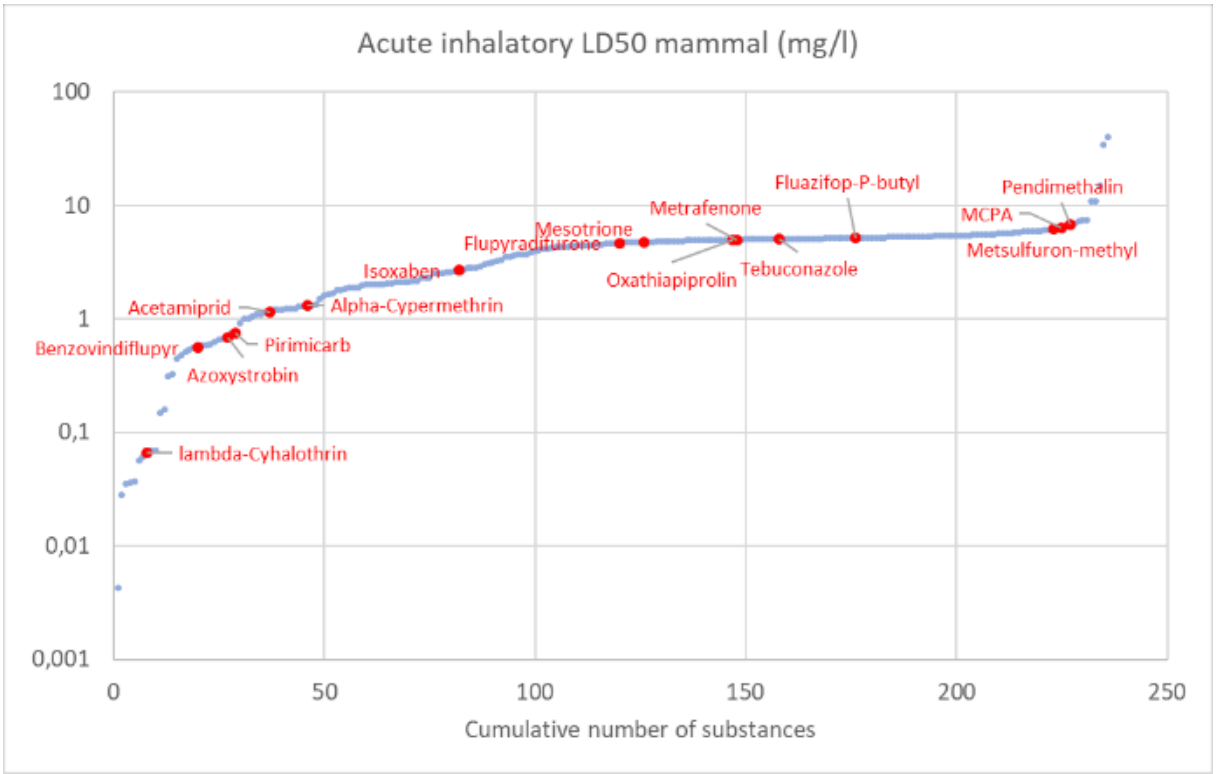

21d-NOEC Daphnia (mg/l)
1000
100
10
1
0,1
0,01
0,001
0,0001
0,00001
0,000001
0
50
100
150
200
250
Cumulative number of substances
Mesotrione
MCPA
Acetamiprid
Metsulfuron-methyl
Flupyradifurone
Isoxaben
Oxathiapiprolin
Metrafenone
Azoxystrobin
Tebuconazole
Benzovindiflupyr
Pendimethalin
Pirimicarb
Alpha-Cypermethrin
lambda-Cyhalothrin


24/72h-contact LD50 honeybee (ug/bee)
1000
100
10
1
0,1
0,01
0,001
0
50
100
150
200
250
Cumulative number of substances
Tebuconazole
MCPA
Fluazifop-P-butyl
Azoxystrobin
Mesotrione
Isoxaben
Pendimethalin
Metsulfuron-methyl
Pirimicarb
Benzovindiflupyr
Oxathiapiprolin
Metrafenone
Flupyradifurone
Acetamiprid
lambda-Cyhalothrin
Alpha-Cypermethrin


24/72h-oral LD50 honeybee (ug/bee)
10000
1000
100
10
1
0,1
0,01
0,001
0
50
100
150
200
250
Cumulative number of substances
Fluazifop-P-butyl
Pendimethalin
Tebuconazole
MCPA
Oxathiapiprolin
Isoxaben
Metrafenone
Azoxystrobin
Metsulfuron-methyl
Benzovindiflupyr
Mesotrione
Acetamiprid
Pirimicarb
Flupyradifurone
lambda-Cyhalothrin
Alpha-Cypermethrin


14d-LC50 earthworm (mg/kg soil)
10000
1000
100
10
1
0,1
0
50
100
150
200
250
Cumulative number of substances
Acetamiprid
Alpha-Cypermethrin
Flupyradifurone
Benzovindiflupyr
Azoxystrobin
MCPA
lambda-Cyhalothrin
Fluazifop-P-butyl
Isoxaben
Metrafenone
Pirimicarb
Pendimethalin
Oxathiapiprolin
Mesotrione
Tebuconazole
Metsulfuron-methyl


Acute dermal LD50 mammal (mg/kg bw)
10000
1000
100
10
1
0
50
100
150
200
250
Cumulative number of substances
lambda-Cyhalothrin
Alpha-Cypermethrin
Mesotrione
Acetamiprid
Pirimicarb
Tebuconazole
Benzovindiflupyr
Isoxaben
Azoxystrobin
Flupyradifurone
Metsulfuron-methyl
MCPA
Fluazifop-P-butyl
Oxathiapiprolin
Metrafenone
Pendimethalin


Acute inhalatory LD50 mammal (mg/l)
100
10
1
0,1
0,01
0,001
0
50
100
150
200
250
Cumulative number of substances
Acetamiprid
Alpha-Cypermethrin
Benzovindiflupyr
Pirimicarb
Azoxystrobin
lambda-Cyhalothrin
Isoxaben
Flupyradifurone
Mesotrione
Metrafenone
Oxathiapiprolin
Tebuconazole
Fluazifop-P-butyl
Pendimethalin
MCPA
Metsulfuron-methyl

B Appendix. QuPath scripts for digital image analysis

B.1 QuPath's built-in Watershed Cell Detection:

```
selectObjectsByClassification("Region*")

runPlugin('qupath.imagej.detect.cells.WatershedCellDetection',
'{"detectionImageBrightfield":"Optical density
sum","requestedPixelSizeMicrons":0.5,"backgroundRadiusMicrons":13.0,"backgroundByReconstru
ction":true,"medianRadiusMicrons":1.1,"sigmaMicrons":1.5,"minAreaMicrons":10.0,"maxAreaMic
rons":100.0,"threshold":0.1,"maxBackground":2.0,"watershedPostProcess":true,"cellExpansionMi
crons":0.0,"includeNuclei":true,"smoothBoundaries":true,"makeMeasurements":true}')
```

B.2 StarDist deep-learning-based plugin for Cell Detection:

```
selectObjectsByClassification("Region*")

import qupath.ext.stardist.StarDist2D

import qupath.lib.scripting.QP

def modelPath = "PATH TO he_heavy_augment.pb"

def stardist = StarDist2D

    .builder(modelPath)

    .normalizePercentiles(1, 99) // Percentile normalization

    .threshold(0.45)            // Probability (detection) threshold

    .pixelSize(0.4)             // Resolution for detection

def pathObjects = QP.getSelectedObjects()

def imageData = QP.getCurrentImageData()

if (pathObjects.isEmpty()) {

    QP.getLogger().error("No parent objects are selected!")

    return

}

stardist.detectObjects(imageData, pathObjects)

stardist.close()

println('Done!')
```

C Appendix. Sublethal effects

Appendix C1: Body condition and food intake rate measured per experiment and treatment

Mean ± SD values per experiment, batch (if applicable) and treatment are given for body condition at the beginning of the assays and on days 7 and 21 after treatment, and for the food intake rate (FIR, in number of eaten crickets per individual per day). The latter parameter is not displayed for experiments of the 7th batch, as in those experiments there were no variation in the FIR.

Substance	Lab	Batch	Treatment	Condition day 0		Condition day 7		Condition day 21		FIR	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD
Metrafenone	IREC	3rd	Control	854.38	54.60	835.70	71.43	1135.42	78.23	1.78	0.09
			Solvent	845.78	64.80	880.43	78.19	1091.73	57.33	1.77	0.10
			0.1x AR	864.26	97.60	881.71	103.31	1044.49	120.75	1.71	0.10
			1x AR	866.78	72.36	864.60	65.57	964.60	62.13	1.69	0.26
			10x AR	915.27	107.76	905.79	44.81	1187.54	82.15	1.77	0.09
Oxathiapiprolin	IREC	3rd	Control	867.46	66.13	842.92	50.51	1031.90	66.18	1.47	0.27
			Solvent	947.13	114.60	834.66	84.85	993.27	178.18	1.46	0.27
			0.1x AR	908.64	124.07	883.95	98.73	988.84	-	1.43	0.32
			1x AR	957.89	105.26	813.50	53.56	1118.69	61.50	1.46	0.28
			10x AR	959.15	99.12	891.80	43.88	949.66	89.98	1.47	0.27
Benzovindiflupyr	IREC	3rd	Control	906.53	74.82	916.66	108.30	990.82	182.40	1.12	0.28
			Solvent	875.26	100.85	844.91	75.79	1010.09	150.15	1.11	0.27
			0.1x AR	936.79	84.03	883.31	20.50	1063.78	80.52	1.08	0.25

Substance	Lab	Batch	Treatment	Condition day 0		Condition day 7		Condition day 21		FIR	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD
			1x AR	849.15	59.37	872.64	32.81	1045.11	96.93	1.10	0.26
			10x AR	964.75	133.31	944.40	79.65	1071.66	114.00	1.10	0.26
			Soil	848.58	86.98	827.03	56.24	1050.86	63.74	1.10	0.26
			Soil+overspray	894.79	65.26	877.56	65.74	1077.68	94.58	0.90	0.64
Azoxystrobin	IREC	2nd	Control	2035,37	225,48	2099,33	217,45	2044,87	183,42	1,27	0,09
			Solvent	2039,61	273,90	1850,93	418,31	2163,77	202,51	1,21	0,10
			0.1x AR	1863,51	190,33	1784,82	82,00	1943,02	50,75	1,23	0,07
			1x AR	1931,85	294,32	1970,81	163,32	1912,47	323,70	1,22	0,06
			5.44x AR	1918,41	368,50	2346,06	273,47	2047,17	362,45	1,20	0,07
			Soil	1857,61	134,82	1899,97	163,82	2113,37	158,71	1,22	0,06
			Soil+overspray	1895,37	112,83	1983,96	307,85	2006,41	272,15	1,19	0,06
Tebuconazole	IREC	2nd	Control	2035,37	225,48	2099,33	217,45	2044,87	183,42	1,27	0,09
			0.1x AR	1829,68	171,00	1887,41	58,05	1960,69	227,43	1,33	0,13
			1x AR	1762,65	176,21	1829,36	128,01	2090,16	152,42	1,36	0,11
			3.2x AR	1949,35	197,80	1945,48	180,80	2161,76	191,77	1,40	0,03
			Soil	1875,25	229,42	2024,12	106,04	2103,66	162,91	1,36	0,10
			Soil+overspray	1892,89	107,29	1937,19	155,60	2141,72	99,90	1,37	0,08

Substance	Lab	Batch	Treatment	Condition day 0		Condition day 7		Condition day 21		FIR	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD
MCPA	UA	6th	Control	296.66	26.71	296.12	38.05	349.92	28.01	3.95	0.21
			Solvent	280.61	21.74	295.19	17.11	341.54	25.96	3.74	0.32
			0.1x AR	301.52	36.37	308.86	31.24	357.07	33.21	3.95	0.20
			1x AR	303.61	30.41	284.24	20.11	351.18	16.54	3.82	0.33
Fluazifop-p-butyl	UA	7th	Control	323.93	22.26	318.71	18.00	381.90	36.87		
			Solvent	328.97	21.45	330.35	15.35	391.15	33.82		
			0.1x AR	318.30	17.03	333.90	16.47	372.87	15.17		
			1x AR	327.53	25.69	360.24	13.55	419.31	15.79		
			Soil	310.47	22.19	355.79	38.88	378.72	9.50		
			Soil+overspray	323.26	24.08	352.73	27.15	395.87	22.20		
Isoxaben	UA	6th	Control	303.74	30.82	257.64	22.61			4.14	0.00
			Solvent	288.68	39.80	234.36	-			4.07	0.08
			0.1x AR	283.09	26.72	244.50	12.47			4.14	0.00
			1x AR	289.98	12.73					4.14	0.00
			Soil	305.75	55.06	326.51	-			4.79	0.88
			Soil+overspray	308.02	25.46					4.00	0.00
Pendimethalin	UA	5th	Control	449,00	71,04	580,74	215,66	1092,98	631,25	1,69	0,54

Substance	Lab	Batch	Treatment	Condition day 0		Condition day 7		Condition day 21		FIR	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD
			Solvent	437,31	82,86	356,21	127,30	762,16	191,14	1,86	0,84
			1x AR	412,57	115,64	266,08	61,91	391,62	3,32	2,00	0,33
			Soil	465,44	72,74	228,25		417,05		2,00	0,40
			Soil+overspray	431,25	45,75	185,39		390,39		2,00	0,40
Metsulfuron-methyl	IREC	1st	Control	740,84	112,22	825,85	56,10	1031,52	248,95	1,87	1,25
			0.1x AR	752,23	98,16	886,27	20,83	1245,33		1,32	0,28
			1x AR	724,43	112,33	954,26	106,14	988,16		1,59	0,78
			10x AR	794,99	62,20	910,65	82,06	1132,31	39,50	1,68	0,25
		2nd	Control	2035,37	225,48	2099,33	217,45	2044,87	183,42	1,27	0,09
			0.1x AR	2017,96	138,77	2014,30	192,39	2098,79	471,80	1,24	0,07
			1x AR	2068,07	116,61	1941,53	57,37	2215,48	98,10	1,26	0,03
			10x AR	2008,53	239,08	2029,41	147,82	2108,45		1,24	0,05
	UA	5th	Control	450,87	79,86	450,56	78,68	603,42	91,87	1,81	0,59
			0.1x AR	415,49	105,64	463,43	59,98	684,10	28,06	1,92	0,34
			1x AR	435,95	93,11	416,74	111,34	648,08	145,29	1,83	0,48
			10x AR	463,56	76,15	600,41	137,55	792,75	217,18	1,64	0,44
Mesotrione	UA	5th	Control	403,80	66,88	561,88	191,07	1006,35	597,84	1,65	0,60

Substance	Lab	Batch	Treatment	Condition day 0		Condition day 7		Condition day 21		FIR	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD
			Solvent	441,82	51,40	329,71	128,54	729,02	364,51	1,87	0,55
			0.1x AR	388,81	74,18	466,36	96,54	724,75	145,54	1,67	0,55
			1x AR	428,97	69,81	414,22	135,71	1420,08	369,09	1,67	0,55
			2.49x AR	499,45	95,79	558,08	207,00	840,65	192,15	1,66	0,54
Pirimicarb	UA	4th	Control	303,49	53,83	369,54	120,19	583,36	198,09	1,89	0,54
			0.1x AR	276,58	64,44	615,54	228,11	956,91	91,56	1,75	0,55
			1x AR	348,00	226,25	416,79		543,88	250,98	1,36	0,69
			10x AR	388,47	118,58	438,85	118,71	910,88	59,47	1,61	0,74
Acetamiprid	UA	4th	Control	388,16	79,00	418,61	100,66	744,85	304,96	2,00	0,30
			0.1x AR	353,31	111,70	473,29	75,99	732,53	197,37	1,92	0,32
			1x AR	470,05	77,35	404,58	82,83	628,29	164,77	2,00	0,30
			10x AR	455,61	115,22	396,96	35,06	783,66	173,06	1,97	0,32
Flupyradifurone	IREC	3rd	Control	854.38	54.60	835.70	71.43	1135.42	78.23	1.78	0.09
			Solvent	845.78	64.80	880.43	78.19	1091.73	57.33	1.77	0.10
			0.1x AR	872.21	104.48	828.06	47.25	1147.58	92.82	1.78	0.10
			1x AR	898.66	44.70	914.77	108.29	1129.51	99.14	1.74	0.19
			10x AR	918.45	102.20	876.81	80.77	1120.40	40.34	1.79	0.10

Substance	Lab	Batch	Treatment	Condition day 0		Condition day 7		Condition day 21		FIR	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD
			Soil	956.50	95.00	850.62	41.69	1059.28	57.47	1.80	0.10
			Soil+overspray	911.78	54.90	912.18	97.58	1083.81	54.25	1.95	0.36
Alpha-cypermethrin	IREC	1st	Control	740,84	112,22	825,85	56,10	1031,52	248,95	1,87	1,25
			Solvent	726,56	110,35	890,17	120,45	1062,14	13,07	1,68	0,45
			0.1x AR	759,84	127,28	868,29	146,51	1060,74	18,78	1,41	0,21
			1x AR	790,68	87,61	859,85	108,33	926,21	48,74	1,36	0,26
			10x AR	751,21	91,35	881,71	114,98	1185,10	85,38	1,36	0,23
Lambda-cyhalothrin	UA	7th	Control	355.03	28.68	354.57	61.67	400.95	19.43		
			Solvent	337.88	19.20	360.81	33.94	411.35	36.71		
			0.1x AR	368.07	22.62	391.78	34.44	404.32	41.41		
			1x AR	348.71	27.98	390.21	71.59	405.96	22.19		
			Soil	348.60	18.20	355.56	49.95	415.82	15.41		
			Soil+overspray	351.91	47.63	390.19	58.30	429.96	26.06		
Vivando	IREC	3rd	Control	942.08	68.36	889.77	103.44	1031.22	162.96	1.93	0.70
			1x AR	930.17	104.42	837.44	94.89	1072.42	132.99	1.69	0.64
			10x AR	869.09	84.25	858.34	87.72	1098.73	64.75	1.90	0.10
Quadris	IREC	1st	Control	733,78	99,29	849,30	122,95	944,90	229,52	1,78	1,05

Substance	Lab	Batch	Treatment	Condition day 0		Condition day 7		Condition day 21		FIR	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD
			0.1x AR	654,99	79,15	767,51	114,12	962,64	72,00	1,31	0,36
			1x AR	676,94	90,52	782,32	62,97			1,25	0,28
			5.44x AR	798,33	42,08	799,66	27,30	963,25	96,35	1,71	0,00
			Soil	724,79	51,83	840,14	32,31	937,12	119,61	1,43	0,18
			Soil+overspray	676,08	121,53	812,95	40,37	890,64	83,15	1,35	0,32
Folicur 25 EW		1st	Control	733,78	99,29	849,30	122,95	944,90	229,52	1,78	1,05
			0.1x AR	763,07	101,40	842,85	56,93	1040,34	69,29	1,97	0,42
			1x AR	695,52	196,19	751,98	10,80	838,75		1,83	0,66
			3.2x AR	634,15	9,19	720,63				0,93	1,31
			Soil	692,52	67,72	851,12	113,28			1,76	0,24
			Soil+overspray	695,31	85,73	739,63	84,94	952,17		1,64	0,24
		2nd	Control	2035,37	225,48	2099,33	217,45	2044,87	183,42	1,27	0,09
			1x AR	1870,56	21,24	1923,56		2320,92		1,43	0,00
			3.2x AR	1916,82	167,56	1988,62	167,35	2217,91	151,13	1,48	0,07
			Soil	2000,89	142,61	2127,36		2035,86		1,48	0,07
			Soil+overspray	1767,28	168,32	2057,16	16,20	2240,64	434,20	1,44	0,02
	UA	4th	Control	283,37	66,14	366,03	5,72	462,65	92,93	1,73	0,69

Substance	Lab	Batch	Treatment	Condition day 0		Condition day 7		Condition day 21		FIR	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD
			1x AR	357,89	154,28	476,05	34,91	552,85	25,91	0,99	1,19
			Soil	351,91	162,47	475,27	189,76	685,97	92,29	1,79	0,71
			Soil+overspray	363,05	117,48					0,00	0,00
Fusilade Max	UA	7th	Control	357.05	26.43	334.33	26.63	403.40	9.22		
			0.1x AR	326.06	28.86	338.04	29.09	376.11	13.65		
			1x AR	331.70	21.32	307.67	9.59	369.68	36.66		
			10x AR	368.52	23.11	344.27	26.00	404.58	29.86		
Sivanto Prime	IREC	3rd	Control	942.08	68.36	889.77	103.44	1031.22	162.96	1.93	0.70
			1x AR	957.87	103.65	945.44	55.08	1110.20	61.15	1.88	0.14
			10x AR	948.64	94.28	894.30	79.17	1067.53	143.95	1.78	0.27
			Soil	972.69	117.65	878.94	54.66	1071.88	114.32	1.90	0.11
			Soil+overspray	910.12	89.78	861.04	92.75	1137.23	178.78	1.88	0.14
Fasthrin 10 EC	IREC	1st	Control	740,84	112,22	825,85	56,10	1031,52	248,95	1,87	1,25
			0.1x AR	662,44	109,43	872,96	120,42			1,17	0,28
			1x AR	768,04	58,51	789,50	20,53	988,98	16,27	1,48	0,18
			10x AR	721,64	59,44	987,74	208,88	1151,39		1,35	0,38
Naphtha	UA	7th	Control	359.57	41.61	341.59	60.73	399.63	23.30		

Substance	Lab	Batch	Treatment	Condition day 0		Condition day 7		Condition day 21		FIR	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD
			0.1x AR	343.12	16.06	360.23	18.22	406.14	11.47		
			1x AR	326.72	25.09	329.67	40.45	385.61	16.56		
			10x AR	333.39	22.73	356.30	21.73	413.48	22.83		
N,N-dimethyldecanamid	UA	7th	Control	352.18	26.46	371.20	17.21	421.97	21.79		
			Solvent	349.12	17.44	390.24	24.94	403.68			
			0.1x AR	346.79	25.50	381.84	17.92	426.91	41.37		
			1x AR	342.07	66.66	396.66	40.36	413.78	32.07		

Appendix C2: Best-fitted model parameters explaining significant effects on body condition

Experiment	Variable	Origin	Wald's Chi-sq	df	p
Metrafenone	Condition day 21	Intercept	37.265	1	<0.001
		Treatment	25.487	4	<0.001
		Treat*cond_ini	56.323	5	<0.001
		Treat*FIR	25.968	5	<0.001
Oxathiapiprolin	Condition day 7	Intercept	4..890	1	0.027
		Treatment	17.056	4	0.002
		Treat*cond_ini	31.526	5	<0.001
	Condition day 21	Intercept	11.557	1	<0.001
		Treatment	39.376	2	<0.001
		Treat*FIR	89.610	3	<0.001
Benzovindiflupyr	Condition day 7	Intercept	16.484	1	<0.001
		Treatment	6.595	6	0.360
		Treat*cond_ini	19.937	7	0.006
	Condition day 21	Intercept	.178	1	0.673
		Treatment	19.579	5	0.001
		Treat*cond_ini	16.818	7	0.019
		Treat*cond_ini*FIR	23.669	6	<0.001
Azoxystrobin	Condition day 21	Intercept	11.026	1	0.001
		Treatment	39.838	6	<0.001
		Treat*cond_ini	29.34	5	<0.001
		Treat*cond_ini*FIR	28.417	5	<0.001
Tebuconazole	Condition day 7	Intercept	4742.713	1	<0.001
		Treatment	10.449	5	0.063
Fluazifop-p-butyl	Condition day 21	Intercept	8864.155	1	<0.001
		Treatment	13.433	5	0.020
Isoxaben	Condition day 7	Intercept	3389.658	1	<0.001
		Treatment	50.111	3	<0.001

Experiment	Variable	Origin	Wald's Chi-sq	df	p
Pendimethalin	Condition day 7	Intercept	96.889	1	<0.001
		Treatment	4.075	2	0.13
		Treat*cond_ini*FIR	21.403	3	<0.001
	Condition day 21	Intercept	2.56	1	0.11
		Treatment	6.828	2	0.033
		Treat*cond_ini*FIR	21.445	3	<0.001
Metsulfuron-methyl (UA)	Condition day 7	Intercept	0.152	1	0.697
		Treatment	3.63	3	0.304
		Treat*cond_ini	21.32	4	<0.001
Metsulfuron-methyl (IREC)	Condition day 7 (1st batch)	Intercept	20.145	1	<0.001
		Treatment	26.973	3	<0.001
		Treat*cond_ini	20.606	3	<0.001
		Treat*cond_ini*FIR	20.277	3	<0.001
Mesotrione	Condition day 7	Intercept	0.087	1	0.768
		Treatment	17.446	4	0.002
		Treat*cond_ini	32.808	5	<0.001
Pirimicarb	Condition day 7	Intercept	0.596	1	0.44
		Treatment	6.443	2	0.04
		Treat*cond_ini	29.751	3	<0.001
	Condition day 21	Intercept	4.342	1	0.037
		Treatment	5.922	1	0.015
		Treat*cond_ini	14.426	2	0.001
		Treat*cond_ini*FIR	6.463	2	0.039
Flupyradifurone	Condition day 21	Intercept	.065	1	0.799
		Treatment	21.412	6	0.002
		Treat*cond_ini	38.505	7	<0.001
		Treat*cond_ini*FIR	25.592	7	<0.001
Alpha-cypermethrin		Intercept	1682.143	1	<0.001

Experiment	Variable	Origin	Wald's Chi-sq	df	p
	Condition day 7	Treatment	17.061	4	0.002
		Treat*cond_ini	16.411	5	0.006
		Treat*cond_ini*FIR	16.558	5	0.005
	Condition day 21	Intercept	33.778	1	<0.001
		Treatment	16.805	4	0.002
		Treat*cond_ini	43.364	5	<0.001
Lambda-cyhalothrin	Condition day 21	Intercept	16.638	1	<0.001
		Treatment	16.913	5	0.005
		Condition initial	7.523	1	0.006
		Treat*cond_ini	16.105	5	0.007
Fusilade Max	Condition day 21	Intercept	5427.970	1	<0.001
		Treatment	8.992	3	0.029
Folicur 25 EW (IREC)	Condition day 7 (1st batch)	Intercept	2089.836	1	<0.001
		Treatment	2.908	4	0.573
		Treat*cond_ini	12.709	5	0.026
	Condition day 21 (2nd batch)	Intercept	3.133	1	0.077
		Treatment	7.969	2	0.019
		Treat*cond_ini	31.772	3	<0.001
Folicur 25 EW (UA)	Condition day 21	Intercept	76.267	1	<0.001
		Treatment	78.118	1	<0.001
		Treat*cond_ini	63.752	2	<0.001
		Treat*cond_ini*FIR	58.053	2	<0.001
Fasthrin 10 EC	Condition day 7	Intercept	4332.298	1	<0.001
		Treatment	8.953	3	0.03
		Treat*cond_ini*FIR	18.927	4	0.001
N,N-dimethyldecanamid	Condition day 7	Intercept	17.562	1	<0.001
		Treatment	27.403	3	<0.001
		Condition initial	8.221	1	0.004

Experiment	Variable	Origin	Wald's Chi-sq	df	p
		Treat*cond_ini	27.943	3	<0.001

D Appendix. Active ingredients below the threshold value

Appendix D: Active ingredients below the calculated threshold value, based on EC50 in Daphnia, for lethal effects on amphibians condition and food intake rate measured per experiment and treatment

Active ingredient	48h-EC_{50} in *Daphnia* (mg/l)
Acrinathrin	0.000022
Gamma-cyhalothrin	0.000045
Tefluthrin	0.00007
Cypermethrin	0.00021
Pirimiphos-methyl	0.00021
Alpha-cypermethrin	0.00022
Lambda-cyhalothrin	0.00023
Esfenvalerate	0.00027
Deltamethrin	0.00056
Meptyldinocap	0.000579
Malathion	0.0007
Pyridaben	0.001
Etofenprox	0.0012
Triflumuron	0.0016
Formetanate hydrochloride	0.0017
Phosmet	0.002
Diflubenzuron	0.0026
Fenpyroximate	0.00328
Pyridalyl	0.0038
Fenazaquin	0.0041
Etoxazole	0.0071

Active ingredient	48h-EC_{50} in *Daphnia* (mg/l)
tau-Fluvalinate	0.0089
Trifloxystrobin	0.011
Chlorantraniliprole	0.0116
Pyraclostrobin	0.016
Pirimicarb	0.017
Dodine	0.018
Cyantraniliprole	0.02